‘Candidatus Phytoplasma lycopersici’, a phytoplasma associated with ‘hoja de perejil’ disease in Bolivia

Yaima Arocha,1 Olivia Antesana,2 Ernesto Montellano,2 Pablo Franco,3 G. Plata4 and Phil Jones5

1National Centre for Animal and Plant Health (CENSA), Apdo 10, San José de Las Lajas, Havana, Cuba
2Ladiplantas Community Plant Clinic, Comarapa, Bolivia
3CIAT, Santa Cruz, Bolivia
4PROINPA, Cochabamba, Bolivia
5Global Plant Clinic, Plant Pathology and Microbiology Department, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK

New diseases known locally as ‘hoja de perejil’ of tomato (Lycopersicon esculentum Mill) and ‘brotes grandes’ of potato (Solanum tuberosum L.) were first recognized in surveys of production fields in Bolivia during 2000–2003. Alfalfa (Medicago sativa) witches’ broom and little leaf diseases of native weeds Morrenia variegata and mora-mora (Serjania perulacea) were also identified near to production fields. Phytoplasma aetiology was attributed to each of these diseases following detection and initial identification of aster yellows group (16SrI) phytoplasmas in all five diseased plant species. While potato, alfalfa and mora-mora plants contained indistinguishable 16SrI-B strains, ‘hoja de perejil’ (THP) and morrenia little leaf (MVLL)-associated phytoplasma strains shared 97.5 % 16S rRNA gene sequence similarity with ‘Candidatus Phytoplasma asteris’ and related strains and 95 % similarity with all other ‘Candidatus Phytoplasma’ species. Phylogenetic analysis of 16S rRNA gene sequences indicated that the THP and MVLL phytoplasmas represent a novel lineage within the aster yellows (16SrI) group and, on the basis of unique 16S rRNA gene sequences, we propose that THP and MVLL phytoplasmas represent ‘Candidatus Phytoplasma lycopersici’, with THP as the reference strain.

INTRODUCTION

‘Hoja de perejil’ (parsley leaf) of tomato (THP) and ‘brotes grandes’ (big bud) of potato (PBG) are two new phytoplasma diseases reportedly affecting these crop species (Jones et al., 2005a, b). PBG was associated with phytoplasmas of 16SrI group, ‘Candidatus Phytoplasma asteris’ and related strains (Jones et al., 2005a), while phytoplasmas in association with THP and nearby Morrenia variegata with little leaf (MVLL) disease were tentatively identified as representatives of a novel phytoplasma group (Jones et al., 2005b).

Tomato plants affected with ‘hoja de perejil’ disease are characterized by adventitious sprouting of axillary buds and rapid elongation of side shoots showing small and fern-like leaves, becoming large bushy plants as the season progresses.

Big bud diseases of tomato have been attributed to at least four distinct phytoplasma strains worldwide (Lee et al., 1993; Marcone et al., 1997; Davis et al., 1997b; Shaw et al., 1993). These include aster yellows subgroup (16SrI-A), peanut witches’ broom subgroup (16SrI-E), clover proliferation subgroup (16SrVI-A) and stolbur subgroup (16SrXII-A) strains. A tomato yellow is associated with a subgroup 16SrI-B strain (Okuda et al., 1997).

Surveys in Italy have also revealed the presence of 16SrV group, ‘Candidatus Phytoplasma ulmi’, in tomato plants showing stunting, yellowing, proliferation of lateral shoots,
adventitious roots and a reduced number of fruits (Del Serrone et al., 2001).

The present work reports results on the molecular character-

ization of phytoplasmas associated with THP and PBG

from surveys carried out during 2000–2003 in potato and
tomato fields located in Sucre and Santa Cruz provinces in

Bolivia. Results of a study involving 16S rRNA gene PCR

amplification, RFLP, sequence and phylogenetic analysis of

THP and PBG compared with 39 other reference phyto-

plasmas representing the current classification scheme are

reported, including the proposal of THP as a novel

‘Candidatus Phytoplasma’ species.

METHODS

Disease survey locations. Tomato and potato production fields in
east-central Bolivia were the focus of disease surveys conducted
during 2000–2003. Survey sites included both tomato and potato
fields located in the Chilon, Saipina, Pulquina and Comarapa
valleys in Santa Cruz province and tomato fields at Limon Pampa, Rio
Chico, in Sucre province.

