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A novel phytoplasma associated with witches' broom disease of *Ligustrum ovalifolium* in Turkey

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Abstract California privet (*Ligustrum ovalifolium* Hassk.) plants exhibiting leaf yellowing, witches' broom, dieback and decline symptoms were observed for two years (2010–2011) in three gardens at Adana region (Turkey). DNA isolated from symptomatic and healthy plants was used to amplify 16S rDNA fragments by direct and nested-PCR. Phytoplasmas were detected in 21 symptomatic plants, out of 30 samples collected, whilst no PCR amplifications were obtained from asymptomatic plants. BLAST analysis of the 16S rDNA showed that the phytoplasma found in *L. ovalifolium* from Turkey, denoted as Turkish Ligustrum witches' broom phytoplasma (TuLiWB), most closely resembled members of group 16SrII (peanut witches' broom group) and shared up to 92 % sequence identity. Based on in silico 16S rDNA RFLP analysis and automated calculation of the pattern similarity coefficient, TuLiWB showed molecular characteristics different from all previously described phytoplasma species to represent a new taxon. Similar indication also emerged from the phylogenetic tree which allocated it in a novel discrete subclade within the phytoplasma clade. This is the first report on the presence of a phytoplasma affecting *L. ovalifolium* and whether this novel phytoplasma is the same agent reported as a mycoplasma-like organism (MLO) and associated with witches' broom disease of Ligustrum in Korea (1989) is yet to be determined.

Keywords *L. ovalifolium* · Phytoplasma · PCR · Sequencing · Phylogenetic analysis

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Ligustrum ovalifolium L. (family *Oleaceae*), known commonly as "California Privet", is an ornamental widely used for edging in Turkey and in other countries of Europe, Asia, Australia and America (Agamirov 1989; Sawayanagi et al. 1999; Huche-Thelier et al. 2006).

During July 2010 and 2011, surveys were conducted in Adana region (southern Turkey) in three gardens planted with *L. ovalifolium* plants which showed small and discoloured leaves, short internodes, bushy growth, stunting and dieback. These symptoms seemed to expand over time and eventually led to decline and death of the plants. However, the symptoms encountered in the fields recalled those of Ligustrum witches' broom disease reported from Korea in 1984 by Chai and Kim (1989). According to this report, the disease was successfully transmitted by grafting to *L. ovalifolium*, *L. obtusifolium* and *L. japonicum* and by the leafhopper *Hishimonus sellatus* to *L. ovalifolium* and *L. obtusifolium*, but the nature of its agent was not determined.

In this study, a molecular investigation was carried out on symptomatic *L. ovalifolium* plants of Turkish origin for which a phytoplasma was suspected to be the causal agent.

Samples from seven symptomatic and three asymptomatic plants were collected from each of three gardens. DNA was extracted from fresh leaves according to

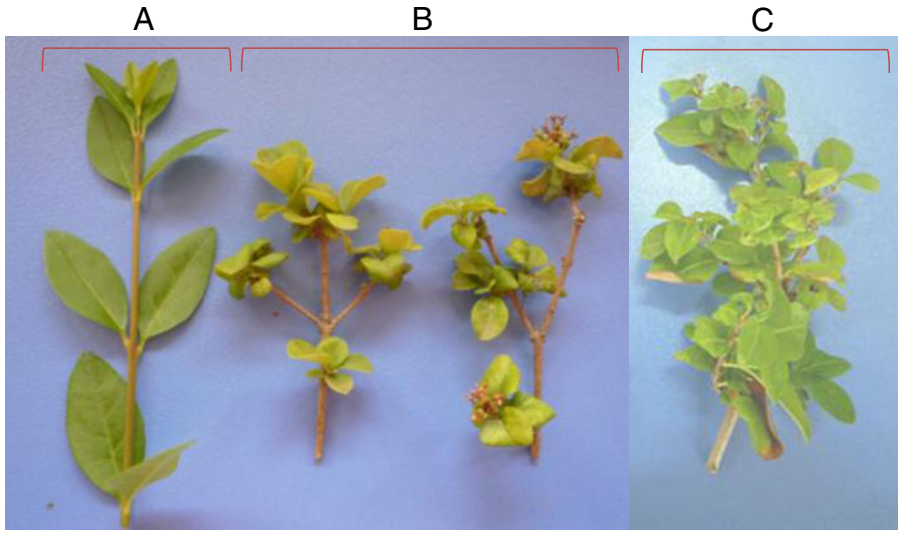


Fig. 1 California privet (*L. ovalifolium*) plants collected from affected gardens in Adana region showing typical phytoplasma symptoms: B: chlorotic curly leaf, short internodes; C: bushy growth and stunting. A: healthy plant

