Eur J Plant Pathol (2011) 130:457–462
DOI 10.1007/s10658-011-9773-7

Detection of '*Candidatus* Phytoplasma brasiliense' in a new geographic region and existence of two genetically distinct populations'

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Accepted: 2 March 2011 / Published online: 22 March 2011

Abstract '*Candidatus* Phytoplasma brasiliense', a phytoplasma taxon associated with hibiscus witches' broom disease was first described in 2001 in Brazil. In September 2007, a peach tree (*Prunus persica*) displaying yellowing symptoms reminiscent of phytoplasma infection was sampled in Guba region of Azerbaijan. A phytoplasma was detected in the diseased peach tree by nested PCR amplification of its 16S rDNA with universal primers for phytoplasmas. Phylogenetical analyses of the amplified 16S rDNA showed that the phytoplasma infecting the peach tree corresponded to '*Ca.* P. brasiliense', a species never reported in Euro-Mediterranean area. To set up a detection assay, cloning of a '*Ca.* P. brasiliense' DNA fragment was undertaken by comparative RAPD. The amplified *dna*K-*dna*J genetic locus was used to design a nested PCR assay able to amplify all '*Ca.* P. brasiliense' isolates of the subgroup 16SrXV-A without amplifying the related members of the group 16SrII. This assay also allowed confirming the first detection of '*Ca.* P. brasiliense' in diseased basil collected in south Lebanon.

Keywords Phloem-restricted bacteria · Nested-PCR, RAPD · Prunus persica

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Phytoplasmas are plant pathogenic bacteria belonging to the class *Mollicutes*, a group of wall-less microorganisms phylogenetically related to low G + C content, Gram-positive bacteria (Weisburg et al. 1989). They cause hundreds of diseases worldwide and are transmitted from plant to plant by sap-feeding hemipteran insects (Lee et al. 2000; Weintraub and Beanland 2006). As of today, the many diseases induced by phytoplasmas cannot be cured and the control of disease spread consist of implementing prophylactic measures, such as quarantine, destruction of infected plant material and pesticide treatment against the insect vectors. Implementing disease control requires the taxonomic characterization of the phytoplasmal agent, the determination of its plant host range and the identification of its insect vector(s) (Lee et al. 1998). All these studies necessitate the development of methods for diagnosis which are relying on the molecular detection of phytoplasma

DNA (Kirkpatrick et al. 1987; Deng and Hiruki 1991). Phytoplasmas have been classified according to 16S-rDNA phylogeny and RFLP profiles into 30 phylogenetic groups and 28 '*Candidatus* Phytoplasma' species (Zhao et al. 2010). Among these species, '*Ca.* P. brasiliense' has been described as the agent of hibiscus witches' broom in Brazil. During a survey of temperate fruit tree orchards of the North of Azerbaijan, a phytoplasma could be detected by 16SrDNA PCR in a chlorotic peach tree (*Prunus persica*). '*Ca.* P. prunorum', the agent of European stone fruit yellows in the Euro-Mediterranean basin (Ahrens et al. 1993; Jarausch et al. 1998) and '*Ca.* P. pruni' the agent of peach western-X in North America (Purcell et al. 1981) are the two main phytoplasma damaging peach orchards in the world. We report in this paper the identification of the peach isolate from Azerbaijan as an isolate of '*Ca.* P. brasiliense' and the development of a PCR detection test based on the '*Ca.* P. brasiliense' *dna*K gene cloned by comparative RAPD.

