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# Phytoplasmas infecting sour cherry and lilac represent two distinct lineages having close evolutionary affinities with clover phyllody phytoplasma

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**Abstract** Phytoplasmas infecting sour cherry and lilac in Lithuania were found to represent two lineages related to clover phyllody phytoplasma (CPh), a subgroup 16SrI-(R/S)C (formerly 16SrI-C) strain exhibiting rRNA interoperon sequence heterogeneity. 16S rDNAs amplified from the cherry bunchy leaf (ChBL) and lilac little leaf (LcLL) phytoplasmas were identical or nearly identical to those of operon *rrnA* and operon *rrnB*, respectively, of CPh. There was no evidence of 16S rRNA interoperon sequence heterogeneity in either LcLL or ChBL phytoplasma. Based on collective RFLP patterns of 16S rDNA, ChBL was classified in subgroup 16SrI-R, and LcLL was classified in new subgroup 16SrI-S. The ribosomal protein (rp) gene sequences from LcLL phytoplasma were identical to those of CPh, and strain LcLL was classified in rp subgroup rpI-C. By contrast, rp gene sequences from ChBL phytoplasma differed from those of subgroup rpI-C; based on RFLP patterns of rp gene sequences, ChBL was classified in new rp subgroup rpI-O. Single nucleotide polymorphisms (SNPs), designated here by a new SNP convention, marked members of rp subgroup rpI-C, and distinguished LcLL and CPh from ChBL and other non-rpI-C phytoplasmas in group 16SrI. The results raise questions concerning phytoplasma biodiversity assessment based on rRNA genes alone and encourage the supplemental use of a single copy gene in phytoplasma identification and classification, while drawing attention to a possible role of horizontal gene transfer in the evolutionary history of these lineages.

**Keywords** Phytoplasma classification · Genetic diversity · Phylogeny

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## Introduction

Phytoplasmas are minute, cell wall-less plant pathogenic bacteria that are limited to phloem in infected plants and are transmitted from plant-to-plant by phloem-feeding insects, mainly leafhoppers and psyllids. In their descent from low G+C, walled bacterial ancestors, phytoplasmas underwent extensive genome reduction, discarding genes encoding diverse metabolic pathways in an on-going process of adaptation to intracellular parasitism (Davis et al. 2005). In spite of numerous attempts for over 40 years, it has not been possible to cultivate phytoplasmas in cell-free media. Consequently, characters used in the identification and classification of cultivable bacteria have

been largely unattainable for phytoplasmas, and analysis of conserved gene sequences has been adopted for phytoplasma classification (Lee et al. 1998; Martini et al. 2007).

Based on RFLP analysis of 16S rDNA, phytoplasmas have been classified in over 30 groups, and 28 '*Candidatus* Phytoplasma' species have been described (Zhao et al. 2010). Adaptation to diverse ecological niches in different geographic and climatic regions of the globe prompts expectations that many more phytoplasma species and strain lineages are yet to be discovered. In assessing biodiversity of phytoplasmas through study of rRNA genes, it will be important to take into account the presence in a phytoplasma genome of two, sometimes sequence heterogeneous, rRNA operons. The rRNA interoperon sequence heterogeneity can create potential for overestimates of biodiversity (Acinas et al. 2004; Jomantiene et al. 2010), and when used as the sole genetic marker, the conserved nature of 16S rRNA genes can pose difficulties in resolving closely related phytoplasmas. To address such problems, more variable genetic markers, including those based on single copy ribosomal protein (rp), *secY,* and *tuf* gene sequences, can be adopted for enhanced identification and classification (Lee et al. 2006; Marcone et al. 2000; Martini et al. 2007).

In the present study, we found two unusual '*Candidatus* Phytoplasma asteris'-related lineages in diseased sour cherry (*Prunus cerassus*) and lilac (*Syringa vulgaris*). Analyses of rDNA indicated that the phytoplasmas each contained 16S rDNA highly similar to that of only one or the other of the two sequence-heterogeneous rRNA operons of clover phyllody (CPh) phytoplasma, a member of 16S rDNA RFLP subgroup 16SrI-C [renamed 16SrI-(R/S)C in the present study]. Unlike CPh, there was no evidence of 16S rRNA interoperon sequence heterogeneity in either the sour cherry- or the lilac-infecting phytoplasma. The rp gene from one of the phytoplasmas differed remarkably from that of CPh, raising the possibility of horizontal gene transfer in the evolutionary history of these lineages. Here we report analyses of rDNA and rp gene sequences, classification of the phytoplasmas, and discovery of single nucleotide polymorphisms (SNPs) that allow prediction of a group 16SrI phytoplasma's subgroup rpI-C affiliation. The findings impact studies of biodiversity based solely on multicopy rRNA genes and encourage the supplemental use of a single copy gene.

