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Molecular identification of 16S rII phytoplasma group in commercial pistachio cultivars in Iran

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Evaluation of phytoplasmas infection was conducted in the pistachio-growing areas of Iran (Rafsanjan in Kerman province) in early autumn of 2011. A total of 30 pistachio trees collected from a pistachio orchard in Rafsanjan showing *Psylla* damage symptoms and 10 samples with different abnormal symptoms from miscellaneous orchards were tested for the presence of phytoplasma. By using nested PCR with primer pairs P1/P7 and internal primer sets R16F2N/R16R2 and fU3-rU5, amplified fragment of expected size was observed in some trees with deformation and yellowing symptoms. On the basis of nucleotide sequence analysis of 16S rDNA amplified by PCR, this phytoplasma was classified in group 16S rII. False positive reaction observed in Ahmadaghaei cultivar, using primer set R16F2N/R16R2 sequence analysis of PCR products determined, amplified fragment related to Plant Genome's; a species of flowering plant in the Anacardiaceae family.

Keywords: *Pistacia vera*; nested PCR; detection; pathogen

Introduction

Pistacia vera, a small tree, is one of the most important nut-producing crop of Iran. According to FAO statistics, Iran is the number one exporter of this crop in the world. Several diseases affecting pistachio that reduce the quantity and quality of products have been identified. Yellowing of pistachio trees, a disease that kills the trees, has emerged and spread throughout pistachio-producing areas in Iran in the last decade. A number of biotic or abiotic factors may lead to yellowing and leaf malformation in pistachio, such as toxicity or deficiency of different soil elements and plant pathogens. Frequent soil analysis and improving orchard management could not recover the trees all the time. Phytoplasmas are bacteria without cell wall and infect wide range of plants (Weintraub & Jones 2010). Recently published reports indicate that one of the agents that affect the health of the pistachio tree is phytoplasma. Positive tests for phytoplasma in pistachios tree with witches' broom, yellowing and leaf malformation symptoms have been reported in Yazd, Ghazvin, Khorasan province of Iran (Esmaeilzadeh-Hosseini 2001; Karimi et al. 2010; Zamharir & Mirabolfathi 2011). Sequencing of 16S rRNA gene amplified products of the phytoplasma isolated from Ghazvin province indicated that pistachio witches' broom phytoplasma is in a separate 16S rRNA group of phytoplasmas

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and maybe it is a representative of a new taxon (Zamharir & Mirabolfathi 2011). But collected phytoplasma from Khorasan province that was positive in nested PCR could belong to 16S rI, 16S rII, 16S rIX or 16S rXII groups (Karimi et al. 2010). The expression of phytoplasmas symptoms are varied based on cultivar, stage of tree development and infection, latent infection, weather conditions and significant changes in the production practices. This indicates a risk of hidden source of infection which can be transmitting either with grafting or other methods especially in newly established orchards in areas with high disease occurrence. Therefore, it is necessary to use sensitive methods for early detection of phytoplasmas in different plant species (Mehle et al. 2010). Since latent phytoplasma infection could be transmitted by grafting from non-symptomatic plant (Weintraub & Jones 2010), in this study polymerase chain reaction-based approach was used to attempt sensitive detection of phytoplasma from symptomic and asymptomic plants and we were screening phytoplasma occurrence in six major pistachio cultivars in the city of Rafsanjan (in Kerman province), the most important pistachio-production site in Iran. Thus, the objective of this study was to determine the presence of phytoplasma in pistachio cultivars and to characterise the phytoplasma associated with disease by using PCR-RFLP and sequencing.

Materials and methods

Plant material

Pistachio trees in commercials orchards were investigated for yellowing, malformation and proliferation symptoms in Kerman and Khorasan Razavi provinces during spring and autumn of 2011. Overall, 40 samples (cultivars: Akabri, Ohadi, Kalleghuchi, Akbari, Momtaz, Fandogii, Ahmadaghaei and Badami) were taken from twigs showing the symptoms. Each sample consisted of 5–10 twigs from each tree canopy. Leaf veins were isolated, splitted into 0.5 g aliquots and kept at −80 °C for further analysis. Samples from naturally infected potato and lime witches' broom were used as positive controls and healthy pistachio was used as negative control.

Extraction of nucleic acid

Frozen samples were ground under liquid nitrogen using mortar and pestle. DNA was extracted using DNA Extraction Kit (Fermentase, Germany). The quality and quantity of DNA was checked using gel electrophoresis and spectrophotometry assays. The concentration of extracted DNA was determined in each sample triplicate and adjusted to 50 ng/µl for further experiments (Ausuble et al. 1992).

