'*Candidatus* Phytoplasma lycopersici', a phytoplasma associated with 'hoja de perejil' disease in Bolivia

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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 (16SrII-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,

Abbreviations: MVLL, *Morrenia variegata* little leaf; PBG, potato 'brotes grandes'; THP, tomato 'hoja de perejil'.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of '*Candidatus* Phytoplasma lycopersici' isolate Santa Cruz is EF199549.

Details of the phytoplasmas used to draw the phylogenetic tree, a matrix of similarity coefficients derived from RFLP and 16S rRNA gene sequence similarities are available as supplementary material with the online version of this paper.

adventitious roots and a reduced number of fruits (Del Serrone *et al.*, 2001).

The present work reports results on the molecular characterization 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 phytoplasmas 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, Río 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 (presumably 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 Firrao. American aster yellows (AAY) 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 phytoplasma 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 previously (Arocha *et al.*, 2005). Nested PCR products were analysed by single-restriction endonuclease digestion with *Hae*III, *Hinf*I (both from Boehringer Mannheim), *Alu*I, *Rsa*I (both from Sigma), *Sau*3AI (Amersham Biosciences) or *Kpn*I (Promega) according to the manufacturers' 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 version 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 nucleotide 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 *Alu*I, *Rsa*I, *Sau*3AI, *Kpn*I and *Hinf*I, according to the previously described formula $F = 2N_{xy} / (N_x + N_y)$ (Lee *et al.*, 1998; Nei & Li, 1979), in which x and y are two given phytoplasmas under investigation, N_x and N_y are the total of number of fragments resulting from enzymic digestion in strains x and y , respectively, and N_{xy} 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 palmarum* ATCC 49389^T was used as an outgroup to root 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 symptomless, 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. *Hae*III profiles of 120 and 1000 bp (data not shown) were yielded by all positive PCR samples, confirming the presence of phytoplasma DNA. *Hinf*I, *Alu*I and *Rsa*I 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 *Kpn*I and *Sau*3AI profiles collectively differentiated THP and MVLL from the rest of the phytoplasmas analysed as a novel phytoplasma related to the 16SrI group.

Fig. 1. RFLP analysis of 16S rRNA genes amplified by nested PCRs with enzymes *HinfI*, *KpnI*, *AluI*, *RsaI* and *Sau3AI*. (a) Lanes: 1, 100 bp ladder (MBI Fermentas); 2–4 and 14–16, PBG, AlfWB and MMLL; 5–6 and 17–18, THP and MVLL; 7 and 19, AAY; 8 and 20, ACLR; 9 and 21, STOL; 10 and 22, VK; 11 and 23, AP; 12 and 24, VWB; 13 and 25, FBP. (b) Lanes: 1, 100 bp ladder (MBI Fermentas); 2–4, PBG, AlfWB and MMLL; 5–6, THP and MVLL; 7, AAY; 8, ACLR; 9, STOL; 10, VK; 11, AP; 12, VWB; 13, FBP.

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 displacement of two *Sau3AI* sites in the 16S rRNA gene sequence of THP and MVLL, which are specified 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 *RsaI* and *AluI* sites for the latter and one additional *HinfI* 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 *RsaI* and *AluI* sites present in '*Ca. Phytoplasma asteris*', as well as the additional *HinfI* site of the 16S rRNA gene of THP and MVLL.

Similarity coefficients

The similarity coefficients of the RFLP patterns of the novel phytoplasma (Supplementary Table S2) derived from

putative restriction map analysis of 16S rRNA gene sequences using five restriction enzymes were 91–96 % with 16SrI phytoplasmas, including '*Ca. Phytoplasma asteris*', 96 % with '*Ca. Phytoplasma caricae*', 95 % with '*Ca. Phytoplasma japonicum*', 93 % with '*Ca. Phytoplasma australiense*', 92 % with '*Ca. Phytoplasma graminis*', 88 % with '*Ca. Phytoplasma fragariae*', 84 % with '*Ca. Phytoplasma americanum*', 77 % with '*Ca. Phytoplasma allocasuarinae*' and 82 % with '*Ca. Phytoplasma brasiliense*'. These results support recognition of THP and MVLL as a novel phytoplasma of the 16SrI group.

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 AshWB, 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

Fig. 2. Putative restriction sites of phytoplasma 16S rRNA gene sequences. Maps were generated using the REsearch program (Rothamsted Research) for comparison of recognition sites for the restriction endonucleases *AluI*, *HinfI*, *KpnI*, *RsaI* and *Sau3AI*. Downward arrows indicate sites that are common between the Bolivian phytoplasmas and the reference strains analysed. Frames enclose different putative restriction sites.

analysis indicated that THP and MVLL and the remaining Bolivian phytoplasmas have a common ancestor with '*Ca. Phytoplasma japonicum*', '*Ca. Phytoplasma australiense*', '*Ca. Phytoplasma graminis*', '*Ca. Phytoplasma caricae*', '*Ca. Phytoplasma fragariae*' and '*Ca. Phytoplasma americanum*' (Valiunas *et al.*, 2006).

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

Fig. 3. Phylogenetic tree of 16S rRNA gene sequences constructed by the neighbour-joining method showing relationships between the phytoplasmas identified in THP, MVLL, PBG, AlfWB and MMLL and reference phytoplasmas from GenBank. Numbers above or below branches are bootstrap values obtained for 1000 replicates. Branch lengths are proportional to the number of inferred character-state transformations. *A. palmarum* ATCC 49389^T was used as an outgroup. Abbreviations of phytoplasma names are defined in Supplementary Table S1.

229–231, 5'-TTGGAGGAA-3' at positions 234–242, 5'-CACG-3' at positions 302–305, 5'-TCT-3' at positions 315–317, 5'-GCT-3' at positions 334–336, 5'-TAT-3' at positions 336–338, 5'-TAC-3' at positions 413–415 and 5'-AGC-3' at positions 434–436.

DISCUSSION

Based on RFLP results, sequence similarity coefficients and phylogenetic analysis of 16S rRNA gene sequences, THP, MVLL and the remaining Bolivian phytoplasmas were definitively 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 *RsaI* 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 *Phytoplasma*' 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. Phytoplasma asteris*' (Jones *et al.*, 2005b). From our study, phylogenetic analysis showed that THP, MVLL and '*Ca. Phytoplasma 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. Phytoplasma asteris*' and 95% or less when compared with the closest relative, '*Ca. Phytoplasma fragariae*', and other previously described 'Candidatus *Phytoplasma*' 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. Phytoplasma*' 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 Phytoplasma lycopersici*', with the following description.

'*Candidatus Phytoplasma 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'-AATGGT-3' (198–203), 5'-ATA-3' (229–231), 5'-TTGGAGGAA-3' (234–242), 5'-CACG-3' (302–305), 5'-TCT-3' (315–317), 5'-GCT-3' (334–336), 5'-TAT-3' (336–338), 5'-TAC-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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