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First Detection and Characterization of *Ca. P. brasiliense* From Yellowing Peach Tree in Guba Region of Azerbaijan

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***"Candidatus Phytoplasma brasiliense"*, a new phytoplasma taxon associated with hibiscus witches broom disease was firstly described in 2001 in Brazil. The disease was characterized by symptoms of witches' broom, i.e. leaf yellowing and malformation.Yellowing peach tree (*Prunus persica*) samples reminiscent of phytoplasma infection were collected in September 2007 in Guba region, which is an important fruit and vegetable growing area in Azerbaijan. A phytoplasma was detected in the diseased peach tree by amplification of its 16 S rDNA by nested PCR with universal primers for phytoplasmas. It was furthermore characterized by RFLP and nucleotide sequence analyses of 16S rDNA. It was shown that a phytoplasma infected peach tree is *Candidatus Phytoplasma brasiliense*. This report constitutes the first detection of this phytoplasma in a plant other than hibiscus and elsewhere than in Brazil. To set up a specific detection test, 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 specific PCR test that amplify all '*Ca. P. brasiliense*' isolates of the group 16SrXV-A without amplifying the related members of the group 16SrII.**

Keywords: *"Ca. P. brasiliense", RFLP, RAPD, cloning, DNA sequencing, phylogenetic tree*

INTRODUCTION

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 hemiteran 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 phytoplasma-induced diseases control requires the taxonomic characterization of the 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 its identification as an isolate of *'Ca. P. brasiliense'* and the development of a specific PCR detection test developed from a '*Ca. P. brasiliense*' sequence cloned after comparative RAPD.

MATERIALS AND METHODS

Plant material and DNA extraction. Yellowing peach tree (*Prunus persica*) samples reminiscent of phytoplasma infection were collected in September 2007 in Guba region. The DNAs were extracted from 1g fresh leaf midribs of diseased and healthy plants as

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control, following the CTAB extraction protocol (Maixner et al., 1995). In this study we also used the DNA of reference phytoplasma isolates (Table 1).

DNA amplification and RFLP analysis. Detection of phytoplasma infection was performed on 2µl of each DNA extracts tested by 16S-rDNA nested PCR with the universal primers for phytoplasmas R16mF2/R16mR1 and R16F2n/R16R2. PCR mixtures and cycling conditions were as described in the original article (Gundersen and Lee, 1996). The PCR products (7 µL) were analyzed by electrophoresis in the 1ÇTBE buffer through 1% agarose gel, stained with ethidium bromide, and DNA bands visualized using a UV transilluminator. Nested PCR products were analysed by single-restriction endonuclease digestion. 4µL PCR products were digested with 2.5 U *Alu*I (Promega) according to the manufacturer's instructions. The restriction fragments, together with the 1-kb-plus DNA size marker (Invitrogen), were separated by 2% agarose gel electrophoresis in 1ÇTBE buffer, stained with ethidium bromide and visualized under UV.

DNA sequencing. The PCR product obtained from peach tree were also sequenced by COGENICS (Grenoble, France) on MegaBACE capillary sequencing instruments. The raw sequence chromatograms were assembled and edited using an assembling programs (GAP4 Staden package) and were compared with reported sequences in GenBank using BlastN program (Altschul et al., 1997). Multiple sequences alignments were performed with the edited 16S sequence of the phytoplasma detected on peach tree and the 16S reference phytoplasma sequences using Clustal W program (Thompson et al., 1994). Phylogenetic analyses were conducted using MEGA version 4 (Tamura et al., 2007) using maximum parsimony with randomized bootstrapping evaluation of branching validity.

RAPD amplification. The 48 RAPD 15-mer primers ATGCATGWSSWWS (called CATG1 to CATG48) with 40% GC contents were designed and used for random PCR on 100 ng of DNA extracted from healthy periwinkle and Surinam virescence-infected periwinkle. 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 mM and 2 U of *Taq* polymerase in a 50 ml reaction mixture containing PCR Buffer 1Ç, $MgCl_2$ 2 mM, and 200µM of dNTP mix. Amplification results were analyzed on ethidium bromide-stained 1% agarose gels. DNA size marker was 1kb DNA ladder plus from Invitrogen.