## Materials and methods

### Plant samples, PCR, RFLP and sequence analysis, and phytoplasma classification

Samples of leaves and petioles from symptomatic lilac and from symptomatic and apparently healthy sour cherry were collected in the Klaipeda region of Lithuania. DNA was extracted from the collected tissues as previously (Jomantiene et al. 2010) and used as a template in polymerase chain reactions (PCRs) for amplification of 16S ribosomal (r) RNA and ribosomal protein gene sequences. Primer pairs P1/16S-SR, P1A/16S-SR, and R16F2n/R16R2n (F2n/R2n) were used in semi-nested and nested PCRs for amplification of rRNA gene sequences as previously described (Lee et al. 1998, 2004). Ribosomal protein (rp) gene sequences (*rpsS* [*rps19*], *rplV* [*rpl22*], *rpsC* [*rps3*]) were amplified using primer pair rp(I)F1A/rp(I)R1A according to Lee et al. (2004). 16S rDNA products (1.2 kbp, F2n/R2n segments) of the nested PCR were subjected to restriction fragment length polymorphism (RFLP) analysis as previously (Jomantiene et al. 2010). Phytoplasmas were classified in rRNA subgroups in accordance with Lee et al. (1998). Ribosomal protein gene products of PCR were analyzed by *in silico* digestion, using *i*PhyClassifier (Zhao et al. 2010), with *Alu*I, *Mse*I, and *Tsp*5091 for initial assignment of strains to subgroups in group rpI according to Lee et al. (2004); digestion with *Hae*II and *Taq*I was used for further differentiation. Nucleotide sequences of rRNA and rp genes were determined by automated sequencing of both strands to achieve a minimum of 3-fold coverage per base position and deposited in the GenBank database. Nucleotide sequence alignments were generated, sequence similarities evaluated, and phylogenetic analyses of nucleotide sequences, obtained in this study and from GenBank (Table 1 and Figs. 5 and 6), were carried out as described (Jomantiene et al. 2010; Zhao et al. 2010).

## Results

### Detection and identification of phytoplasmas in diseased sour cherry and lilac

Sour cherry trees exhibited disease symptoms that included mild yellowing of leaves, bunchy little leaf

**Table 1** Phytoplasma strains related to subgroup 16SrI-C, source host plants, source countries, 16Sr group and subgroup, ribosomal protein subgroup, composite 16S rDNA RFLP patterns, and GenBank accession numbers of nucleotide sequences used in this study

| Phytoplasma strain | Source host, country | 16Sr/rp subgroup | Composite pattern[a] | rRNA operon[b] | rDNA GenBank no. | rp GenBank no.[c] | Reference(s) |
|---|---|---|---|---|---|---|---|
| Cherry bunchy leaf (ChBL) | *Prunus cerasus*, Lithuania | I-R/rpI-O | No | rrnA | HM067754 | HM067756 | This study |
| Lilac little leaf (LcLL) | *Syringa vulgaris*, Lithuania | I-S/rpI-C | No | rrnB | HM067755 | HM067757 | This study |
| Clover phyllody (CPh, CPh-C) | *Trifolium sativum,* Canada | I-C/rpI-C | Yes | rrnA | AF222065 | AY264862 | Lee et al. 1998 |
| | | | | rrnB | AF222066 | | |
| Clover phyllody (CPh-L) | *T. sativum*, Lithuania | I-C/NA[d] | Yes | NA | NA | NA | Staniulis et al. 2000 |
| Clover phyllody (KVG, KV) | *T. repens*, Germany | I-C/rpI-C | Yes | rrnA | X83870 | AY264860 | Schneider et al. 1997 |
| Clover phyllody (KVE) | *T. repens*, England | I-C/rpI-C | Yes | rrnB | AY265217 | AY264861 | Marcone et al. 2000 |
| Cirsium yellows (CirY) | *Cirsium arvense*, Lithuania | I-R/NA | No | rrnA | AF200431 | NA | Jomantiene et al. 2000 |
| Poa stunt (PoaS) | *Poa pratensis*, Lithuania | I-C | Yes | rrnA | DQ640501 | NA | Valiunas et al. 2007 |
| | | | | rrnB | DQ640502 | | |
| Gaillardia yellows (GaiY) | *Gaillardia* sp., Lithuania | I-C | Yes | rrnA | EF583065 | HM164408 | Valiūnas et al. 2008 |
| | | | | rrnB | EF583066 | | |
| Festuca yellows (FesY) | *Festuca arundinacea*, Lithuania | I-C | Yes | rrnA | DQ640503 | NA | Valiunas et al. 2007 |
| | | | | rrnB | DQ640504 | | |
| Polish tomato phyllody (PTPh) | *Lycopersicon esculentum*, Poland | I-C?[e] | Unknown | rrnA | EF164961 | NA | Pospieszny et al. 2007 |
| Strawberry phylloid fruit (StrawbPhF) | *Fragaria x ananassa*, USA | I-R | No | rrnA | AY102275 | NA | Jomantiene et al. 2002b |
| Strawberry green petal (SGP) | *F. x ananassa*, Canada | I-C | Yes | NA | NA | HM164409 | This study |
| Ribes rubrum (RibR) | *Ribes rubrum*, Czech Republic | I-C | Yes | rrnA | AY669063 | NA | Navrátil et al. 2007 |
| Echinacea phyllody (CzEchPh) | *Echinacea purpurea*, Czech Republic | I-C/rpI-C | Yes | rrnA | EF546778 | EF546779 | Fránová et al. 2009 |
| Echinacea phyllody (SlEchPh) | *E. purpurea*, Slovenia | I-C? | Unknown | rrnB | EU416172 | NA | Radisek et al. 2009 |
| Willow yellow | *Salix* sp., China | I-C? | Unknown | rrnB | FJ179166 | NA | NA |
| Black currant reversion (BCRev) | *Ribes nigrum*, UK | I-C? | Unknown | rrnA | EU168788 | NA | Hodgetts et al. 2008 |

