'*Candidatus* Phytoplasma fragariae', a novel phytoplasma taxon discovered in yellows diseased strawberry, *Fragaria* × *ananassa*

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Symptoms of general stunting and yellowing of leaves were observed in diseased cultivated strawberry (*Fragaria* × *ananassa* Duchesne) in Lithuania. Analysis of 16S rRNA gene sequences amplified by PCR indicated that the symptoms were associated with infection by a phytoplasma, designated strawberry yellows (StrawY) phytoplasma. Phylogenetic analysis of 16S rRNA gene sequences indicated that StrawY phytoplasma, '*Candidatus* Phytoplasma australiense', '*Candidatus* Phytoplasma asteris', stolbur phytoplasma and '*Candidatus* Phytoplasma japonicum' shared a common ancestor, but were mutually distinct. Nucleotide sequence alignments of a 1·3 kb 16S rRNA gene sequence fragment revealed that StrawY phytoplasma shared 97·4 % or less similarity with previously described '*Candidatus* Phytoplasma' species. These results, in addition to natural host and geographical occurrence, support the recognition of StrawY phytoplasma as a representative of a novel taxon, '*Candidatus* Phytoplasma fragariae'.

Phytoplasmas are wall-less, plant-pathogenic bacteria that are classified in the class *Mollicutes*. In diseased plants, phytoplasmas reside in the sieve elements of phloem tissue. Phloem-feeding insects, mainly leafhoppers, transmit phytoplasmas from plant to plant (Davis & Lee, 2000). Fifteen phytoplasma groups (16Sr groups) and more than 40 subgroups have been delineated on the basis of results from RFLP analysis of 16S rRNA gene sequences (Lee *et al.*, 1998; Marcone *et al.*, 2000; Davis *et al.*, 2001; Jomantiene *et al.*, 2002a). Thus far, three 16Sr groups (16SrI, 16SrIII and 16SrV) and eleven subgroups have been reported in Lithuania (Jomantiene *et al.*, 2000, 2002a, b; Staniulis *et al.*, 2000; Valiunas *et al.*, 2000, 2001a, b, 2004; Urbanaviciene *et al.*, 2005). Based on 16S rRNA gene sequences, 20 '*Candidatus* Phytoplasma' species have been described (IRPCM, 2004; Schneider *et al.*, 2005).

In this communication, we propose that a phytoplasma associated with a disease of strawberry be considered as a novel '*Ca.* Phytoplasma' species. Symptoms of general stunting and yellowing of leaves were observed in diseased cultivated garden strawberry (*Fragaria* × *ananassa* Duchesne) in Lithuania, suggesting possible infection by a phytoplasma. Evidence of an association of phytoplasma with the disease, termed strawberry yellows (StrawY), was obtained by the use of PCR primed by phytoplasma-specific oligonucleotides to amplify phytoplasma 16S rRNA gene sequences. Results from comparative analysis of the 16S rRNA gene sequences indicated that StrawY phytoplasma is taxonomically distinct from previously described '*Ca.* Phytoplasma' species.

Three separate samples of leaf tissue were collected from a naturally infected symptomatic strawberry plant growing at a farm in Kavarskas, Anyksciai region, Lithuania, in 2004. Nucleic acid for use as a template in PCR was extracted from each sample according to Jomantiene *et al.* (1998). The three nucleic acid samples were used in three separate PCRs in which two pairs of oligonucleotides were used to prime the amplification of rRNA gene sequences. P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995) and R16F2n/R16R2 (Gundersen & Lee, 1996) are oligonucleotide pairs that prime the amplification of sequences from phytoplasma rRNA operons as described previously (Lee *et al.*, 1993). Products from nested PCR, primed by R16F2n/R16R2 according to Gundersen & Lee (1996), were analysed by single enzyme digestion with *Alu*I, *Mse*I, *Kpn*I, *Hae*III, *Hha*I, *Hpa*II, *Rsa*I, *Hin*fI and *Taq*I (MBI Fermentas) according to the manufacturer's instructions. The resulting RFLP patterns were compared with previously published data (Lee *et al.*, 1998; Marcone *et al.*, 2000). The 16S rRNA gene products from the three PCRs primed by P1/P7 were cloned

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Abbreviation: STOL, stolbur phytoplasma group.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of phytoplasma strain StrawY is DQ086423.