Plant samples and reference phytoplasma strains. During the
course of surveys, leaf samples for analysis were collected from a total
of 44 diseased plants. These included 11 potato plants with typical
PBG symptoms (Jones et al., 2005b), 13 tomato plants with THP
symptoms (Jones et al., 2005b), eight Morrenia variegata (Griseb.)
T. Mey. vines (Jones et al., 2005b) and six mora-mora (Serjania
perulacea Radlk.) vines, both with little leaf symptoms, as well as six
alfalfa (Medicago sativa L.) plants with witches’ broom and little leaf.
Morrenia and mora-mora plants were found growing in hedgerows
near tomato crops near San Rafael, Santa Cruz, and potato fields in
La Tranca, Sucre, respectively. Leaf samples from nine symptomless
(symptomatic healthy) plants, including at least one plant of each
species, were also collected for comparative purposes.

Phytoplasma strains belonging to five 16S rRNA RFLP groups (Lee
et al., 1998) were included in the study for reference purposes. The
strains and their respective group affiliations are as follows. Stolbur
(STOL), grapevine yellows (VK) and Bois Noir (BN) (16SrXII, stolbur
group) were kindly provided by Dr Giuseppe Firraro. American aster
yellows (AYY) and apple chlorotic leaf roll (ACLR) (16SrI, aster
yellows group), apple proliferation (AP) (16SrX, apple proliferation
group), faba bean phyllody (FBP) (16SrII, peanut witches’ broom
group) and vaccinia witches’ broom (VWB) (16SrIII, X-disease group)
were all obtained from the phytoplasma collection at Rothamsted
Research, UK.

DNA amplification and RFLP analysis. DNA was extracted from
1.5 g samples of leaf tissue by the method of Doyle & Doyle (1990)
and used as the template for a nested PCR assay primed by phyto-
plasma universal primer pairs P1 (Deng & Hiruki, 1991) and P7
(Schneider et al., 1995) for the first reaction and R16F2n/R16R2
(Gundersen & Lee, 1996) for the nested reaction, as described pre-
viously (Arocha et al., 2005). Nested PCR products were analysed by
single-restriction endonuclease digestion with HaeIII, Hinfl (both
from Boehringer Mannheim), Alul, Rsal (both from Sigma), Sau3AI
(Amersham Biosciences) or KpnI (Promega) according to the manu-
facturers’ instructions. Digestion products were electrophoresed
through 2.5–3 % agarose gels and visualized after staining with
ethidium bromide by UV transillumination. The resulting RFLP
patterns were compared with those described previously (Schneider
et al., 1995; Gibb et al., 1996; Davis et al., 1997a; Lee et al., 1998, 2004;
Arocha et al., 2005).

DNA sequencing. P1/P7 amplicons were purified on spin columns
(QIAquick gel extraction kit; Qiagen) and sequenced in the forward
and reverse directions with primer pair P1/P7 by the Sequencing
Service of the School of Life Sciences, University of Dundee, UK
(http://www.dnaseq.co.uk), using Applied Biosystems Big-Dye ver-
sion 3.1 chemistry on a 3730 automated capillary DNA sequencer.

16S rRNA gene sequence similarity, putative restriction sites
and phylogenetic analysis. Comparison of 16S rRNA gene sequences
of Bolivian phytoplasmas with those archived in the nucle-
otide sequence database of the National Center for Biotechnology
Information (NCBI) (Supplementary Table S1) was performed using
the BLASTN program (Altschul et al., 1990). Sequence similarities
were evaluated and putative restriction site maps of 16S rRNA genes
were constructed using the RESearch program (Rothamsted Research).
RFLP patterns of 16S rRNA genes were compared and similarity
coefficients (F) for each pairwise comparison were calculated from
virtual (in silico) restriction site analysis of rRNA gene sequences with
enzymes Alul, Rsal, Sau3AI, KpnI and Hinfl, according to the
previously described formula F=2Nxy/(Nxx+Nyy) (Lee et al., 1998; Nei
& Li, 1979), in which x and y are two given phytoplasmas under
investigation, Nxx and Nyy are the total number of fragments
resulting from enzymic digestion in strains x and y, respectively, and
Nxy is the number of fragments shared by the two strains.