[a] Composite pattern; composite RFLP pattern, observed in gel electrophoretic analysis of rDNA fragments after digestion of PCR product with *Alu*I, indicating presence of two sequence-heterogeneous rRNA operons characteristic of subgroup 16SrI-C. We recommend that, if actual gel electrophoretic analysis of digested PCR product is not carried out, and 16S rDNA from only one rRNA operon is sequenced and that sequence is highly similar to either rrnA or rrnB of CPh, an *Alu*I RFLP actual gel analysis be done to determine whether a composite RFLP pattern is obtained that would indicate presence of two sequence-heterogeneous rRNA operons. In addition, if no rRNA interoperon sequence heterogeneity is observed, based on the *Alu*I RFLP pattern of digested PCR product, it is recommended to sequence the rp genes as in the present study, to characterize a previously unidentified phytoplasma

[b] rRNA operon of nucleotide sequence in the indicated GenBank accession. In the case of a strain for which the nucleotide sequence for rDNA is available from only a single rRNA operon, the operon (rrnA or rrnB) listed is the CPh-C rRNA operon whose nucleotide sequence shares greater sequence identity with the strain's rDNA

[c] rp, ribosomal protein

[d] NA, not available

[e] I-C?, affiliation with subgroup 16SrI-C is not certain, because composite RFLP patterns, rDNA sequences from both rRNA operons, or other evidence for interoperon rRNA sequence heterogeneity as found in CPh phytoplasma, was not published, leaving open the possibility of lineages having two sequence-homogeneous 16S rRNA genes, a second rRNA operon having a sequence differing from that of either rRNA operon of CPh, and/or presence in the same genome of another conserved gene (such as rp protein gene) having a nucleotide sequence substantially divergent from that of CPh

growths, and general leaf drop (Fig. 1, left). DNA extracted from leaves of two symptomatic trees and three healthy trees was used as templates in nested PCRs primed by primer pairs P1/16S-SR and F2n/R2n. Whereas, samples from the apparently healthy trees yielded no observable DNA amplicons, samples from the two symptomatic trees yielded 1.2 kb DNA amplicons. The two amplicons yielded mutually indistinguishable collective RFLP patterns, consistent with infection of both trees by the same phytoplasma, designated the cherry bunchy leaf (ChBL) phytoplasma. RFLP patterns of the amplified 16S rDNA indicated that ChBL phytoplasma was a member of group 16SrI (Fig. 2 and data not shown). The *Mse*I and *Hae*III RFLP profiles differed from those characterizing nearly all known representatives of group 16SrI, but were indistinguishable from those reported for the subgroup 16SrI-R strawberry phylloid fruit (StrawbPhF) phytoplasma and the unclassified Cirsium yellows (CirY) phytoplasma, in both of which no evidence of sequence heterogeneous 16S rRNA was observed (Jomantiene et al. 2000). Such RFLP profiles were previously documented also for one of the two sequence heterogeneous rRNA operons (operon *rrnA*) of clover phyllody (CPh) phytoplasma (Fig. 2), but like StrawbPhF and CirY and unlike CPh, there was no evidence for 16S rDNA interoperon sequence-heterogeneity in ChBL phytoplasma.

At the same location, several lilac trees exhibited disease symptoms that included general yellowing, reduced leaf size, rolling of leaves (Fig. 1, right), and drying of flowers. The disease was termed lilac little leaf (LcLL). RFLP analysis of PCR-amplified rDNA revealed that all of three symptomatic lilac plants