A supplementary table showing the unique regions in the 16S rRNA gene sequence of '*Candidatus* Phytoplasma fragariae' compared with other '*Candidatus* Phytoplasma' species is available in IJSEM Online.

as described previously (Jomantiene *et al.*, 1998) and the three cloned DNA fragments were sequenced by automated sequencing of both strands to achieve a minimum of 4× coverage per position. The nucleotide sequence determined in this study was deposited in GenBank. Other sequences used in the study were obtained from GenBank and their accession numbers are given in Figs 2 and 3. Maps of putative restriction sites were constructed by the use of the MapDraw program in the DNASTAR software package. For calculations of sequence similarities, sequences were aligned by using the ALIGN program in the same software package. For phylogenetic analysis, 16S rRNA gene sequences from 20 '*Ca.* Phytoplasma' species, StrawY phytoplasma and *Acholeplasma palmae* were aligned using CLUSTAL_X, version 1.63b (Thompson *et al.*, 1997). A phylogenetic tree was constructed by the neighbour-joining method and the tree was viewed by using TREEVIEWPPC (Page, 1996). *A. palmae* was selected as the outgroup to root the tree. Bootstrapping was performed 1000 times for estimation of stability and support for the clades.

Amplification of phytoplasmal 16S rRNA gene fragments in three separate PCRs primed by phytoplasma universal primer pairs indicated that the strawberry plant affected by strawberry yellows disease in Kavarskas, Lithuania, was infected by a phytoplasma, designated strain StrawY (data not shown). Results from comparative analysis of collective RFLP patterns of a 1·2 kb segment of the 16S rRNA gene sequence, corresponding to the DNA fragment amplified in PCR primed by R16F2n/R16R2, indicated that the StrawY phytoplasma was related to group 16SrI and 16SrXII phytoplasmas and '*Candidatus* Phytoplasma japonicum', but was distinct from the these phytoplasmas (Figs 1 and 2). For example, StrawY phytoplasma was distinguishable from '*Ca.* Phytoplasma japonicum' by the presence of *Mse*I, *Rsa*I, *Kpn*I, *Eco*RI and *Alu*I sites that were absent in the '*Ca.* Phytoplasma japonicum' 16S rRNA gene fragment. In addition, a *Hha*I site in the '*Ca.* Phytoplasma japonicum' 16S rRNA gene sequence was absent in StrawY. The 16S rRNA gene sequence of StrawY phytoplasma was distinguished from that of the stolbur (STOL) phytoplasma (group 16SrXII) by the presence of *Mse*I and *Hha*I sites that were absent in the STOL 16S rRNA gene sequence and by *Hae*III, *Taq*I and *Mse*I sites in STOL phytoplasma 16S rRNA gene sequences that were absent in StrawY. The 16S rRNA gene sequence of '*Candidatus* Phytoplasma australiense' (group 16SrXII) lacked *Mse*I, *Alu*I and *Hha*I sites that were present in StrawY and had *Hae*III and two *Mse*I recognition sites that were absent in StrawY 16S rRNA gene sequences. StrawY 16S rRNA gene sequences differed from those of '*Candidatus* Phytoplasma asteris' (group 16SrI) by the presence of three sites for *Mse*I, one *Hin*dIII and one *Alu*I site in StrawY and by the lack of a *Hae*III site in '*Ca.* Phytoplasma asteris' that was present in StrawY. The 16S rRNA gene sequence of StrawY phytoplasma had one unique *Mse*I restriction site that was absent in these other phytoplasmas. Thus, StrawY, '*Ca.* Phytoplasma japonicum', STOL phytoplasma, '*Ca.* Phytoplasma australiense' and '*Ca.* Phytoplasma asteris' phytoplasmas could be distinguished from one another by RFLP analysis of 16S rRNA gene sequences.