Cloning and sequence analysis. When a DNA band was consistently observed in RAPD profile of "Surinam viresence"-infected periwinkle but not in healthy plant control, a PCR product obtained from "Surinam virescence"-infected periwinkle were ligated into pGEMt easy vector (Promega, Madison, Wis.). Plasmids were electroporated into *Escherichia coli* DH10B cells, and inserts were sequenced. Sequences were edited with two different sequence-editing and assembling programs (Chromas and GAP4 Staden package) and were compared with reported sequences in GenBank using Blast program.

Group specific PCR test. The non ribosomal dnaK gene based PCR test was carried out with 100 ng of plant DNA 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

Table 1. List of used reference phytoplasma isolates

Isolate	Disease	Host	Country	16Sr Group
SV	Virescence	*Catharanthus roseus*	Surinam	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
CRP	Crotolaria phyllody	*Catharanthus roseus*	Thailand	II-C
CLP	Cleome phyllody	*Catharanthus roseus*	Thailand	II-A
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
ESFY	Apple proliferation	*Catharanthus roseus*	Italy	X-B
MOL	Stolbur	*Catharanthus roseus*	France	XII-A
PTL	Tomato proliferation	*Catharanthus roseus*	Lebanon	VI-A

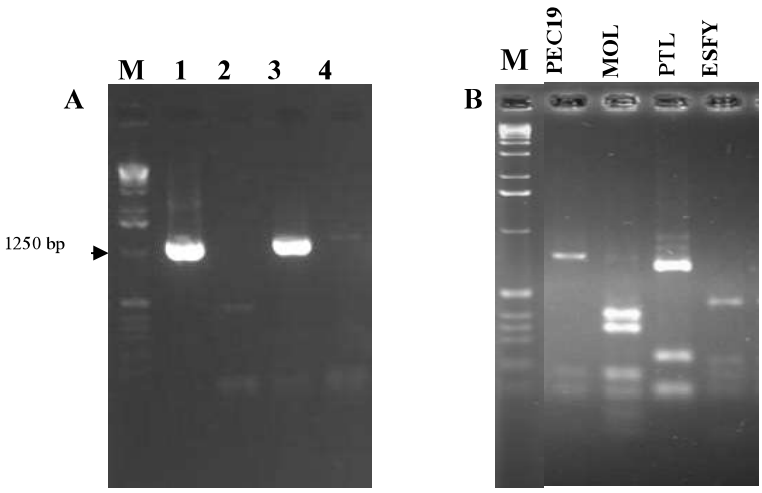

Figure 1. PCR-RFLP analysis. A - Nested PCR amplification of phytoplasma 16S rDNA fragment with the universal primers for phytoplasmas R16mF2/R16mR1 and R16F2n/ 16R2. Lane M, 1-Kb DNA ladder (Invitrogen), lane 1-the diseased peach tree *Prunus persica*, lane 2 - healthy peach tree, lane 3 - Stolbur Moliere-infected periwinkle, and lane 4 - healthy reference strain maintained in periwinkle hosts. B - RFLP analysis with restriction enzyme *Alu*I. Lane M, 1-Kb DNA ladder (Invitrogen), lane PEC19, the diseased peach tree *Prunus persica*, lanes MOL., PTL and ESFY, reference phytoplasma isolates maintained in periwinkle host.

(TGG AGA TTC TTC TGA AGT AG). PCR was performed in 50 µL containing 2µl of nucleic acid (about 100 ng), PCR Buffer 1Ç, $MgCl_2$ 2 mM, 0.5 mM of each primer, 200µM of dNTP mix, and 50 units/ml *Taq* polymerase. Following PCR conditions were used: initial denaturation step at 94°C for 4min, followed by 35 cycles consisting of denaturation at 94ºC for 30 sec, annealing (hybridation) at 55ºC during 30 sec, and primer extension (elongation) at 72ºC for 1 min and 10 min in the final cycle. The PCR products (7 µL) were analyzed by electrophoresis in the 1ÇTBE buffer troughs 1% agarose gel, stained with ethidium bromide, and DNA bands visualized using a UV transilluminator.