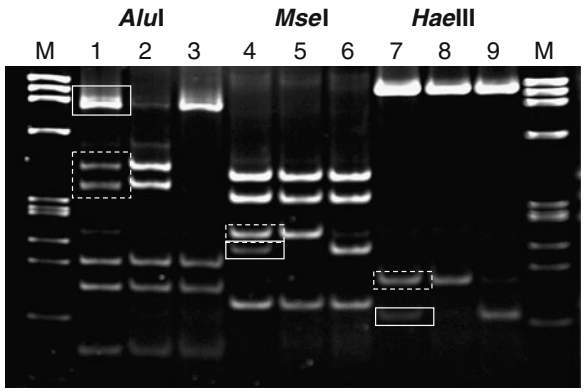


**Fig. 2** RFLP analysis of 16S rDNA amplified from phytoplasma strains CPh (lanes 1, 4, and 7), ChBL (lanes 2, 5, and 8), and LcLL (3, 6, and 9) in nested PCR primed by R16F2n/R16R2n, and digested with *Alu*I, *Mse*I, and *Hae*III.. CPh bands containing DNA fragments common to strains CPh and LcLL are enclosed within *solid line boxes*; bands containing DNA fragments common to strains CPh and ChBL are enclosed within *dashed line boxes*. M, size marker ΦX174 RFI DNA *Hae*III digest; fragment sizes (bp) from *top to bottom*: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72

were infected by a phytoplasma also belonging to group 16SrI. The *Alu*I and *Mse*I RFLP profiles of the amplified rDNA (F2n/R2n segment) differed from those that defined all previously reported subgroups in group 16SrI, but these and other profiles were identical to the collective RFLP profiles that characterize operon *rrnB* of CPh phytoplasma (Fig. 2 and profiles not shown). As in the case of ChBL phytoplasma, and unlike CPh, there was no evidence of a composite RFLP profile that would indicate 16S rDNA interoperon sequence-heterogeneity in LcLL phytoplasma.

**Fig. 1** Symptoms caused by cherry bunchy leaf (ChBL) and lilac little leaf (LcLL) phytoplasmas: *left*, sour cherry exhibiting mild yellowing of bunchy little leaf growths on the branches and general leaf drop; *right*, lilac exhibiting general yellowing, reduced leaf size, and curling of leaves

Since ChBL RFLP (pattern type R) profiles were indistinguishable from those of subgroup 16SrI-R and LcLL RFLP (pattern type S) profiles of rDNA differed from those defining all previously reported subgroups in group 16SrI, we classify the ChBL and LcLL phytoplasmas in subgroups 16SrI-R and new subgroup 16SrI-S, respectively, and classify strain CirY in subgroup 16SrI-R. These assignments follow the use of 16S rDNA RFLP patterns for phytoplasma classification (Lee et al. 1998). In accordance with designations for subgroups that are characterized by 16S rRNA interoperon sequence heterogeneity, in which 16S rDNA RFLP pattern types (of the two rDNA copies per genome) are noted within parentheses (Zhao et al. 2010), the designation of the CPh subgroup 16SrI-C becomes 16SrI-(R/S)C. Designations of the subgroups represented by ChBL and LcLL hence could be written as 16SrI-(R)R and 16SrI-(S)S, respectively, since each is characterized by collective RFLP patterns characteristic of a single rDNA copy.

## Nucleotide sequence analyses of 16S rDNAs

The nucleotide sequences of rDNAs (1500 bp products of P1A/16S-SR-primed PCRs) from strains ChBL and LcLL were deposited in the GenBank database under accession numbers HM067754 (ChBL phytoplasma) and HM067755 (LcLL phytoplasma). The 16S rDNA sequence (F2n/R2n segment) from phytoplasma ChBL was nearly identical to that of operon *rrnA* from CPh phytoplasma, as well as to that of StrawbPhF (AY102275) and CirY (AF200431) phytoplasmas; the nucleotide sequence of the F2n/R2n segment from phytoplasma LcLL was identical to that of operon *rrnB* of CPh phytoplasma. Since the 16S rDNA RFLP and nucleotide sequence analyses implied that ChBL and LcLL phytoplasmas represented distinct lineages, we next analyzed additional gene sequences to further characterize the unique lineages.

## Analysis of ribosomal protein gene sequences

We examined rp gene sequences, which provide enhanced differentiation and classification of phytoplasmas (Martini et al. 2007). The results indicated that ChBL and LcLL phytoplasmas belonged to two different rp subgroups in group rpI. The products from rpF1A/R1A-primed PCR revealed that the strain LcLL rp sequence (1111 bp, GenBank no. HM067757) shared 100% identity with, and displayed the same *Alu*I, *Mse*I, *Tsp*5091, and *Taq*I RFLP patterns as, the comparable region of the sequence (GenBank no. AY264862) from strain CPh, a member of rp subgroup rpI-C (Fig. 3). Thus, strain LcLL is also a member of the subgroup rpI-C lineage.