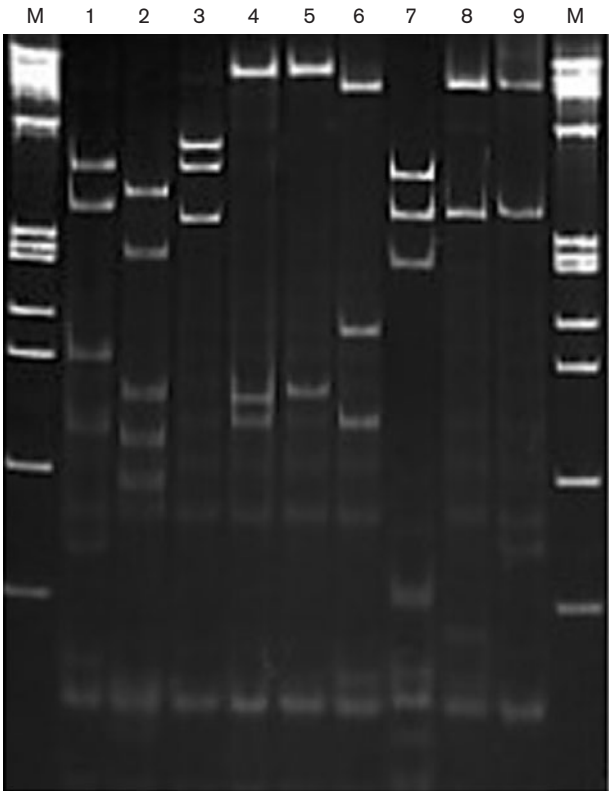

Fig. 1. RFLP analysis of 16S rRNA gene sequences amplified in nested PCR primed by oligonucleotide pair R16F2n/R16R2 from '*Ca.* Phytoplasma fragariae'. Lanes: 1, *Alu*I; 2, *Mse*I; 3, *Kpn*I; 4, *Hha*I; 5, *Hae*III; 6, *Hpa*II; 7, *Rsa*I; 8, *Hin*fI; 9, *Taq*I; M, Marker, a *Phi*X174 DNA/*Bsu*RI (*Hae*III) digest size standard with fragment sizes of 1353, 1078, 872, 692, 310, 281, 271, 234, 194, 118 and 72 bp.

A phylogenetic tree was constructed based on 16S rRNA gene sequences from all previously described '*Ca.* Phytoplasma species', StrawY phytoplasma and *A. palmae* (Fig. 3). The branching order of the tree is in good agreement with that of previously published trees (IRPCM, 2004; Lee *et al.*, 2004). The phylogenetic analysis indicated that StrawY phytoplasma (shown as the proposed species '*Ca.* Phytoplasma fragariae'), '*Ca.* Phytoplasma japonicum', '*Ca.* Phytoplasma australiense' and '*Ca.* Phytoplasma asteris' shared a common ancestor. '*Ca.* Phytoplasma fragariae' formed a new, well-supported branch representing a distinct lineage.

The nucleotide sequences of three cloned DNA fragments were in agreement. Alignment of 1333-base segments of 16S rRNA gene sequences indicated that StrawY phytoplasma contained nucleotide sequences previously reported as unique to phytoplasmas (Gundersen *et al.*, 1994; IRPCM, 2004). In addition, the StrawY phytoplasma 16S rRNA gene sequence contained unique nucleotide sequences that

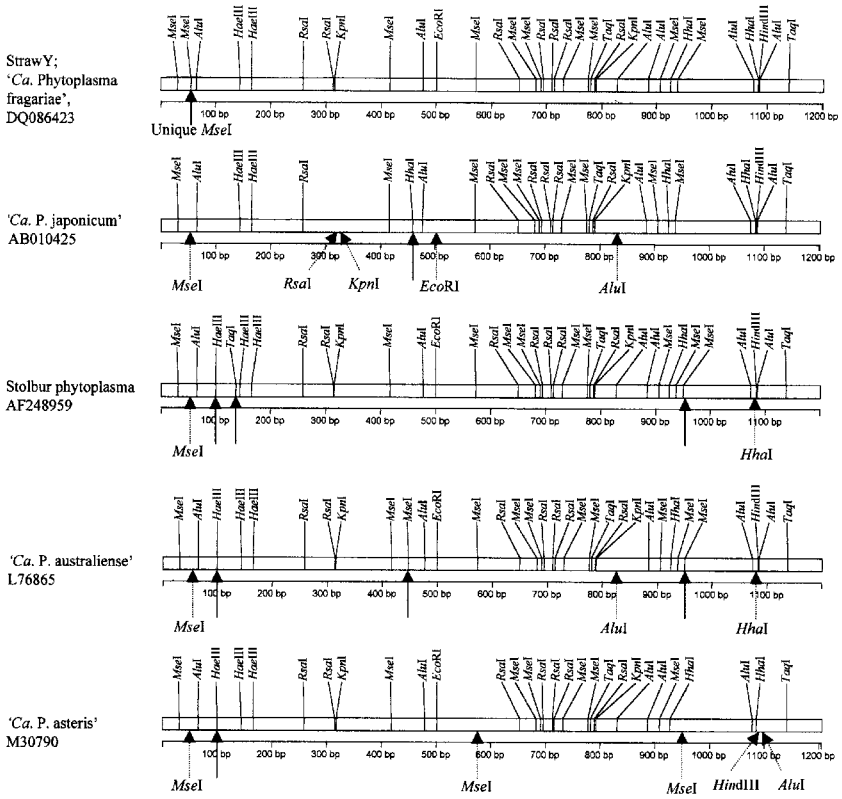

Fig. 2. Comparative analysis of putative restriction sites in 16S rRNA gene sequences corresponding to DNA amplified from '*Ca.* Phytoplasma fragariae' phytoplasma and its closest known relatives in PCRs primed by oligonucleotide pair R16F2n/R16R2. Arrows indicate restriction sites that differentiate '*Ca.* Phytoplasma fragariae' from other phytoplasmas. Broken arrows indicate sites present in the 16S rRNA gene sequence of '*Ca.* Phytoplasma fragariae' and absent in other 16S rRNA gene sequences.

