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A subgroup 16SrIII-B phytoplasma identified in honeyweed plants with leaf deformation in Brazil

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Abstract *Leonurus sibiricus* (honeyweed) plants displaying small, shriveled and mildly chlorotic leaves were observed in commercial cassava fields. Based on nested PCR assays using a group-specific primer pair, computer-simulated RFLP analysis, similarity coefficient calculation and phylogenetic analysis, the phytoplasma identified in these plants was classified as a representative of subgroup 16SrIII-B. This is the first report of honeyweed as a host of phytoplasmas.

Keywords Leaf deformation · Mollicutes · Weed diseases · Yellows

Leonurus sibiricus, commonly called honeyweed, is a widespread and broadly adapted weed species of annual and perennial crops in all tropical Brazilian ecosystems (Lorenzi 2000). Phytoplasmas are wall-less prokaryotes that inhabit plant phloem and naturally transmitted by insects. These organisms are classified into groups and subgroups based on the diversity of 16S rRNA gene sequences (Bertaccini 2007).

Honeyweed plants exhibiting small, shrivelled and mildly chlorotic leaves, resulting in malformation of the upper parts of the plants, were observed in cassava fields in the state of São Paulo, Brazil (Fig. 1). These symptoms are typical of a phytoplasma infection. In a literature search, no reference was found to phytoplasma-associated diseases in honeyweed. Since this plant species commonly occurs in agricultural areas, this study explored whether the plant was a host for phytoplasmas. This study investigated the association of phytoplasmas with plants exhibiting disease, the molecular identity and classification of the phytoplasma, and the implications of honeyweed as a new host of phytoplasmas.

Total DNA extracts were obtained from the leaves of six symptomatic and two asymptomatic honeyweed plants growing spontaneously in cassava fields using the Dneasy Plant Mini Kit (Qiagen). DNA from chayote infected with a group 16SrIII-J phytoplasma (GenBank: AF147706) served as a positive control. Each phytoplasma identified in each plant was considered an isolate. Nested PCR assays were performed according to Lee et al. (1998) using the primers P1/Tint and R16F2n/R16R2. Identification by nested PCR was performed using P1/Tint, followed by the group-specific primers R16(III)F2/R16(III)R1 (Lee et al. 1994).

The 16S rDNA PCR product obtained from all isolates were cloned (Amaral Mello et al. 2011) and DNA fragments of three clones from each isolate sequenced, aligned and analyzed using the Bioedit program. A computer-simulated RFLP analysis of the 16S rRNA gene (Wei et al. 2007) was performed using a consensus sequence of the honeyweed phytoplasma and the sequences of other phytoplasmas belonging to distinct subgroups of the 16SrIII group. A phylogenetic tree was generated by the Neighbor-Joining method using the software MEGA4.

Nested PCR using the primer pair R16F2n/R16R2 generated 1.2 Kb DNA fragments, revealing the presence of phytoplasma in all symptomatic plants. The disease was termed honeyweed leaf deformation (HwLD). No amplification occurred when DNA from asymptomatic plants was used as a template in nested PCR. Identification using nested PCR with the specific primers R16(III)F2/R2 demonstrated the presence of a group16SrIII phytoplasma in all six symptomatic samples through the amplification of

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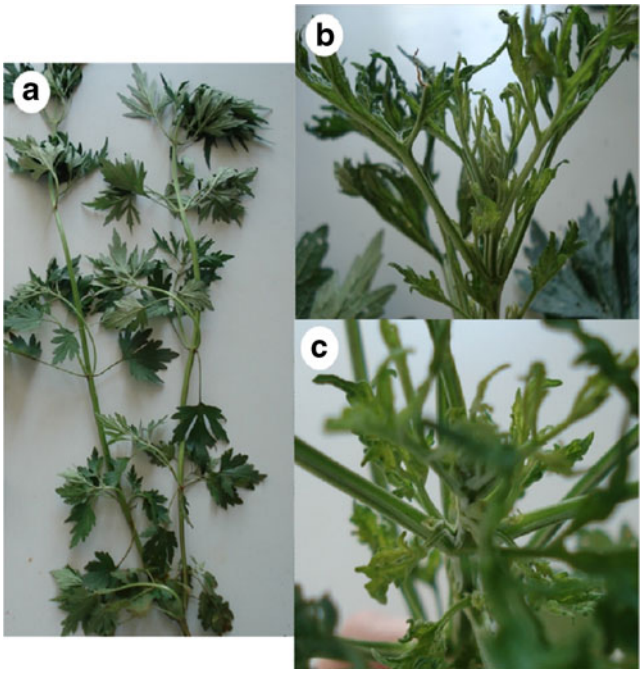

Fig. 1 **a** Honeyweed plants asymptomatic; **b** and **c** exhibiting small, shriveled and mildly chlorotic leaves resulting in malformation of the upper part of the plants

typical 0.8 Kb fragments. PCR of DNA from chayote plants, used as a positive control, amplified the expected 1.2 Kb and 0.8 Kb DNA fragments with the R16F2n/R16R2 primers and the specific primers, respectively.