PCR amplification

To test the presence of phytoplasma, the purified DNA was used in PCR with 25 µl reaction mixtures consisting of: 3 µl template DNA, 300 µ1M dNTPs, 1 × PCR buffer, 4 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and 0.8 µ1M of primers. The temperature profile for PCR amplification was 94 °C pre-denaturation for 4 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and final extension at 72 °C for 7 min (Deng & Hiruki 1991). The PCR products obtained with primers P1/P7 (Deng & Hiruki 1991) were reamplified in nested PCR with the universal primer pair R16F2n/R16R2

(Gundersen & Lee 1996) and fU5/RU3 (Lorenz et al. 1995). Nested PCR was carried out in a volume of 25 µl as follows: 1 µl PCR products, 300 µ1M dNTPs, 1 × PCR buffer, 5 mM $MgCl_2$, 1.5 U Taq polymerase and 0.8 µ1M of each primers. Parameters for 35 cycles of nested PCR were denatured at 94 °C for 1 min (4 min for cycle 1) followed by annealing at 54 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C 7 min.

Sequencing and phylogenetic analysis

The PCR products were resolved by gel electrophoresis (Ausuble et al. 1992) and purified using Qiagen gel extraction kit (Qiagen, USA). Purified DNA was sequenced by the MWG Company (Germany). Sequences were aligned, using the program Clustal W, with the sequences of some other phytoplasmas in 16S rII group and isolated from Iran.

Restriction Fragment Length polymorphism

Ten µl of PCR product was digested using *Hpa*II *Taq*I and *Alu*I restriction enzymes for R16F2/R16R2 and fU5/RU3 amplicons following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The comparison of restriction patterns obtained with those of control strains was carried out after electrophoresis through a 3% Agarose followed by staining with ethidium bromide and visualisation under an UV transilluminator.

Results

The present study illustrated detection of phytoplasma by amplification of 16S rDNA on various pistachio cultivars with deformation and yellowing symptoms from miscellaneous orchards. But among 40 samples, collected from pistachio orchard of Kerman province, that showed *Psylla* injured symptoms, only three trees conduct to Ahmadaghaei cultivar showed positive response in phytoplasma infection (Figure 1) by using primer sets R16F2N/R16R2. But we observed false positive reaction in these trees of Ahmadaghaei cultivar. Sequence and RFLP analysis of ~1250 bp PCR product indicated that amplified fragment was similar to *Schinus terebinthifolius* sequence, species of flowering plant in the Anacardiaceae family. In the other hand, any of these 30 cultivars did not respond positively, when amplifications were attempted with fU5/rU3 primer

Figure 1. Ethidium bromide-stained gel of PCR amplification products obtained by using primer pair R16F2N/R16R2 from DNA extracted of following cultivars with *Psylla* injured symptoms: Ohadi (lane 1–5), Kalleghuchi (lane 6–10), Akbari (lane 11–15), Momtaz (lane 16–20), Fandogii (lane 21–25) and Ahmadaghaei (lane 26–30). Phytoplasma isolated from Khorasan province and *Candidatus* phytoplasma aurantifolia used as positive controls (+). Health sample from pistachio used as negative control (–p). M: 1 kb DNA ladder marker.

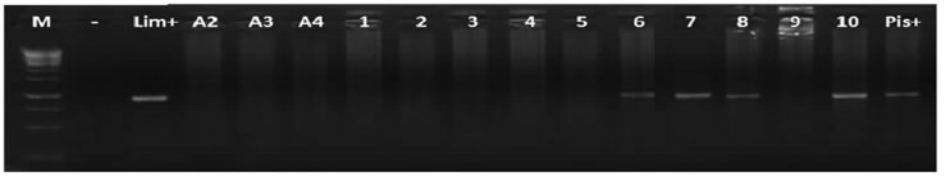


Figure 2. Ethidium bromide-stained gel of PCR amplification products obtained by using primer pair fU3-rU5 from DNA extracted from pistachio trees with various symptoms. M: 1 kb DNA ladder marker. Lane 2: negative control, lane 3: Candidatus phytoplasma aurantifolia used as positive controls (Lim+), A2, A3 and A4: Ahmadaghaei cultivar (belong to lane 27–29 in Figure 1 that was positive with R16F2N/R16R2), lanes 1–10: pistachio with deformation symptoms, phytoplasma isolated from Khorasan province (Pis+).

pair. Also, PCR using primer pairs R16F2n/R16R2 and fU5/rU3 produced an amplified fragment of expected size in pistachio samples derived from Khorasan province as Karimi described (Karimi et al. 2010) and other positive controls. No amplification was observed from healthy plant (Figure 2).