Nucleotide sequence analysis of the products from rpF1A/R1A-primed PCR revealed that the strain ChBL rp sequence (1124 bp, GenBank no. HM067756) differed by 26 bases (one base in *rpsS*, three in *rplV*, and 22 in *rpsC*) from the strain CPh subgroup rpI-C nucleotide sequence (GenBank no. AY264862). Putative recognition sites for three of the enzymes used for rp subgroup differentiation (*Alu*I, *Mse*I, and *Tsp*5091) were the same as those characterizing the rp sequence reported for paulownia witches'-broom phytoplasma strain PaWB (GenBank no. AY264857), but ChBL was distinguished from PaWB based on recognition sites for *Hae*II (data not shown) and *Taq*I (Fig. 3); ChBL and PaWB rp gene sequences differed from one another by two bases in *rplV* and 8 bases in *rpsC*. Based on these findings, we classified ChBL in a new rp subgroup lineage, designated rpI-O (GenBank no. HM067756). Several of these differences between ChBL and CPh phytoplasmas were reflected in the deduced amino acid sequences of the S3 rp proteins encoded by the two rp gene sequences, although the proteins do share a high level of amino acid sequence

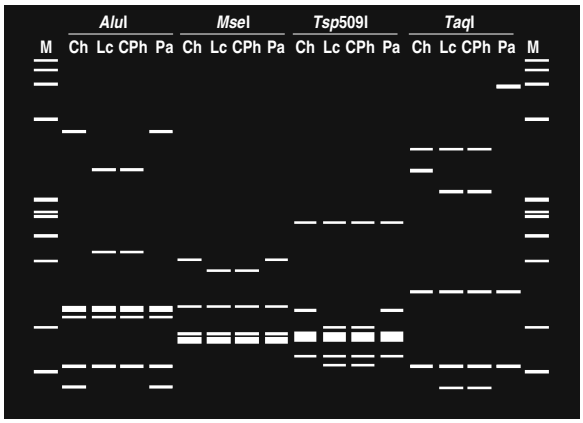


**Fig. 3** *In silico* RFLP analysis of ribosomal protein operon genes amplified in PCR primed by primer pair rp(I)F1A/rp(I)R1A from cherry bunchy leaf (Ch), lilac little leaf (Lc), clover phyllody (CPh), and paulownia witches'-broom (Pa) phytoplasmas. GenBank nos. of the sequences are given in Table 1. M, size marker ΦX174 RFI DNA *Hae*III digest; fragment sizes (bp) from *top to bottom*: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72

conservation. The marked differences between the rp gene sequences in LcLL vs ChBL phytoplasma underscore the concept that these two strains represent distinct 16SrI subgroup level lineages. The presence in ChBL, of lineage-disparate rDNA and rp gene sequences, may have implications for rRNA or rp gene horizontal transfer resembling phenomena previously proposed for other microbes (Mylvaganam and Dennis 1992; Yap et al. 1999).

## Single nucleotide polymorphisms (SNPs) mark sister rRNA operons and uniqueness of subgroup rpI-C

Alignment of 16S rDNA sequences from phytoplasma strains classified in subgroup 16SrI-C revealed a single nucleotide polymorphism (SNP) that corresponded to position 1204 in the sequence derived from strain LcLL (GenBank no. HM067755) (Fig. 4). In order to refer to a specific base in a given SNP position, we propose a convention as follows: letters SNP followed by gene designation, [GenBank no. of reference sequence for SNP position], position of SNP in reference sequence, (base identity in SNP position of sequence under study). For example, the SNP in LcLL 16S rRNA gene is designated LcLL SNP16Sr[HM067755]1204(T); that in ChBL is designated SNP16Sr[HM067755] 1204(C); and that in KVG is designated SNP16Sr [HM067755]1204(C). The identity (C or T) of the base at this position was the same in both rRNA operons of a given phytoplasma genome of the strains examined. For example, both operons of strain CPh and both operons of strain PoaS contained T in the SNP position, whereas, both operons of strain FesY and both operons of strain GaiY contained C in the SNP position. These results are consistent with the previously reported conclusion that the composite 16S rDNA RFLP patterns characterizing strains CPh, PoaS, FesY, GaiY each represent two sequence-heterogeneous rRNA operons from the same genome (Lee et al. 1998; Staniulis et al. 2000; Valiunas et al. 2007, Valiūnas et al. 2008), and with the hypothesis that the rDNA sequences from strains ChBL and LcLL represent two distinct 16S rDNA SNP lineages.

Alignment of rp gene sequences from strains LcLL and ChBL, eight phytoplasma strains previously classified in subgroup 16SrI-C, and 14 strains classified in other group 16SrI subgroups, revealed at least 11 rp gene SNPs that distinguished LcLL and the subgroup 16SrI-C strains from ChBL and all other group 16SrI strains examined (Table 2). In the proposed convention for designation of SNPs (this paper), one of the rp gene SNPs listed for strain LcLL is designated SNPrp[HM067757]137(A), and the corresponding SNP base for ChBL is designated SNPrp[HM067757]137(C). Thus, the base identities in the positions indicated in Table 2 delineate subgroup rpI-C among group 16SrI phytoplasmas. Therefore, one should be able to predict a phytoplasma strain's subgroup rpI-C affiliation based on rp gene SNPs alone, underscoring the potential of SNPs for enhancing phytoplasma identification and classification.