Phytoplasma 16S rRNA gene sequences were aligned and edited using
the multiple alignment program CLUSTAL W (Thompson et al., 1994)
included in the MEGA program version 3.1 (Kumar et al., 2004). A
phylogenetic analysis of the aligned sequences was performed using
MEGA version 3.1 and a tree was constructed by the neighbour-joining
method. Acholeplasma palmae ATCC 49389T was used as an outgroup
root to the tree. Bootstrap analyses (1000 replicates) were performed
to estimate the stability and support for the inferred clades.

RESULTS

DNA amplification, analysis of RFLP profiles and
putative restriction sites in phytoplasma rRNA
operon sequences

Nested PCR products of about 1250 bp in size were
amplified from all samples of each diseased plant species,
whereas no products were amplified from any of the
symptomatic, presumably healthy plants included during
this study. Sequence analysis of amplification products by
comparison with the NCBI nucleotide sequence database
confirmed their phytoplasma origin. As such, S. perulacea and
M. variegata represent newly identified phytoplasma
hosts.

Based on the combined RFLP data, no differences were
evident between THP and MVLL; thus they appear to be
very similar or identical strains. HaeIII profiles of 120 and
1000 bp (data not shown) were yielded by all positive PCR
samples, confirming the presence of phytoplasma DNA.
Hinfl, Alul and Rsal profiles (Fig. 1) shown by THP and
MVLL were similar to those of AAY, STOL, VK and the
remaining Bolivian phytoplasmas, classifying them all as
members of group 16SrI. However, the KpnI and Sau3AI
profiles collectively differentiated THP and MVLL from the
rest of the phytoplasmas analysed as a novel phytoplasma
related to the 16SrI group.
Putative restriction maps of rRNA operon sequences support results obtained from actual digestion of phytoplasma rRNA gene products amplified by PCR. The differences in the KpnI profile between THP and MVLL on the one hand and the remaining Bolivian phytoplasmas are reflected by the addition and displacement of KpnI sites in the 16S rRNA gene sequence of THP and MVLL when compared with those of PBG, AlfWB and MMLL, besides AshWB, BD, AAY, ‘Ca. Phytoplasma asteris’ and ‘Ca. Phytoplasma fragariae’ phytoplasmas, except for ACLR (Fig. 2). Similarly, the differences in the Sau3AI RFLP profile exhibited by THP and MVLL when compared with the Bolivian and 16SrI group phytoplasmas are reflected by the downward arrows in the 16S rRNA gene sequences of the Bolivian and 16SrI group phytoplasmas in Fig. 2.

In addition, comparisons of putative restriction sites between the THP and MVLL 16S rRNA gene sequences with that of ‘Ca. Phytoplasma asteris’ exhibited additional Rsal and AluI sites for the latter and one additional Hinfl site for THP and MVLL. Comparison with the closest relative, ‘Ca. Phytoplasma fragariae’, showed one and two additional AluI sites for the latter when compared with ‘Ca. Phytoplasma asteris’ and with THP and MVLL, respectively. The 16S rRNA gene sequence of ‘Ca. Phytoplasma fragariae’ also lacked the Rsal and AluI sites present in ‘Ca. Phytoplasma asteris’, as well as the additional Hinfl site of the 16S rRNA gene of THP and MVLL.

**Similarity coefficients**


**Sequence similarity**

Comparisons of 16S rRNA gene sequences revealed that those of novel phytoplasmas THP and MVLL were co-identical and shared only 96.5 % similarity with those of Bolivian strains PBG, AlfWB and MMLL, 97.26 % similarity with that of BD and AAY, 96 % with that of AAY and ACLR, 97.5 % with that of ‘Ca. Phytoplasma asteris’ and less than 95 % with the remaining ‘Candidatus Phytoplasma’ species. Sequence similarity values are tabulated in Supplementary Table S3.

**Phylogenetic analysis**

The phylogenetic relatedness of THP and MVLL and the rest of the phytoplasmas analysed, including A. palmae, is depicted in Fig. 3. The tree is in good agreement with previous findings according to the bootstrapping values that support most branches, indicating the robustness of the branching order (Arocha et al., 2005; Lee et al., 2006). THP and MVLL were included in the same branch corresponding to PBG, AlfWB and MMLL and the rest of the phytoplasmas of group 16SrI. In addition, the phylogenetic

**Sequences unique to phytoplasmas in the 16S rRNA gene of the new phytoplasmas identified**

The 16S rRNA gene sequence from THP and MVLL was aligned with sequences from 39 phytoplasmas representing the current phytoplasma grouping (IRPCM, 2004), including those of the remaining Bolivian phytoplasmas.