RESULTS AND DISCUSSION

Detection and taxonomic characterization of phytoplasma. The amplicons of the expected size (1250 bp) were obtained from yellowing peach tree, and from the reference phytoplasma infected periwinkle (as positive control) by Nested PCR with the universal primers for phytoplasmas R16mF2/R16mR1 and R16F2n /R16R2. No PCR products were obtained from healthy control plants (healthy peach tree and healthy reference plant) (results illustrated in Figure 1A). For taxonomic characterization of detected phytoplasma, the PCR products obtained from peach tree and from reference phytoplasma strains were submitted to enzymatic restriction fragment polymorphism (RFLP) analysis with restriction enzyme *Alu*I. Results of RFLP analysis (Figure 1B) shown that the RFLP pattern obtained from peach tree is different from RFLP patterns of the reference phytoplasma strains used.

The PCR product from peach tree was sequenced. The 16S sequence of the phytoplasma detected in the peach tree shared 100% identity with the rDNA-16S sequence of '*Candidatus Phytoplasma brasiliense*' of the phylogenetic group 16SrXV (Hibiscus witches broom phytoplasma, Montano and *al.*, 2001). It is the first detection of this phytoplasma in a plant other than hibiscus and elsewhere than in Brazil.

The 16S sequence of detected peach tree phytoplasma was aligned with 16S sequences of reference phytoplasma *Candidatus* species and a phylogenetic tree constructed using the neighbor joining method. The resulting phylogenetic tree is presented on Figure 2.

Random amplification and cloning of the SVG28 fragment. RAPD patterns primed with 48 15-mer primers were obtained from Surinam virescence infected periwinkle and compared to those obtained from healthy periwinkle of the same cultivar. The G28 15-mer (ATGCATGACTCGAAC) primer allowed amplification of a 1.6 kbp DNA fragment from "Surinam virescence"-infected periwinkle (Figure 3, lane 1) but not from healthy periwinkle (Figure 3, lane 2). This amplicon, called SVG28, was cloned and sequenced. The sequence was 1651 bp long and shared 84% similarity 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 based on non-ribosomal *dna*K gene was developed with the aim of specifically detect phytoplasmas of group 16SrXV-A ('*Ca. P. brasiliense*').

According to the sequence, an 886-bp amplicon is expected. To verify the specificity of Bra-

dnaKF1/R1 and Bra-dnaKF2/R2, they were used on DNAs extracted from diseased peach tree (Figure 4, lane 6), healthy peach tree (Figure 4, lane 7), Surinam virescence infected periwinkle (Figure 4, lane 4), a '*Ca. P. brasiliense*'-infected basil (Figure 4, lane 5), three '*Ca. P. brasiliense*'-infected *Hibiscus rosa-sinensis* plants (Figure 4, lanes 1, 2, 3), and