Leaves of a peach tree (*Prunus persica*) displaying yellows reminiscent of phytoplasma infection were collected in September 2007 in Guba region in the north of Azerbaijan. DNA was extracted from 1 g of fresh leaf midribs of diseased and healthy peach trees following the CTAB extraction procedure (Maixner et al. 1995). Detection of phytoplasma infection was performed on 2 μl of each DNA extract tested by 16SrDNA nested PCR with the universal primers for phytoplasmas R16mF2/R16mR1 followed by R16F2n/R16R2 according to the cycling conditions described in the original publication (Gundersen and Lee 1996). The PCR products (7 μl) were analyzed by electrophoresis in the 1 X TBE buffer through 1% agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator. A PCR product of 1.25 kbp was obtained from the yellowing peach PEACH19 but not from the healthy peach tree. The *Rsa*1 and *Alu*1 RFLP patterns of PEACH19 was different from reference phytoplasmas present in this geographic area such as of '*Ca.* P. prunorum', '*Ca.* P. pyri', '*Ca.* P. trifolii' and stolbur phytoplasma (data not shown). The PEACH19 PCR was therefore sequenced on MegaBACE capillary sequencing instruments (COGENICS, Grenoble, France). The raw sequences were assembled and edited using GAP4 of the Staden package and the consensus sequence deposited at EMBL (FR717540). Multiple sequences alignments were performed with the PEACH19 16S rRNA sequence and 16S rRNA sequences of reference '*Candidatus* Phytoplasma' species using Clustal W program (Thompson et al. 1994). Phylogenetic analyses were conducted using MEGA version 4 (Tamura et al. 2007) using the neighbour joining method with randomized bootstrapping evaluation of branching validity. Results indicated that the PEACH19 16S rRNA was identical to that of '*Ca.* P. brasiliense' of the phylogenetic group 16SrXV-A (Hibiscus witches' broom phytoplasma) (Montano et al. 2001), both sequences clustering together on the same phylogenetic branch supported by a bootstrap value of 100 (Fig. 1). In conclusion, it was clear that PEACH19 phytoplasma belonged to the taxonomic group XV-A and to the species '*Ca.* P. brasiliense'. It is the first detection of this phytoplasma in a plant other than hibiscus and anywhere else other than in Brazil.

No specific diagnosis tool was available for the detection of '*Ca.* P. brasiliense' mainly because except for 16S rRNA, no '*Ca.* P. brasiliense' gene had been cloned. Therefore, the characterization of a '*Ca.* P. brasiliense' non ribosomal genetic locus was undertaken. The strategy was to apply comparative RAPD, a successful strategy for cloning genes of non cultivated bacteria (Chen and Chen 1995; Foissac et al. 2000; Hocquellet et al. 1999). Forty eight 15-mer RAPD primers (CATG1 to CATG48) designed as ATGCATGWSSWWS (40% GC contents) were used for random PCR amplification on 100 ng of DNA extracted from healthy periwinkle and Suriname virescence-infected periwinkle. Suriname virescence isolate is an isolate of '*Ca.* P. brasiliense' maintained in the phytoplasma collection in Bordeaux (Pracros et al. 2002). Samples were amplified through 40 cycles of 30 s at 94°C, 30 s at 37°C, and 1 min at 72°C using a single primer at 1 μM and 2 U of *Taq* polymerase (Promega, Madison, USA) in a 50 μl reaction mixture containing PCR Buffer 1X, $MgCl_2$ 2 mM, and 200 μM of dNTP. Amplification results were analyzed on ethidium bromide-stained 1% agarose gels. The CATG28 15-mer (ATGCATGACTCGAAC) primer allowed amplification of a 1.6 kbp DNA fragment from "Suriname virescence"-infected periwinkle (Fig. 2, lane 1) but not from healthy periwinkle (Fig. 2, lane 2). This amplicon, the SVG28 fragment, was cloned and sequenced. The raw sequences were assembled and edited using GAP4 of Staden package and deposited at EMBL