Ahrens and Seemüller (1992). All samples were subjected to two rounds of polymerase chain reaction assays (PCR and nested-PCR) for the amplification of the 16S rRNA gene, employing primers R16F1/R16R0 followed by primer pair R16F2n/R16R2 (Gundersen and Lee 1996). Ten µl of R16F2n/R16R2 amplicons were electrophoresed in 1 % agarose gel in 1xTBE buffer (67 mM Tris-HCl, 22 mM boric acid, 10 mM EDTA, pH 8.0) together with 100 bp DNA marker (Fermentas, Milano, Italy), stained with ethidium bromide and photographed under UV at 312 nm using a transilluminator. No PCR-positive reactions were obtained from the nine asymptomatic plants (Fig. 1a). The presence of phytoplasmas, hereafter refereed as "Turkish Ligustrum witches' broom" phytoplasma (TuLiWB), was detected in 21 symptomatic *L. ovalifolium* plants (Fig. 1b,c).

Ten 16SrDNA amplicons resulting from nested-PCR assay and originating from 10 different infected plants were selected for sequencing. Accordingly, two different DNA clones for each PCR amplicon product were ligated into StrataClone™ PCR Cloning vector pSC-A (Stratagene, La Jolla, CA, USA), cloned into *Escherichia coli* DH5 or SoloPACK cells, and automatically sequenced from both sides at the insertion site (Primm, Milano, Italy) (Fig. 2).

Search for DNA homologies in Genbank database was done with BLASTn (Altschul et al. 1990) and nucleotide sequences were aligned using CLUSTALX

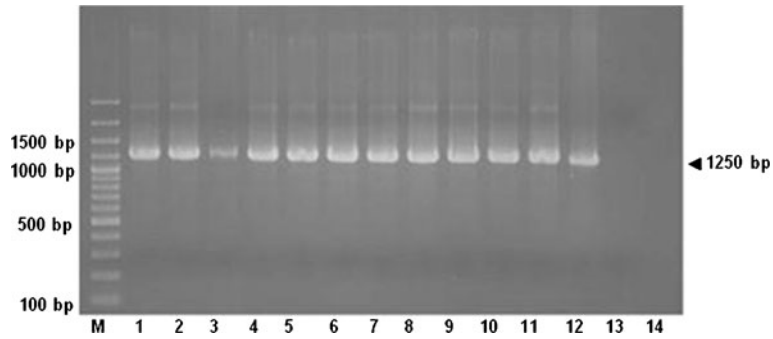


Fig. 2 Electropherogram showing nested-PCR amplicons products (1,250 bp), amplified by R16F2n/R16R2 primers, from *L. ovalifolium* infected plants and phytoplasma used as positive controls for PCR reactions and references to actual-RFLP assays. Lane M: Molecular marker DNA XIV (Fermentas, Milan, Italy), Lanes 1–5: *L. ovalifolium* plants infected with phytoplasma; Lane 6: 16SrII-C (*Chrotalaria phyllody* phytoplasma); Lane 7: 16SrII-C (*Faba bean phyllody* phytoplasma), Lane 8: 16SrII-D (*Tomato Big Bud* phytoplasma); Lane 9: 16SrII-E (*Picirs echioides phyllody* phytoplasma), Lane 10: *Cotton phyllody* phytoplasma); Lane 11: 16SrIII-A (*Western-X* phytoplasma); Lane 12: 16SrV-C (*Flavescence dorée* phytoplasma); Lane 13: healthy *L. ovalifolium*; lane 14: Water control reaction

1.8 (Thompson et al. 1997). Percentage of nucleotide sequence homology among phytoplasmas from different groups was calculated with GeneDoc (Nicholas et al. 1997).