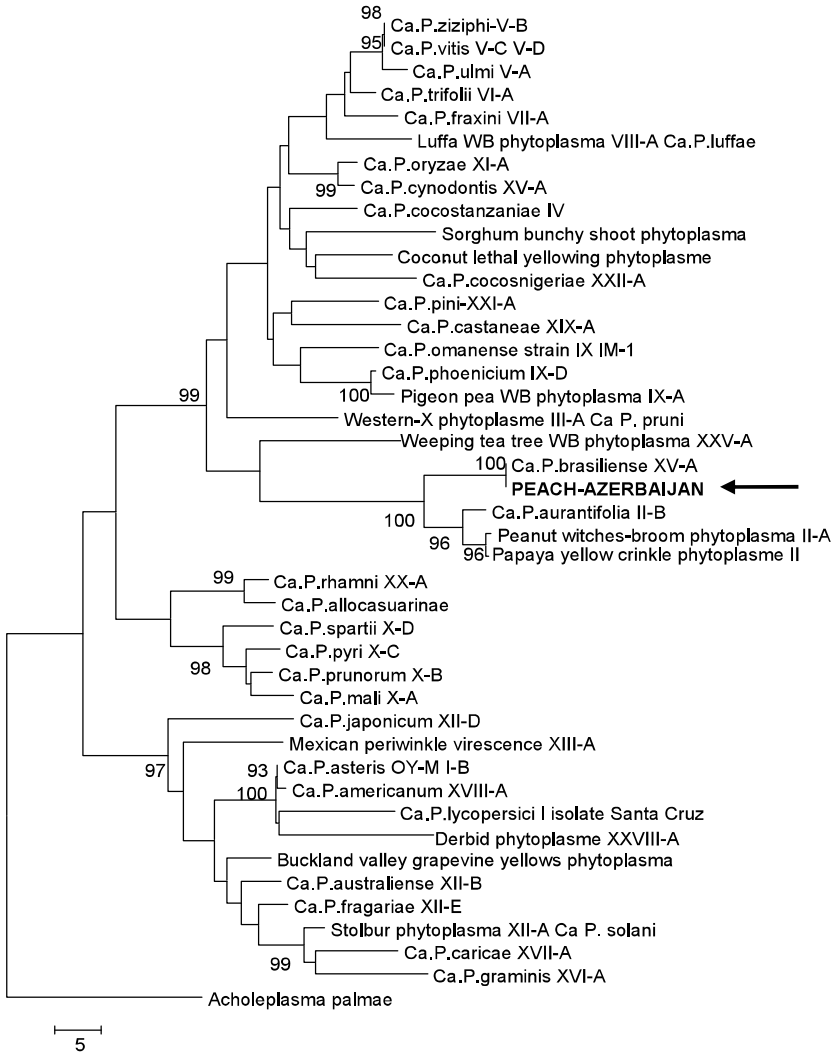

Figure 2. Phylogenetic tree of 16S rRNA gene sequences constructed by the neighbour-joining method showing relationships between the phytoplasma identified in peach tree from Azerbaijan and reference phytoplasmas from GenBank. Numbers above or below branches are bootstrap values obtained for 1000 replicates. Branch lengths are proportional to the number of inferred character-state transformations. Bar represents 5 inferred character state changes. A. palmae ATCC 49389T was used as outgroup.

ten phytoplasma strains belonging to the 16SrII group, a group phylogenetically related to group 16SrXV (Figure 4, lanes 8 to 17). An 886-bp fragment was obtained only with diseased peach tree, Surinam virescence infected periwinkle, basil and the three '*Ca. P. brasiliense*'-infected *Hibiscus* plants.

In conclusion, the detection of '*Ca. P. brasiliense*' was performed for the first time in a plant other than hibiscus and elsewhere than Brazil. This phytoplasma, new for the old world was detected in a peach tree in the Guba region of Azerbaijan. A detection test specific to '*Ca. P. brasiliense*' was developed and was based on the amplification of the non ribosomal dnaK gene.

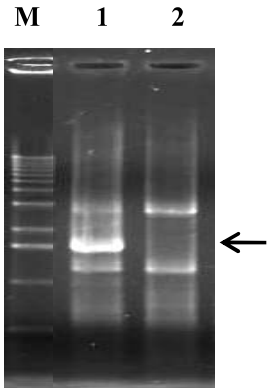

Figure 3. RAPD with 15-mer G28. Lane M, 1-Kb DNA ladder (Invitrogen), lane 1, Surinam virescense infected reference isolate maintained in periwinkle and lane 2, healthy periwinkle control.