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CPh  rrnA    ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGTAAGTTTTTGGCGAATC
CPh  rrnB    ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGTAAGTTTTTGGCGAATC
PoaS rrnA    ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGTAAGTTTTTGGCGAATC
PoaS rrnB    ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGTAAGTTTTTGGCGAATC
GailY rrnA   ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGCAAGTTTTTGGCGAATC
GailY rrnB   ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGCAAGTTTTTGGCGAATC
FesY rrnA    ATGCCCCTTATGACCTGGGCTACAAACGTAATACAATGGCTGTTACAAAGGGTAGCTGAAACGCAAGTTTTTGGCGAATC
FesY rrnB    ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGCAAGTTTTTGGCGAATC
KVG          ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGTAAGTTTTTGGCGAATC
RibR         ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGCAAGTTTTTGGCGAATC
SlEchPh      ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGTAAGTTTTTGGCGAATC
KVE          ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGTAAGTTTTTGGCGAATC
CzEchPh      ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGCAAGTTTTTGGCGAATC
StrawbPhF    ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGCAAGTTTTTGGCGAATC
CirY         ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGTAAGTTTTTGGCGAATC
LcLL         ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGTAAGTTTTTGGCGAATC
ChBL         ATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAACGCAAGTTTTTGGCGAATC
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**Fig. 4** Alignment showing single nucleotide polymorphism (SNP) at position 1204 (boxed) in the rDNA sequence derived from strain LcLL (GenBank no. HM067755). Note that the identity (C or T) of the base at this position is the same in both rRNA operons of a given genome; T for strains CPh and PoaS, and C for strains FesY and GaiY. GenBank accession numbers of rDNAs from the strains are listed in Table 1

**Table 2** Single nucleotide polymorphisms (SNPs) in ribosomal protein (rp) gene sequences distinguishing phytoplasma strain LcLL and subgroup rpI-C strains from ChBL and other non-subgroup rpI-C strains[a]

| Phytoplasma | rpI | SNP position[b] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| strain | subgroup | 137 | 263 | 528 | 598 | 690 | 918 | 986 | 1009 | 1098 | 1100 | 1104 |
| **LcLL** | **C** | **A** | **T** | **T** | **A** | **G** | **T** | **A** | **A** | **T** | **T** | **T** |
| CPh | C | A | T | T | A | G | T | A | A | T | T | T |
| CPh-L | C | A | T | T | A | G | T | A | A | T | T | T |
| GaiY | C | A | T | T | A | G | T | A | A | T | T | T |
| PoaS | C | A | T | T | A | G | T | A | A | T | T | T |
| FesY | C | A | T | T | A | G | T | A | A | T | T | T |
| KVE | C | A | T | T | A | G | T | A | A | T | T | T |
| KVG | C | A | T | T | A | G | T | A | A | T | T | T |
| SGP | C | A | T | T | A | G | T | A | A | T | T | T |
| **ChBL** | **O** | **C** | **C** | **C** | **C** | **A** | **C** | **C** | **G** | **C** | **G** | **C** |
| BB | A | C | C | C | C | A | C | C | G | C | G | C |
| HYDP | A | C | C | C | C | A | C | C | G | C | G | C |
| MIAY | B | C | C | C | C | A | C | C | G | C | G | C |
| AVUT | B | C | C | C | C | A | C | C | G | C | G | C |
| Btsv1CarH11 | B | C | C | C | C | A | C | C | G | C | G | C |
| AV2192 | B | C | C | C | C | A | C | C | G | C | G | C |
| PaWB | D | C | C | C | C | A | C | C | G | C | G | C |
| BBS3 | E | C | C | C | C | A | C | C | G | C | G | C |
| IOWB | F | C | C | C | C | A | C | C | G | C | G | C |
| STRAWB2 | J | C | C | C | C | A | C | C | G | C | G | C |
| MBS | L | C | C | C | C | A | C | C | G | C | G | C |
| GD1 | M | C | C | C | C | A | C | C | G | C | G | C |
| Btsv4Ca2a | N | C | C | C | C | A | C | C | G | C | G | C |
| ACLR | N | C | C | C | C | A | C | C | G | C | G | C |

[a] Broken horizontal line separates LcLL and subgroup 16SrI-C(rpI-C) phytoplasmas (above broken line) from ChBL and phytoplasmas classified in other subgroups of 16S rDNA RFLP group 16SrI and ribosomal protein gene RFLP group rpI. Data for strains reported in this communication are in bold. GenBank nos. of rp gene sequences from the strains are: AVUT, AY264855; IOWB, AY264859; Btsv4Ca2a, AY183683; the remaining GenBank nos. are listed in Table 1 and Fig. 6

[b] Base position in rp gene sequence from phytoplasma LcLL (GenBank no. HM067757). SNPs at positions 137 and 263 are located in the *rplV* gene; the remaining SNPs are located in the *rpsC* gene. In the proposed convention for designation of SNPs (This paper), the first SNP listed for strain LcLL is designated SNPrp[HM067757]137(A), and that for ChBL is designated SNPrp[HM067756]137(C)