Phylogenetic relationships on 16S rDNA sequences

Phytoplasma which were collected in Rafsanjan from trees with deformation symptom were used for 16S rDNA sequences analysis. Blast analysis showed that phytoplasma 16S rDNA sequences of fU5/rU3 PCR fragments were 99% identical to phytoplasma members of group16S rII. Alignment revealed that the 16S rDNA sequences of this phytoplasma were distinguished from Buinzahra isolate that are previously reported by Zamharir and Mirabolfathi (2011) (Figures 3 and 4).

fU5/rU3 nested PCR product allows for virtual and laboratory digestions of DNA fragments with two *Hpa*II and *Alu*I enzymes. The result of the digestion was similar in both virtual and laboratory digestions (Figure 5).

R16F2n/R16R2 nested PCR product of Ahmadaghaei cultivar that showed positive response in phytoplasma infection was used in RFLP analysis (Figure 6). Following *Alu*I and *Taq*I digestion showed unique properties (Lee et al. 1998). In addition, due to the digestion pattern and sequence results, we have false positive reaction in these trees.

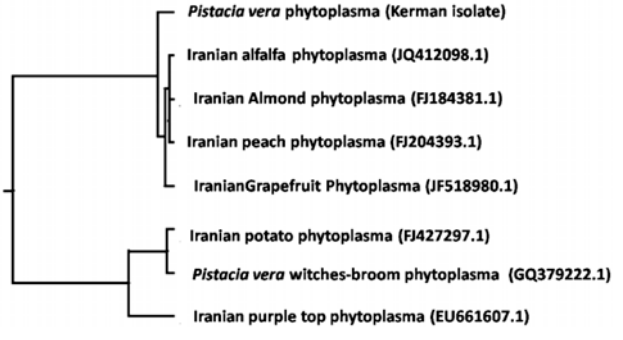


Figure 3. Dendrogram, constructed by the UPGMA method, showing the phylogenetic relationships amongst the eight different Iranian phytoplasma isolated from various crops based on the 16S rRNA gene. GenBank accession numbers for sequences are shown in parentheses alongside the names of the phytoplasma.

Discussion

On the basis of PCR-RFLP and sequencing analysis, the phytoplasma in 16S rII group was obtained from pistachio cultivars with yellowing and deformation symptoms. Based on previous reports, various groups of phytoplasma, specially phytoplasmas related to

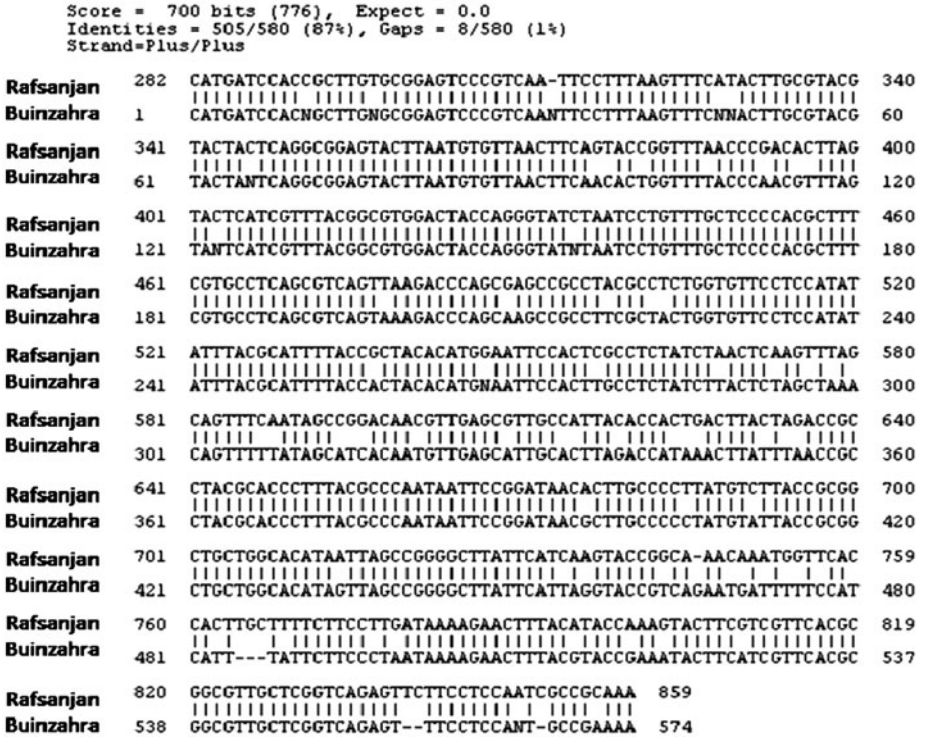
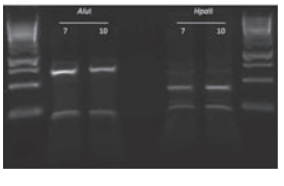


Figure 4. Alignment of 16S rDNA nucleotide sequences phytoplasma isolated from pistachio in Rafsanjan with Buinzahra isolate by Blast analysis F.