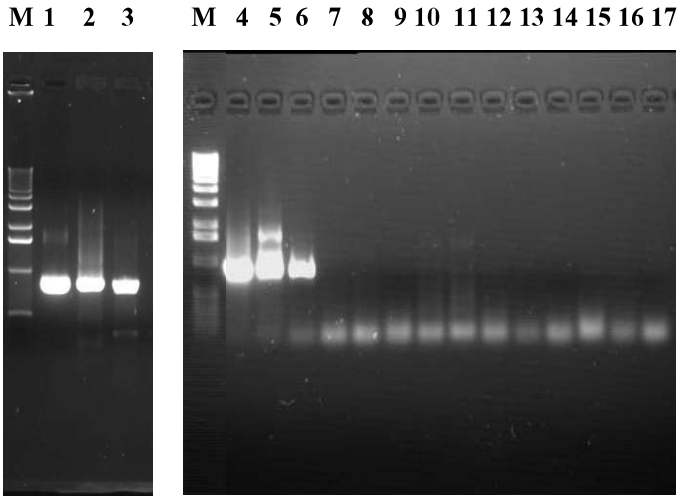

Figure 4. Nested PCR amplification with the specific primers bradnaK F1/ bradnaK R1 and bradnaK F2/bradnaK R2 for phytoplasmas of 16SrXV group based on non-ribosomal sequence *dna*K Lane M, 1-Kb DNA ladder (Invitrogen), lane 1 - Hib121, lane 2 - Hib122, lane 3 - Hib Cb02, lane 4 - Surinam virescence infected perwinkle, lane 5 - Basilic from Liban, lane 6 - diseased peach tree, lane 7 - healthy peach tree, lane 8 - Cr.Ph, lane 9 - Cl.Ph, lane 10 - J.Ph, lane 11 - Cr.W.B., lane 12 - Ce.Ph., lane 13 - TBB, lane 14 - So.Ph., lane 15 - SPLL, lane 16 - WBDL, lane 17 - Co.W.B. Abbreviations of phytoplasma names are defined in Table 1.

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REFERENCES

Ahrens U., Lorenz K.H., Seemuller E. (1993) Genetic diversity among mycoplasmalike organisms associated with stone fruit diseases. Mol. Plant-Micr. Interac. **6:** 686-691.

Altschul S.F., Madden T.L., Schdffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. (1997) Gapped BLAST (Basic Local Alignment Search Tool) and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389-3402.

Deng S.J., Hiruki C. (1991) Amplification of 16S ribosomal-RNA genes from cultivable and nonculturable mollicutes. J. Microbiol. Meth. **14:** 53-61.

Gundersen D.E., Lee I.-M. (1996) Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopath. Mediter. **35:** 114-151.

Jarausch W., Lansac M., Saillard C., Broquaire J.M., Dosba F. (1998) PCR assay for specific detection of European stone fruit yellows phytoplasmas and its use for epidemiological studies in France. Europ. J. Plant Pathol. **104:** 17-27.

Kirkpatrick B.C., Stenger D.C., Morris T.J., Purcell A.H. (1987) Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. Science **238:** 197-200.

Lee I.M., Gundersen-Rindal D.E., Bertaccini A. (1998) Phytoplasma: Ecology and Genomic Diver-

sity. Phytopathol. **88:** 1359-1366.

Lee I.M., Davis R.E., Gundersen-Rindal D.E. (2000) Phytoplasma: phytopathogenic mollicutes. Annu. Rev. Microbiol. **54:** 221-255.

Maixner M., Ahrens U., Seemuller E. (1995) Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. Europ. J. Plant Pathol. **101:** 241-250.

Purcell A.H., Nyland G., Raju B.C., Heringor M.R. (1981) Peach yellow leaf roll epidemic in Northern California: effects of peach cultivar, tree age and proximity to pear orchards. Plant Dis. **65:** 365-368.

Tamura K., Dudley J., Nei M., Kumar S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:** 1596-1599.

Thompson J.D., Higgins D.G., Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673-4680.

Weintraub P.G., Beanland L. (2006) Insect vectors of phytoplasmas. Annu. Rev. Entomol. **51:** 91-111.

Weisburg W.G., Tully J.G., Rose D.L., Petzel J.P., Oyaizu H., Yang D., Mandelco L., Sechrest J., Lawrence T.G., Etten J.V., Maniloff J., Woese C.R. (1989) A phylogenetic analysis of the mycoplasmas: basis for their classification. J. Bacteriol. **171:** 6455-6467.

Zhao Y., Wei W., Davis R.E., Lee I.M. (2010) Recent advances in 16S rRNA gene-based Phytoplasma differenciation, classification and taxonomy. In: Phytoplasmas: genomes, plant hosts and vectors. (Weintraub P.G., Jones P., eds.), CAB International, Wallingford: 64-92.