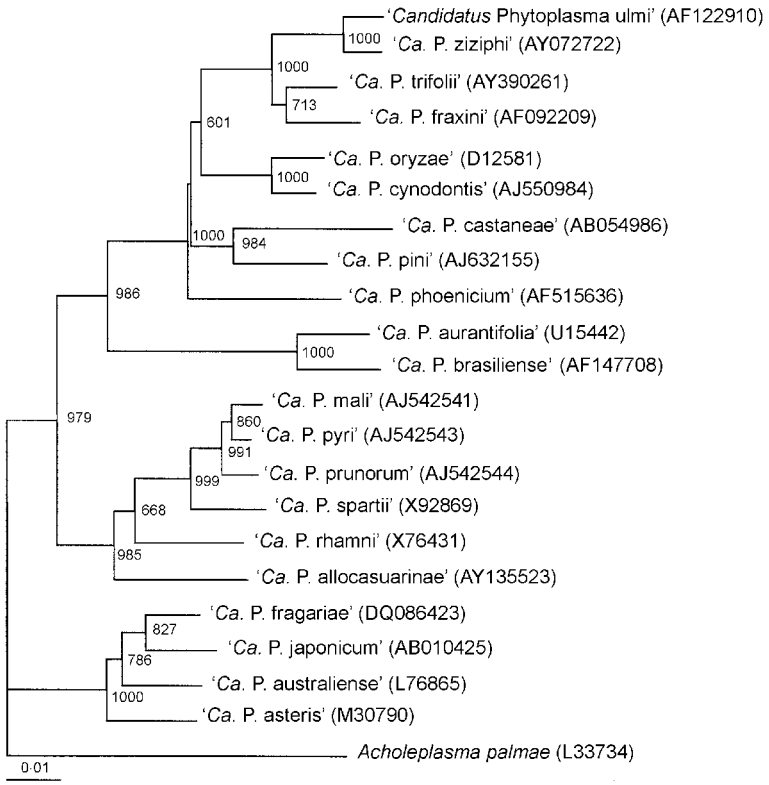

Fig. 3. Phylogenetic tree constructed by the neighbour-joining method of 16S rRNA gene sequences from previously described '*Ca.* Phytoplasma' species, '*Ca.* Phytoplasma fragariae' and *Acholeplasma palmae*, employing *A. palmae* as the outgroup. Bar, 1 substitution in 100 nucleotides. Numbers on the nodes are bootstrap (confidence) values.

distinguished it from all previously described '*Ca.* Phytoplasma' species. A table comparing the nucleotide sequences of unique regions in the 16S rRNA gene sequence of the StrawY phytoplasma is available as Supplementary Table S1 in IJSEM Online. Unique regions in the StrawY phytoplasma 16S rRNA gene fragment are $_{482}$5′-GTGCAATGCTCAACGTTGTGAT-3′$_{503}$, $_{899}$5′-AATTGCA-3′$_{905}$ and $_{1313}$5′-TGAGTAATCAAGAGGGAG-3′$_{1330}$, which differ in from one to eight, from one to six and from two to eleven base positions, respectively, from the corresponding regions in the 16S rRNA gene sequences of previously described '*Ca.* Phytoplasma' species.

Sequence comparisons revealed that the StrawY phytoplasma 1·3 kb 16S rRNA gene sequence segment shared no greater than 97·4 % nucleotide sequence similarity with any previously described '*Ca.* Phytoplasma' species. According to recommendations by the International Research Program for Comparative Mycoplasmology, Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group (IRPCM, 2004), ''a '*Ca.* Phytoplasma' species description should refer to a single, unique 16S rRNA gene sequence (>1200 bp)'' and ''a strain can be recognized as a novel '*Ca.* Phytoplasma' species if its 16S rRNA gene sequence has <97·5 % similarity to that of any previously described '*Ca.* Phytoplasma' species''. Results from rRNA gene sequence analysis, in addition to the natural host and the geographical location, support the recognition of StrawY phytoplasma as a representative of a novel taxon. Thus, we propose that the StrawY phytoplasma be designated a member of a novel, distinct '*Candidatus*' species, '*Candidatus* Phytoplasma fragariae'.

Description of '*Candidatus* Phytoplasma fragariae'

'*Candidatus* Phytoplasma fragariae' (frag′ar.i.ae. N.L. gen. n. *fragariae* of *Fragaria*, the scientific name of strawberry; epithet referring to the plant host).

Reference isolate is strain StrawY^{R}.

[(*Mollicutes*) NC; NA; O; NAS (GenBank accession number DQ086423), oligonucleotide sequences of unique regions of the 16S rRNA gene are 5′-GTGCAATGCTCAACGTTGTGAT-3′, 5′-AATTGCA-3′ and 5′-TGAGTAATCAAGAGGGAG-3′; P (*Fragaria* × *ananassa*, phloem); M]. Valiunas *et al.*, this study.

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