## Phylogenetic analysis of 16S rRNA and rp gene sequences

Phylogenetic analysis of 16S rRNA gene sequences yielded clustering of the ChBL rDNA with subgroup 16SrI-C *rrnA* sequences, and clustering of strain LcLL rDNA with 16SrI-C *rrnB* sequences, confirming the close relatedness of the two newly described 16S rDNAs with subgroup 16SrI-C (Fig. 5). Not unexpectedly, the bootstrap values are

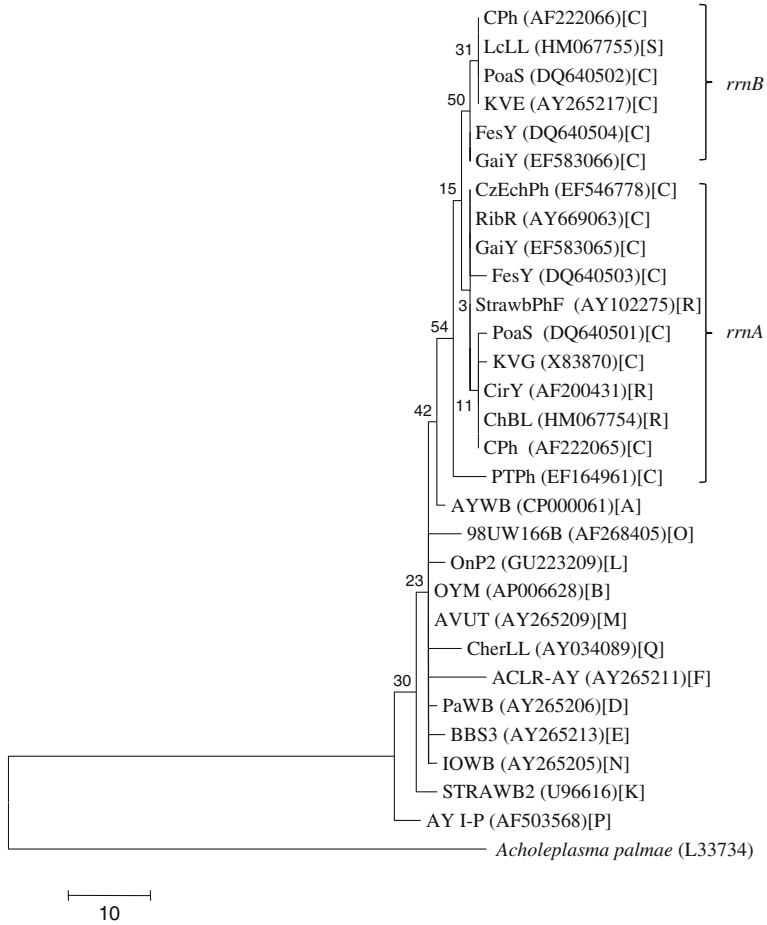


**Fig. 5** Phylogenetic tree of 16S rRNA gene sequences from phytoplasma strains representing diverse subgroups in group 16SrI. Classification of 16S rDNA subgroups are given as *capital letters in brackets*. GenBank accession numbers of nucleotide sequences used for construction of the tree are given in parentheses

low because the sequences mentioned are highly similar, but it should be noted that group and subgroup are based on restriction (site) profiles, and not phylogenetic position.

Phylogenetic analysis of rp gene sequences yielded clustering of group rpI strains according to rp subgroup (Fig. 6). The rp gene sequence from LcLL phytoplasma clustered with that of CPh and other 16SrI-C strains classified in rp subgroup I-C (rpI-C), indicating a close relatedness between LcLL and CPh. By contrast, the rp gene sequence from ChBL phytoplasma formed a branch that was surprisingly distinct from that of phytoplasmas classified in subgroup 16SrI-C (rpI-C), as well as from that of PaWB phytoplasma, supporting the concept that ChBL represents a lineage that is distinct from CPh, LcLL, and PaWB. Subgroup 16SrI-R strains StrawbPhF and CirY may also contain rp gene sequences similar to that of strain ChBL.

## Discussion

Phytoplasma genomes, that have thus far been completely sequenced or otherwise examined sufficiently, contain two sets of ribosomal RNA genes (Bai et al. 2006; Davis et al. 2003; Jomantiene et al. 2002a; Liefting et al. 1996; Marcone et al. 2000; Schneider and Seemüller 1994). RFLP analysis of PCR products has revealed composite RFLP profiles consistent with 16S rRNA interoperon sequence homogeneity in some strains; whereas, such analyses have yielded no evidence of rRNA interoperon sequence heterogeneity for many other phytoplasmas, consistent with a long prevailing view that the multiple copies of rRNA genes in a bacterial genome were identical or nearly so (Asai et al. 1999; Yap et al. 1999). Although rRNA interoperon sequence heterogeneity was first revealed in some phytoplasma genomes by composite RFLP patterns of PCR-amplified 16S rDNA (Davis and Dally 2001; Davis