*Alu*I	*Hpa*II
657 bp	414 bp
192 bp	213 bp
7 bp	82 bp
	74bp
	52 bp
	21 bp

Figure 5. RFLP analysis of phytoplasma isolated infected pistachio. fU5/rU3 nested PCR product were digested with *Alu*I and *Hpa*II by virtual and laboratory digestions. Lane M, 1 kb DNA ladder.

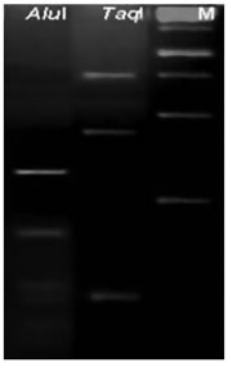


Figure 6. RFLP analysis of PCR product with expected size (~1250 bp) from pistachio Ahmadaghaei cultivars that were digested with *Taq*I and *Alu*I. Lane M, 1 kb DNA ladder.

16S rII group (Peanut Witches broom), exist in Iran and have wide host ranges in various crops in central and southern area of Iran (Salehi et al. 2005); some of them such as lime witches' broom is lethal and economically important. Alfalfa witches' broom from Yazd (Salehi et al. 1995), lime witches broom from central and southern of Iran (Bove et al. 2000), pot marigold phyllody from Yazd (Esmailzadeh-Hosseini et al. 2011) and sweet cherry witches' broom from central region of Iran (Zirak et al. 2010) are reported with various symptoms. This is the first report of phytoplasma detection in pistachio with yellowing and deformation symptoms; several factors which may account for disease and symptom development include strain virulence, strain interference, phytoplasma concentration, toxins, plant hormone imbalance and attachment of phytoplasmas to host cell membrane (Weintraub & Jones 2010). Based on symptoms and molecular identification methods, isolate of Buinzahra pistachio phytoplasma (Zamharir & Mirabolfathi 2011) was different from Kerman isolate. There is increasing evidence that phytoplasma related to Peanut witches' broom have been developed to another plant species in central region of Iran (Salehi et al. 2005; Zirak et al. 2010; Esmailzadeh-Hosseini et al. 2011). Probably, they are vectored by known or unknown phloem-feeding leafhoppers, planthoppers or psyllids (Weintraub & Beanland 2006). On the other hand, the common pistachio *Psylla, Agonoscena pistaciae*, is the most serious pest in the pistachio-producing regions of Iran that usually reaches outbreak levels. There are no records of it acting as a vector of plant diseases. However, the psyllid nymphs ingest large amounts of the phloem sap (Mehrnejad et al. 2011) since important phytoplasma such as pear decline, apple proliferation transmitted by kind of *Psylla* (Seemüller & Schneider 2004). Current research on the biological and molecular background of host specificity of phytoplasmas and their vectors could help to improve our understanding of the mechanisms of host specificity, virulence and distribution of phytoplasmas and provide new means of prevention or control of these agents (Kuske & Kirkpatrick 1992; Constable et al. 2003; Saracco et al. 2006; Weintraub & Jones 2010). In other hand, our results showed false positive detection of phytoplasma by universal primer in some samples. Low concentration, uneven distribution of phytoplasma and variations in titer according to season and plant organ are known from woody hosts and detection level of microscopical methods is not sufficient for diagnosis of these agents (Berges

et al. 2000). So, molecular-based method such as PCR or nested PCR has been employed for phytoplasma detection. Nested PCR with a combination of different universal primers can improve the diagnosis of unknown phytoplasmas present with low titer in the symptomatic host (Firrao et al. 2007). But, sequence similarity of phytoplasma with host plant or other micro-organism's genes is the most important causal agent of false positive reaction. Some primers can react probably with sequences of part of plant genome or dimmers and false positives could be observed (Franova 2011). So, the PCR alone is not sufficient for the survey of phytoplasmas and subsequent confirmation of phytoplasma presence like using several primer combination, sequencing and RFLP analyses must be done.

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