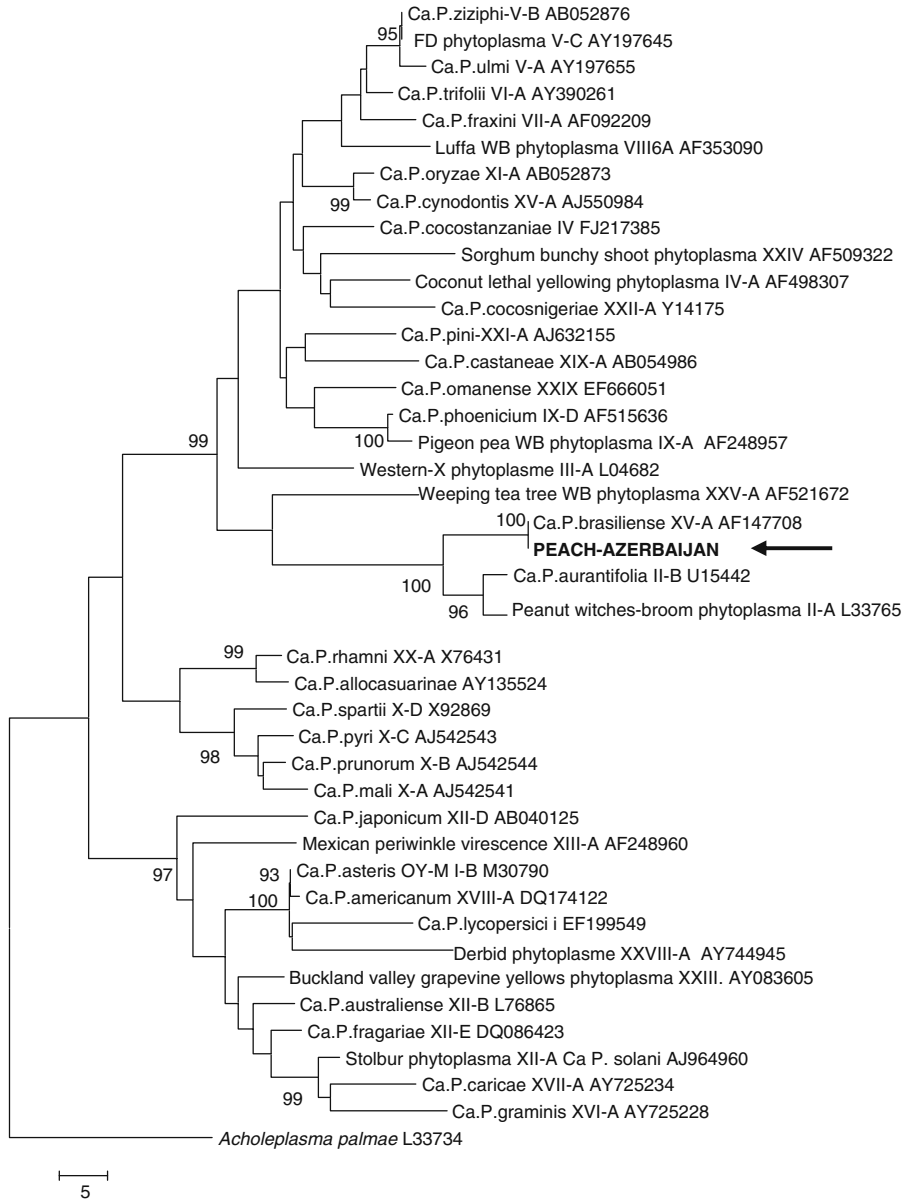

Fig. 1 Phylogenetic tree of 16S rRNA gene sequences constructed by the neighbour-joining method showing relationships between the phytoplasma identified in peach tree from Azerbaijan and reference phytoplasmas from GenBank. Numbers above or below branches are bootstrap values obtained for 1,000 replicates. *Acholeplasma palmae* ATCC 49389T was used as outgroup

(FR717541). The sequence was 1,651 bp long and shared 84% homology with the Peanut witches' broom phytoplasma genes coding for heat shock protein DnaK and heat shock protein DnaJ. Group specific primers Bra-dnaKF1/R1 for first PCR and nested internal primers Bra-dnaKF2/R2 were designed on SVG28 sequence, and a PCR test amplifying the non ribosomal *dna*K gene was developed with the aim of specifically detect phytoplasmas of group 16SrXV-A ('*Ca.* P. brasiliense') but not phytoplasmas of group 16SrII (Fig. 3). Reference phytoplasma isolates of the group 16SrII, a group phylogenetically close to the group 16SrXV, and the isolate Surinam Virescence from group 16SrXV-A were propagated by grafting on *Catharanthus roseus* (Table 1). DNA from '*Ca.* P. brasiliense'—infected hibiscus and basil were kindly provided by Professor Paulo Bedendo and Dr. Elia Choueiri respectively.