Sequence analyses conducted on the 16S rDNA amplicons showed the presence of a signature sequence (5′-CAAGACGATGATGTGTAGCTGGACT-3′), at nucleotide position 126–159, which is unique to the phytoplasmal 16S rRNA gene. BLAST analyses showed that the 16S rDNA of the TuLiWB shares the highest identity (92.4 %) with sequences of *Picris echioides phyllody* phytoplasma from Italy (Y16393)

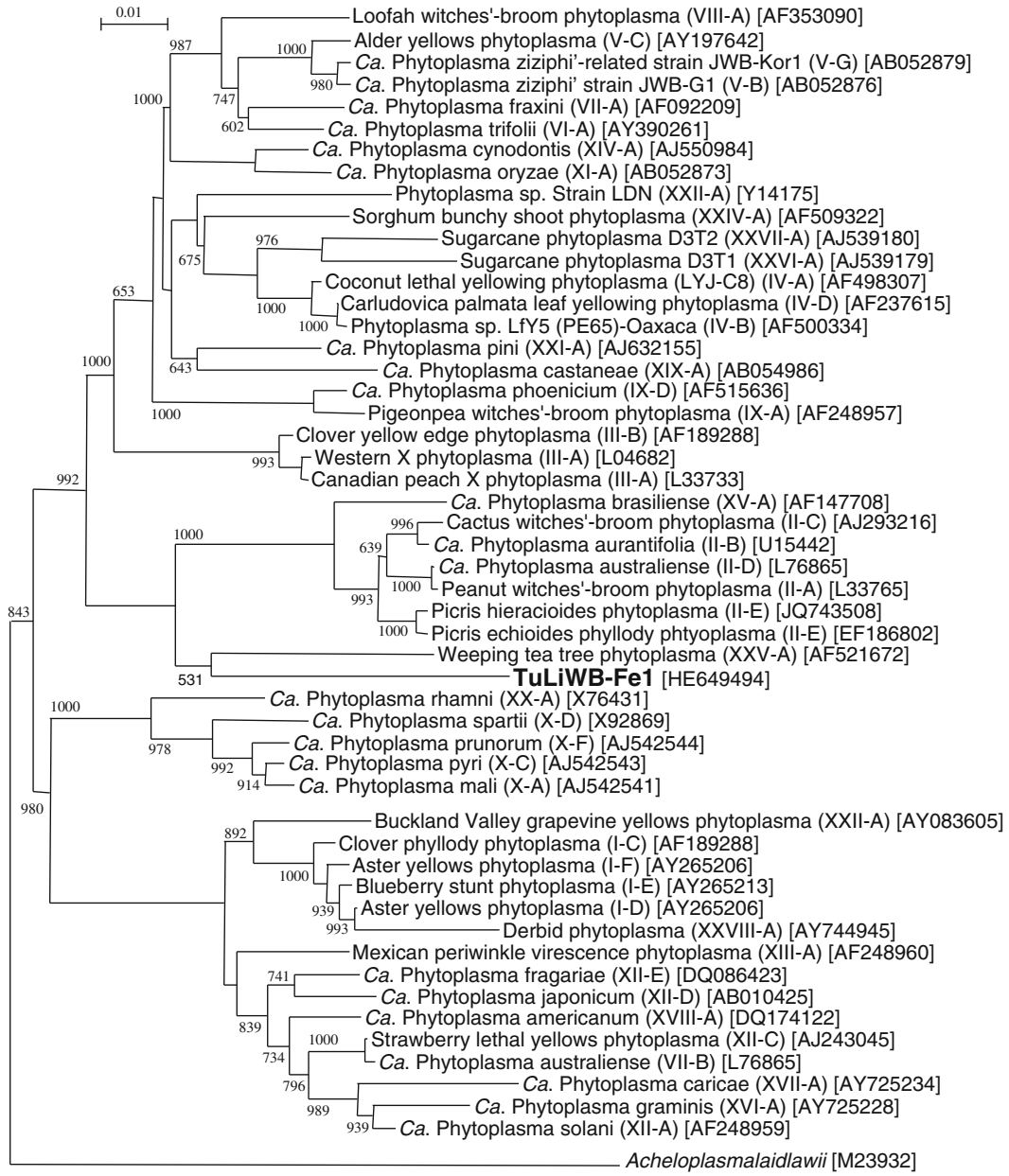


Fig. 3 Dendrogram, constructed by the neighbour-joining method, showing the phylogenetic relationship of TuLiWB-Fe1 with phytoplasmas in database, based on 16S rRNA gene sequences. Groups and subgroups of phytoplasmas are reported between brackets. GenBank accession numbers for sequences are reported between two braces [ ]. The reliability of the analysis was subjected to a bootstrap test with 1,000 repeats. Bar, 0.01 nucleotide substitutions per site. *Acheloplasma laidlawii* was used as an outgroup species to root the tree