This analysis revealed that THP, MVLL and the remaining Bolivian phytoplasmas contained the six sequences previously reported to be unique to phytoplasmas (Gundersen et al., 1994); however, signature sequences unique to THP and MVLL distinguished them from the other phytoplasmas analysed.

Unique sequences not present in the 16S rRNA gene sequences of other phytoplasmas were found in those of THP and MVLL: 5’-CTTA-3’ at positions 175–178, 5’-AATGGT-3’ at positions 198–203, 5’-ATA-3’ at positions...
DISCUSSION

Based on RFLP results, sequence similarity coefficients and phylogenetic analysis of 16S rRNA gene sequences, THP, MVLL and the remaining Bolivian phytoplasmas were definitely placed as members of the 16SrI group, 'Ca. Phytoplasma asteris' and related strains. However, both Sau3AI profiles and putative restriction site analysis distinguished THP and MVLL from PBG, AlfWB and MMLL and the other phytoplasmas analysed. The 16S rRNA gene sequence similarity of THP and MVLL in comparison with those from the remaining Bolivian phytoplasmas was 96.5% and ranged from 96–97.26% in comparison with the other members of the 16SrI group.

THP and MVLL showed clear differences in Rsal and Sau3AI putative restriction sites when compared with 'Ca. Phytoplasma asteris', which also belongs to the 16SrI group. Comparisons of 16S rRNA gene sequences of THP and MVLL reached 97.5% when compared with 'Ca. Phytoplasma asteris'.

The International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of Mollicutes has recommended the inclusion of 16S rRNA gene sequences for any description of a novel mollicute species (Marcone et al., 2004a, b). Novel putative species of uncultured phytoplasmas may be described when their 16S rRNA gene
sequence (1200 bp) has $\leq 97.5\%$ similarity to any previously described 'Candidatus Phytomplasma' species (IRPCM, 2004).

Previous BLAST analysis of the 16S/23S rRNA intergenic sequence of THP and MVLL showed the highest similarity of 91 % with those of other phytoplasmas in the 16SrI group, ‘Ca. Phytomplasma asteris’ (Jones et al., 2005b). From our study, phylogenetic analysis showed that THP, MVLL and ‘Ca. Phytomplasma asteris’ share the same branch of phytoplasmas belonging to the 16SrI group. However, THP and MVLL exhibited 97.5 % 16S rRNA gene sequence similarity to ‘Ca. Phytomplasma asteris’ and 95 % or less when compared with the closest relative, ‘Ca. Phytomplasma fragariae’, and other previously described ‘Candidatus Phytomplasma’ species and phytoplasma strains representing other unnamed phytoplasma groups or subgroups. Differences have been also supported by means of 16S rRNA gene putative restriction maps and sequence similarity coefficients.

In addition, signature sequences not found in the 16S rRNA gene sequences of any other ‘Ca. Phytomplasma’ species described previously have been described from the 16S rRNA gene sequences of THP and MVLL.

We propose to designate to THP as the reference strain of a novel Candidatus, according to the scheme for assigning incompletely described prokaryotes to the provisional status ‘Candidatus’ implemented by the International Committee on Systematics of Prokaryotes (Murray & Stackebrandt, 1995). We propose that THP is designated ‘Candidatus Phytomplasma lycopersici’, with the following description.

‘Candidatus Phytomplasma lycopersici’ (N.L. n. Lycopersicon a botanical genus; N.L. gen. n. lycopersici of Lycopersicon esculentum, referring to the plant host, tomato) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. AY787136)]; oligonucleotide sequences of unique regions of the 16S rRNA gene: 5'-CTTA-3' (positions 175–178), 5'-ATA-3' (229–231), 5'-TGAGAA-3' (234–242), 5'-GAC-3' (302–305), 5'-TCT-3' (315–317), 5'-GCT-3' (334–336), 5'-ATG-3' (336–338), 5'-ACG-3' (413–415) and 5'-AGC-3' (434–436); P (Lycopersicon esculentum, phloem; M), DNA samples from these strains are available from the authors.

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REFERENCES