The non ribosomal *dna*K gene based PCR test was carried out using PCR primers Bra-dnaKF1(CCC TTT AAA ACA GGT GTT AG) and Bra-dnaKR1 (TCT TCA AAC TCG GCA TCA AC) followed by nested PCR primers Bra-dnaKF2 (TTG GTC GGC GGA TCA ACA AG) and Bra-dnaKR2 (TGG AGA

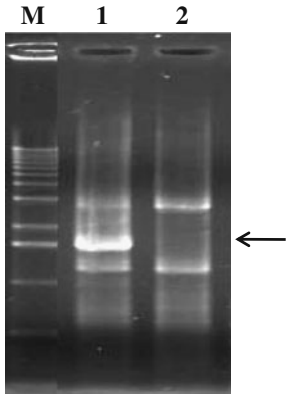

Fig. 2 RAPD with the 15-mer CATG28. Lane M, 1-Kb DNA ladder (Invitrogen), lane 1, Suriname virescense infected reference isolate maintained in periwinkle and lane 2, healthy periwinkle control

TTC TTC TGA AGT AG). PCR was performed in 50 µl containing 2 µl of nucleic acid (about 100 ng), PCR Buffer 1X, $MgCl_2$ 2 mM, 0.5 mM of each primer, 200 µM of dNTP mix, and 50 units/ml *Taq* polymerase. The following PCR conditions were used: initial denaturation step at 94°C for 4 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C during 30 s, and primer extension at 72°C for 1 min and 10 min at 72° C as a final extension step. The PCR products (7 µl) were analyzed by electrophoresis in the 1 X TBE buffer through 1% agarose gel, stained with ethidium bromide and the DNA bands were visualized using a UV transilluminator. According to the sequence, an 886-bp amplicon is expected (Fig. 3). To verify the specificity of Bra-dnaKF1/R1 and Bra-dnaKF2/R2, nested-PCR was performed on DNA extracted from diseased peach tree (Fig. 4a. lane 6), healthy peach tree (Fig. 4a. lane 7), Suriname virescence infected periwinkle (Fig. 4a. lanes 4), a '*Ca.* P. brasiliense'-infected basil (Choueiri et al., unpublished) (Fig. 4a. lane 5), three '*Ca.* P. brasiliense'-infected *Hibiscus rosa-sinensis* plants (Fig. 4a. lanes 1, 2, 3), and ten phytoplasma strains belonging to the 16SrII group (Fig. 4a. lanes 8 to 17). A 886-bp fragment was obtained only with diseased peach tree, Suriname virescence infected periwinkle, basil and the three '*Ca.* P. brasiliense'-infected Hibiscus plants. In conclusion the Bra-dnaK nested-PCR was amplifying phytoplasmas of the subgroup 16SrXV-A, but did not amplify the related phytoplasmas of the group 16SrII. For further characterization of phytoplasmas of group 16SrXV-A, the PCR products obtained with the 16SrXV-A group specific primers Bra-dnaKF2/R2 were submitted to restriction fragment length polymorphism (RFLP) analysis with restriction enzyme *Taq*I. Results of RFLP analysis (Fig. 4b) showed that the restriction of the amplicons using *Taq*I revealed two restriction profiles among the different '*Ca.* P. brasilience' isolates. All Hibiscus witches' broom phytoplasmas gave the same digestion pattern characterized by a DNA band at 0.45 kbp, whereas PEACH19, Surinam virescence and Basil had no 0.45 kbp DNA band but an additional band at 0.3 kbp and a brighter band at 0.15 kbp. These results indicate the existence of two '*Ca.* P. brasiliense' group of strains that are genetically different. This was confirmed by sequencing the six amplicons. Two sequences were obtained. The first sequence (accession number FR775800), that of Hibiscus witches' broom 121, 122 and CB2 exhibited 19 mutations when compared to the sequence of Suriname virescence, basil and peach 19 which were totally identical and constitute a second sequence (accession number FR717541). Especially a G → T mutation at position 689 (Fig. 4c) in the bradnaK PCR product of Hibiscus witches'-broom isolates eliminated a *Taq*1 restriction site, responsible for the difference in restriction profiles with the Suriname virescence, basil and peach19 isolates.