The six isolates from honeyweed were indistinguishable in their RFLP patterns, which were produced by in silico digestions with 17 restriction enzymes. Based on this absence of polymorphism, a consensus sequence, designated HwLD-Br1 (1.246 Kb), was selected to represent the phytoplasma found in honeyweed plants and deposited in GenBank under accession number JX028239. The virtual RFLP analysis also revealed that the HwLD phytoplasma produced restriction patterns identical to those generated by the clover yellow edge phytoplasma (CYE) (AF 175304), which is the reference phytoplasma for subgroup 16SrIII-B (Fig. 2). Alignments of 16S rDNA sequences showed that the sequence similarity between the HwLD phytoplasma and other group16SrIII phytoplasmas was 99 %. When the HwLD phytoplasma was compared to CYE, a similarity coefficient equal to 1 was obtained. These results demonstrate that the honeyweed phytoplasma is affiliated with group 16SrIII and belongs specifically to subgroup 16SrIII-B.

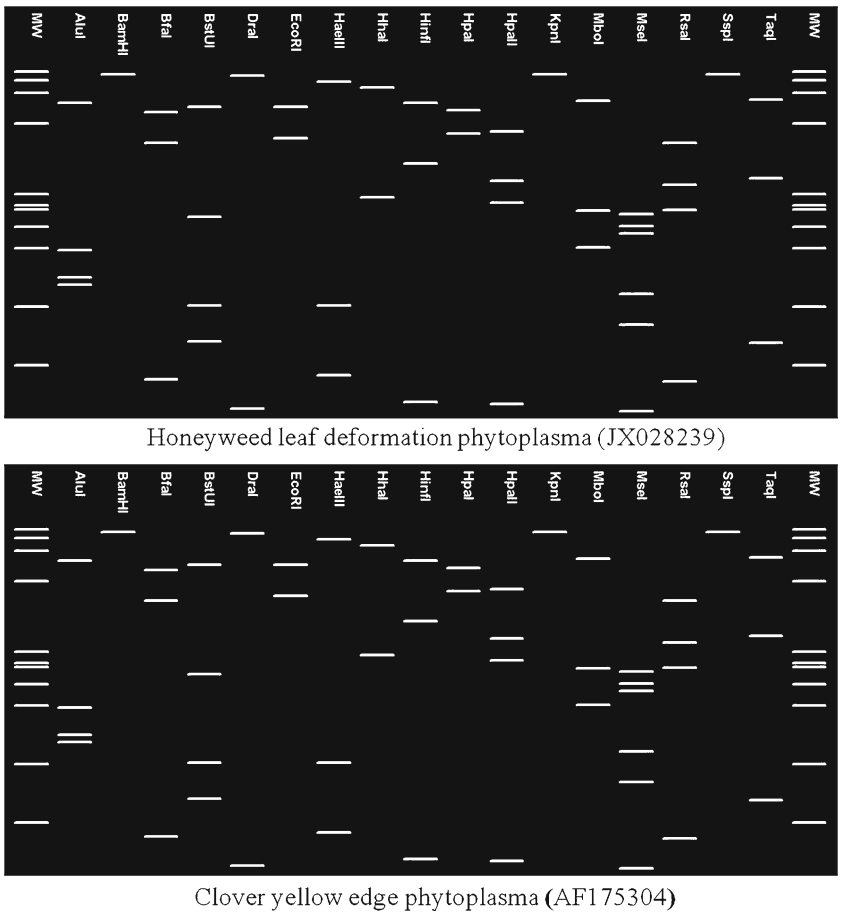

Fig. 2 Virtual RFLP patterns from in silico digestions of 16S rRNA gene R16F2n/R16R2 fragments from phytoplasma HwLD-Br1 and previously known phytoplasma belonging to 16SrIII-B subgroup. The following restrictions enzymes were used: *Alu*I, *BamH*I, *Bfa*I, *Bst*UI, *Dra*I, *EcoR*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I. MW, φX174RFHaeIII digest