and *Picris hieracioides* phytoplasma from Serbia (JF799094), both classified as subgroup 16SrII-E strains (Mitrović et al. 2011; Tolu et al. 2006; Marcone et al. 1997). All sequences of 16S rDNA of the TuLiWB phytoplasma were identical (100 % homology) when compared between them; therefore one sequence, denoted as TuLiWB-Fe1, was deposited in GenBank under the accession number HE649494.

A similarity coefficient was generated using ***i***PhyClassifier software (Wei et al. 2007) applied on the obtained 16S rDNA sequences to illustrate the relationship between the phytoplasma found in *L. ovalifolium* plants with phytoplasmas of other groups (Zhao et al. 2009). The closest pattern to TuLiWB-Fe1 was that of *Weeping tea tree witches' broom* phytoplasma, a 16SrXXV-A subgroup member (AF521672), with a similarity coefficient of 0.60, which is much lower than the conventional threshold (0.85), a value stabilized for the delineation of new and distinct phytoplasma 16Sr subgroup lineages (Wei et al. 2008). It is noteworthy mentioning that the sequence identity between this phytoplasma and TuLiWB-Fe1 was 91 %. Results of actual RFLP analyses, conducted on 16S rDNA nested-PCR products from infected plants, using the same endonucleases, were consistent with those of *in silico* analyses (data not shown). A unique RFLP pattern from all samples was generated thus indicating that a single phytoplasma species was infecting the *L. ovalifolium* plants.

A phylogenetic tree was constructed using the NJPLOT package with the neighbour-joining method (Perrière and Gouy 1996). Sequence of the 16S rRNA gene is widely used to detect the presence of phytoplasmas by PCR in infected plants and for determining of phylogenetic relationships and classification (Zhao et al. 2010; Firrao et al. 2007). On the basis of 16S rRNA sequences of all established groups of phytoplasmas (16SrI through 16SrXXVIII), TuLiWB-Fe1 was allocated close to *Weeping tea tree* phytoplasma in a cluster genetically separated from all groups, well distinct from members of 16SrII-A to F (Fig. 3), whose sequences had shown initially a closer correlation to TuLiWB-Fe1 in BLAST analyses.

The sequence divergence (8 %) was significant at the ribosomal RNA level between TuLiWB-Fe1 and all members of the 16SrII group, and in general with all phytoplasmas present in Genbank. All results of sequence analyses, computer-simulated RFLP analysis and sequence similarity scores determined with ***i***PhyClassifier confirmed the peculiarity of TuLiWB-Fe1, supporting the suggestion that this phytoplasma is a novel putative species. The low similarity coefficient (0.6) emerged from the comparison of TuLiWB-Fe1 with other phytoplasmas furthermore suggests the phytoplasma under study may represent a new 16Sr group.

Our finding is in line with the demarcation criteria recommended by the Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group (IRPCM, 2004), according to which a "*Candidatus* phytoplasma" species should refer to a single, unique 16S rRNA gene sequence (>1,200 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.

Notwithstanding results of Koch's postulates are not yet available, the detection of TuLiWB phtyoplasma in all 21 symptomatic *L. ovalifolium* plants tested and in none of 9 symptomless plants together with the absence of any other phytoplasma in symptomatic plants represent consistent proofs supporting the putative role of TuLiWB-Fe1 in the etiology of the witches' broom disease in Turkey. Whether it is also the same etiological agent of the disease earlier reported in Korea on Ligustrum in 1984 remains yet to be determined. This study reports for the first time the presence of LiWB disease in Turkey; thus measures should be taken to limit the possible expansion to nurseries of this disease, which can constitute a big threat to the landscape industry of the country.

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