In conclusion, detection of '*Ca.* P. brasiliense' was performed for the first time in a plant other than

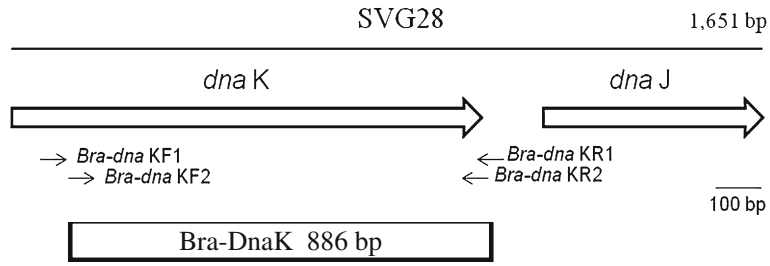

Fig. 3 Gene content of SVG28 DNA fragment. Short arrows indicate the positions of primers used for PCR and white box represent the final nested PCR product Bra-dnaK

Table 1 Phytoplasma isolates tested for this study

Isolate	Disease	Host	Country	16Sr group
SV	Virescence	*Catharanthus roseus*	Suriname	XV-A
Bas	Yellows	*Ocimum basilicum*	Lebanon	XV-A
Hib121	Witches' –broom	*Hibiscus rosa-sinensis*	Brazil	XV-A
Hib122	Witches' –broom	*Hibiscus rosa-sinensis*	Brazil	XV-A
HibCB2	Witches' –broom	*Hibiscus rosa-sinensis*	Brazil	XV-A
PEACH19	Peach yellows	*Prunus persica*	Azerbaijan	XV-A
CRP	Crotalaria phyllody	*Catharanthus roseus*	Thailand	II-C
CLP	Cleome phyllody	*Catharanthus roseus*	Thailand	II-A
JPH	Jute Phyllody	*Catharanthus roseus*	Thailand	II
CWB	Crotalaria witches'- broom	*Catharanthus roseus*	Thailand	II
SEP	Sesame phyllody	*Catharanthus roseus*	Thailand	II-A
TBB	Tomato big bud	*Catharanthus roseus*	Australia	II-D
SOYP	Soybean phyllody	*Catharanthus roseus*	Thailand	II-C
SPLL	Sweet potato little leaf	*Catharanthus roseus*	Australia	II
WBDL	Lime witches' –broom	*Catharanthus roseus*	Oman	II-B
COWB	Cotton witches' –broom	*Catharanthus roseus*	Burkina Faso	II-F