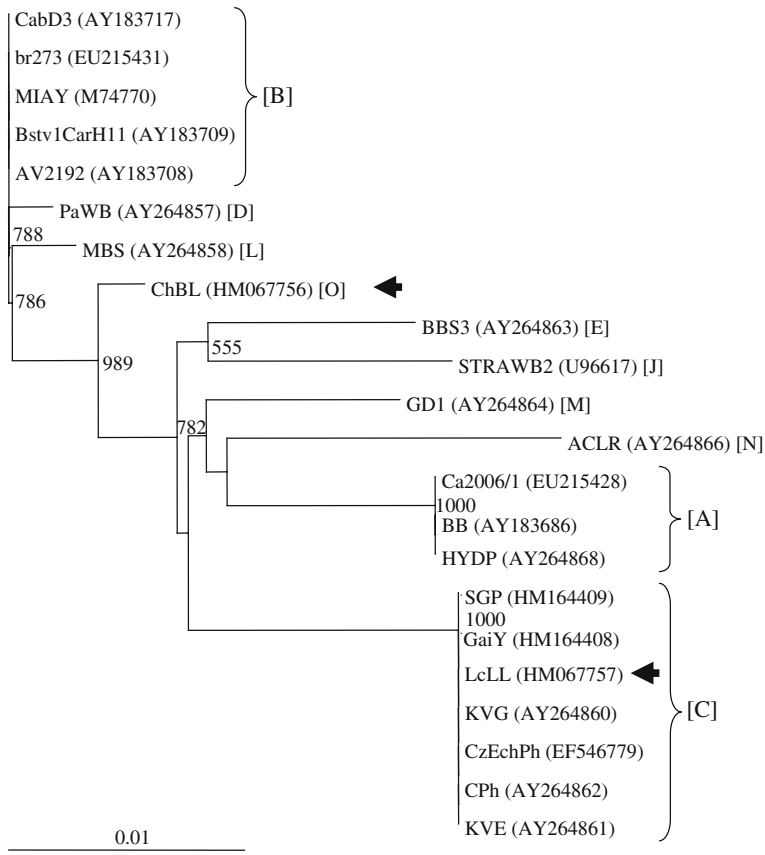


**Fig. 6** Phylogenetic tree of ribosomal protein (rp) gene sequences from phytoplasma strains representative of group 16SrI. Classification of rp subgroups are given as *capital letters in brackets*. GenBank accession numbers of nucleotide sequences used for construction of the tree are given in parentheses. Note that LcLL (arrow) but not ChBL (arrow) phytoplasma clustered with the members of subgroup rpI-C

et al. 2003; Jomantiene et al. 2002a; Lee et al. 1993; Liefting et al. 1996; Marcone et al. 2000), RFLP analysis is often not performed on PCR products potentially containing products from both such operons, and for a number of phytoplasma genomes, the nucleotide sequence has been reported for only one of the two rRNA operons, leaving open the potential for misidentifying phytoplasmas that may represent diverse lineages.

This communication reports infection of sour cherry and lilac plants by two mutually distinct phytoplasma lineages, 16SrI-R and 16SrI-S, both of which possess 16S rDNA having affinity with one or the other rRNA operon of CPh phytoplasma. Interestingly, it is possible that subgroup lineages 16SrI-R and 16SrI-S were previously encountered but went unrecognized. For example, although subgroup 16SrI-(R/S)C strains are characterized by interoperon sequence heterogeneity of 16S rDNA, yielding composite RFLP patterns of mixed rDNA amplicons (from two sequence-heterogeneous operons) in actual gel electrophoretic analyses, the nucleotide sequence of 16S rDNA from just a single rRNA operon has been reported for a number of phytoplasma strains classified in subgroup I-(R/S)C by the respective sequence authors (Table 1). Composite RFLP patterns have been documented by actual gel electrophoresis of digested PCR products for CPh, KVG (KV), KVE, KVM, GaiY, FesY, *Ribes rubrum* phytoplasma, SGP, and CzEchPh, confirming that these strains each contain two sequence-heterogeneous 16S rDNAs, even though in some cases the nucleotide sequence from only one of the two rRNA operons per genome has been reported. Neither nucleotide sequences from both rRNA operons nor composite, actual gel electrophoretic RFLP patterns (characteristic of CPh, for example) have been documented for some other strains classified in subgroup 16SrI-C (Table 1). The present study raises the question of whether such strains might not be members of subgroup 16SrI-(R/S)C, but rather members of subgroup 16SrI-R or 16SrI-S. Understanding the origins and evolutionary relationships of these lineages awaits further research, but findings in this communication, especially the

rDNA and rp gene sequences in ChBL phytoplasma compared to those of CPh, raise the possibility of rRNA or rp gene horizontal transfer.

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