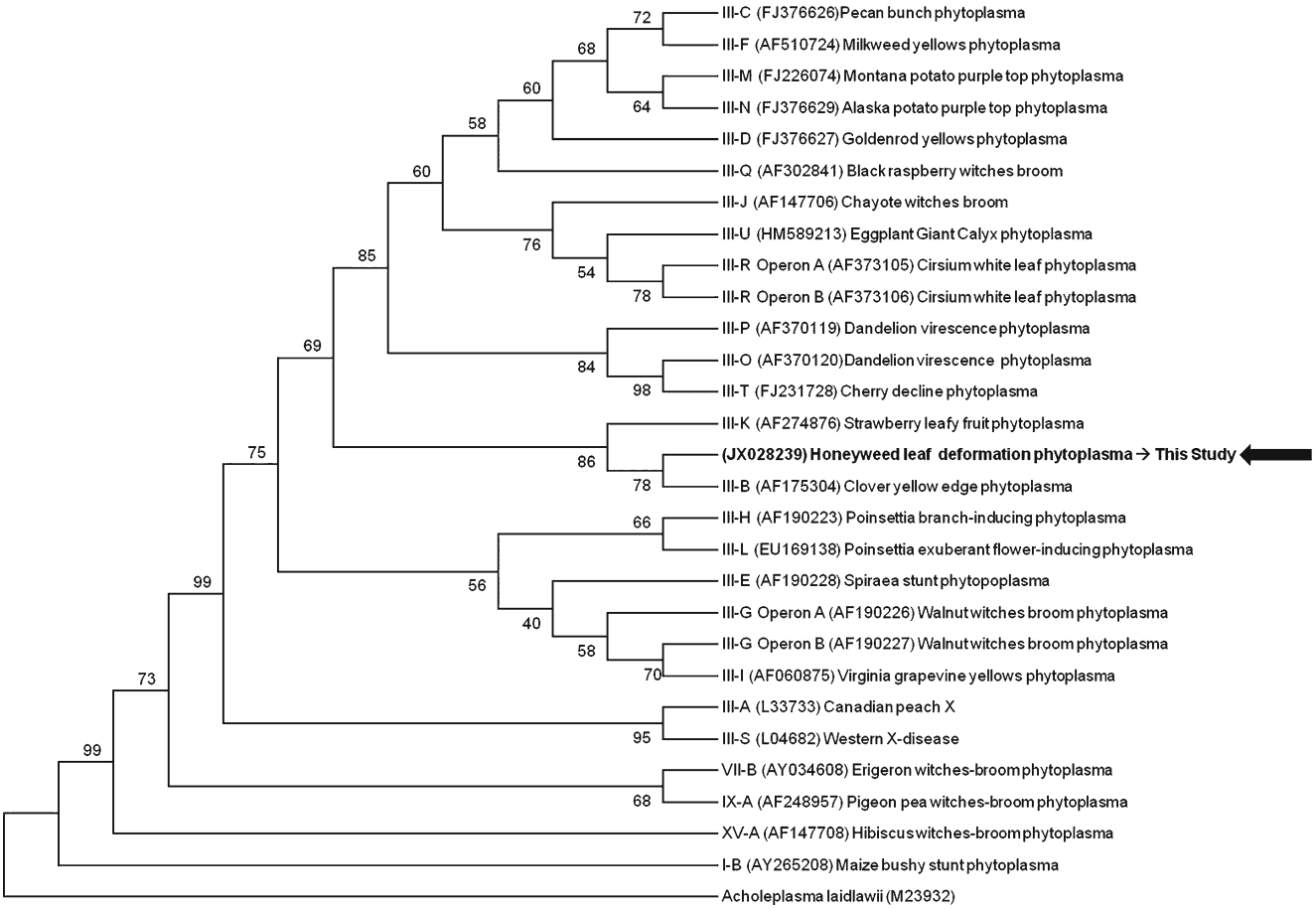

Fig. 3 Phylogenetic tree generated by sequences of 16S rDNA of the phytoplasma associated with Honeyweed leaf deformation (HwLD), phytoplasmas affiliated to distinct groups more frequently reported in Brazil and representatives of subgroups of 16Sr III. *Acholeplasma laidlawii* was included as outgroup and bootstrapping was performed 1,000 times

In agreement with these results, the phylogenetic tree generated using sequences from representatives of groups 16SrI, VII, IX and XV (the groups that are most frequently reported in Brazil), different subgroups of 16SrIII, and the HwLD phytoplasma showed that the latter organism was closely related to group 16SrIII, emerging from the same branch as the reference phytoplasma for subgroup 16SrIII-B (Fig. 3).

In Brazil, group16SrIII phytoplasmas have often been identified in numerous hosts, including apple, begonia, cabbage, chayote, China tree, eggplant, passion fruit, periwinkle, poinsettia, pumpkin, summer squash and tomato (Amaral Mello et al. 2011). Representatives of subgroups 16SrIII-B and 16SrIII-J are the most common and a new subgroup (16SrIII-U) was recently described (Amaral Mello et al. 2011). Honeyweed is indigenous to Asia but is widely distributed in the American and European continents, where it grows in various climates (Carneiro and Irgang 2005). In Brazil, this species is a widespread weed of crop fields, occurring in association with economically important species, including some of those listed previously, as well as other annual crop species such as coffee and fruit trees (Lorenzi 2000).

Our findings implicate honeyweed as a reservoir for a relevant group of phytoplasmas that occur in diverse Brazilian crops. Although the insects involved in transmission are unknown, the high frequency and host diversity of group16SrIII phytoplasmas indicate the presence of vectors. This preliminary investigation has ecological and epidemiological implications suggesting that honeyweed may serve as an alternative host and potential source of inoculum for cultivated species. In addition, to the best of our knowledge, our results reveal for the first time that honeyweed is a host of phytoplasmas.

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