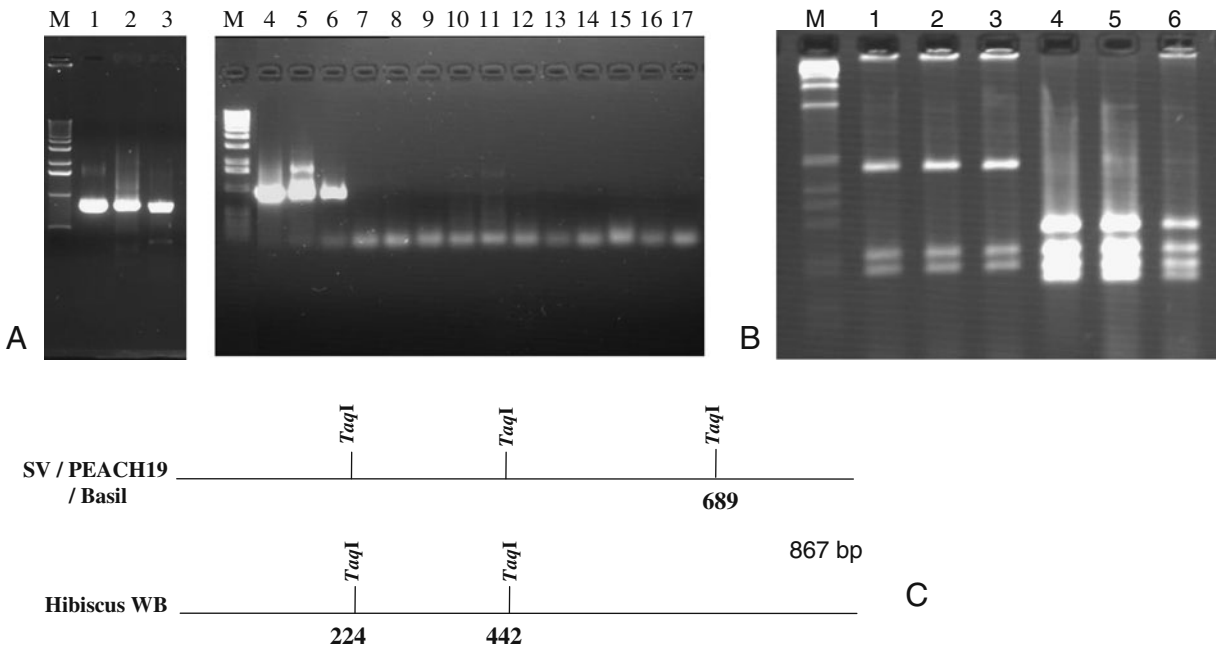

Fig. 4 Nested PCR amplication of '*Ca.* P. brasiliense' *dna*K. **a**: PCR amplification with the specific primers Bra-dnaK F1/Bra-dnaK R1 and Bra-dnaK F2/Bra-dnaK R2 for phytoplasmas of groups 16SrXV and 16SrII. **b**: RFLP analysis with *Taq*I. Digested DNAs were analyzed on 3% agarose gel in 1X TBE. **c**: *Taq*1 restriction map of bradnaK PCR products amplified from '*Ca.* P. brasiliense isolates Lane M, 1-Kb DNA ladder (Invitrogen), lane 1 Hib121, lane2-Hib122, lane3-Hib CB02, lane 4-SV, Suriname virescence infected periwinkle, lane 5- Bas, Basil from Lebanon, lane 6-Pec19.AZ, diseased peach tree, lane 7- Pec20.AZ, healthy peach tree, lane 8-CRP, lane 9-CLP, lane 10-JPH, lane 11-CWB, lane 12-SEP, lane 13-TBB, lane 14-SOYP, lane 15-SPLL, lane 16-WBDL, lane 17-COWB. Abbreviations of phytoplasma names are defined in Table 1

hibiscus and from a location outside of Brazil. This phytoplasma, new for the Old World, was detected in a peach tree in the Guba region of Azerbaijan. Its presence not only in a woody host but also in an annual host (basil) demonstrates it is transmitted locally by insects. A test for the detection of '*Ca.* P. brasiliense' was developed and was based on the amplification of the non ribosomal *dna*K gene which had been cloned upon comparative RAPD. This new detection assay should help surveying for '*Ca.* P. brasiliense' in the Euro Mediterranean area.

Acknowledgements We greatly acknowledge the French Embassy in Baku and the Federation of European Biochemical Societies (FEBS) for their financial support. We thank Professor Ivan Paulo Bedendo for kindly providing phytoplasma isolates from Hibiscus and Dr. Elia Choueiri for providing phytoplasma –infected basil from Lebanon.

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