Identification of a phytoplasma associated with pomegranate little leaf disease in Iran

Short communication

Identification of a phytoplasma associated with pomegranate little leaf disease in Iran

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A B S T R A C T

During 2012—2014 surveys for the presence of phytoplasma diseases in Fars province (Iran), pome-granate little leaf symptoms were observed in several orchards in Khafr and Neyriz areas. Samples collected from symptomatic plants positively reacted in nested PCR assays using P1/P7 followed by R16F2n/R16R2 primer pairs producing the expected 1,250 bp DNA fragments. Real and virtual RFLP analysis showed that the sequences of phytoplasma strains from Khafr and Neyriz (KPLL and NPLL strains, respectively) were identical to each other and belong to 16SrII phytoplasma group, subgroup D. Phylogenetic analysis of the R16F2nR16R2 DNA region confirmed that KPLL and NPLL phytoplasmas were enclosed in the same clade as other 16SrII-D subgroup phytoplasmas. This is the first reported occurrence of a 16SrII phytoplasma infecting pomegranate trees.

Keywords:
Pomegranate disease
16SrII group
Little leaf
Molecular identification

The pomegranate (*Punica granatum* L., Lythraceae) is native to Iran and is one of the oldest known edible fruits (Singh, 1997; Stover and Mercure, 2007), it is grown in many subtropical countries especially in the Mediterranean region, and also extensively in Iran, India, Pakistan, Afghanistan, Saudi Arabia and in the subtropical areas of South America (Elyatem and Kader, 1984). The importance of pomegranate is not only for its taste, but also for its nutritional and medical properties (Miguel et al., 2010; Tehranifar et al., 2010; Hasni Sayyed et al., 2012). Iran is one of the most important pomegranate producers and exporters in the world with an annual production of about 900,000 tonnes (Anonymous, 2013) and annual export of more than 150,000 tonnes (Tehranifar et al., 2010). The main pomegranate cultivation centers of Iran are the provinces of Fars, Markazi, Isfahan, Khorasan, Yazd, Kerman, Semnan, Kermanshah, Tehran, Bakhtiari, Sistan and Baluchistan, Khouzestan, Lorestan, Mazandaran, Zanjan, Kohgilouyeh Boyer-ahmad, Azarbaijan Sharghi, Gilan, Hormozgan, Boushehr and Ilam.

Main biotic diseases of pomegranate are bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* (Benagi et al., 2012) and fruit rot caused by different fungi (Hebert and Clayton, 1963; Sharma and Jain, 1978; Snowdon, 1990; Bardas et al., 2009a, 2009b; Jamadar et al., 2011; Mirabolfathy et al., 2012). Fungal fruit rot and abiotic diseases such as fruit cracks, sunburn and frost damage are the most common causes of pomegranate fruit losses in Iran (Ashkan, 2006).

There is a recent report on phytoplasma presence in pomegranate showing yellows symptoms in Turkey (Gazel et al., 2015) in which two phytoplasma strains belonging to subgroups 16SrI-B and 16SrXII-A were identified. In 2012—2014 surveys for phytoplasma diseases in Fars pomegranate growing areas, a possible phytoplasma associated disease tentatively named pomegranate little leaf (PLL) was observed in Khafr and Neyriz areas. The objective of the present work was to verify phytoplasma presence and identity in Khafr and Neyriz pomegranate plants showing PLL symptoms.

Samples from ten pomegranate trees from Khafr and Neyriz areas (Fars province) (five per each area), showing little leaf and yellowing symptoms, and from four asymptomatic seedlings growing in insect-free greenhouse were collected and employed for phytoplasma detection and identification assays.

Fresh midribs (0.3 g) of symptomatic pomegranate plants were ground in liquid nitrogen and total DNA was extracted with a CTAB (Hexadecyl trimethyl ammonium)-based protocol (Zhang et al., 1998). DNA concentration was determined

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spectrophotometrically (Sambrook et al., 1989) and/or estimated by 1% agarose gel electrophoresis stained with ethidium bromide. DNA extracted from the asymptomatic seedlings of pomegranate and from a periwinkle plant infected with lime witches' broom phytoplasma (Salehi et al., 2002) were used as negative and positive control, respectively.

A direct PCR with P1/P7 universal phytoplasma primer pair (Deng and Hiruki, 1991; Schneider et al., 1995), amplifying 16S rDNA, spacer region between 16S and 23S rDNA and 5′ portion of 23S rDNA, was performed for phytoplasma detection. In nested PCR, P1/P7 amplicons diluted 1: 30 in sterile distilled water were re-amplified with the internal primer pair R16F2n/R16R2 (Gundersen and Lee, 1996) which amplifies about 1,250 bp of 16S rRNA gene. PCR conditions were as described previously (Salehi et al., 2011). PCR products were electrophoresed in 1% agarose gels in TAE buffer and visualized with a UV transilluminator following ethidium bromide staining.

From each sampling area PCR positive samples were selected to perform RFLP analysis. Eight microliters (approximately 250 ng) of nested PCR products from samples of symptomatic pomegranate trees from Khafr and Neyriz were individually digested with 3 μl each of restriction endonucleases *Alu*I, *Hha*I *Hin*fI, *Hpa*II, *Mse*I, *Rsa*I, *Sau*3AI and *Taq*I in 20 μl volumes at 37 °C (65 °C for *Taq*I) overnight following manufacturer instructions (Fermentas, Vilnius, Lithuania). RFLP profiles were analyzed by electrophoresis of digested DNA through 2% agarose gel, staining with ethidium bromide, and visualization with a UV transilluminator. The profiles were compared with those published previously (Lee et al., 1998).

Two nested PCR products, one from each area (Khafr and Neyriz) named KPLL and NPLL phytoplasmas respectively, were ligated

Fig. 1. Yellowing, little leaf, internode shortening and witches' broom in a little leaf affected pomegranate tree from Khafr area (A) compared to a healthy one (B).

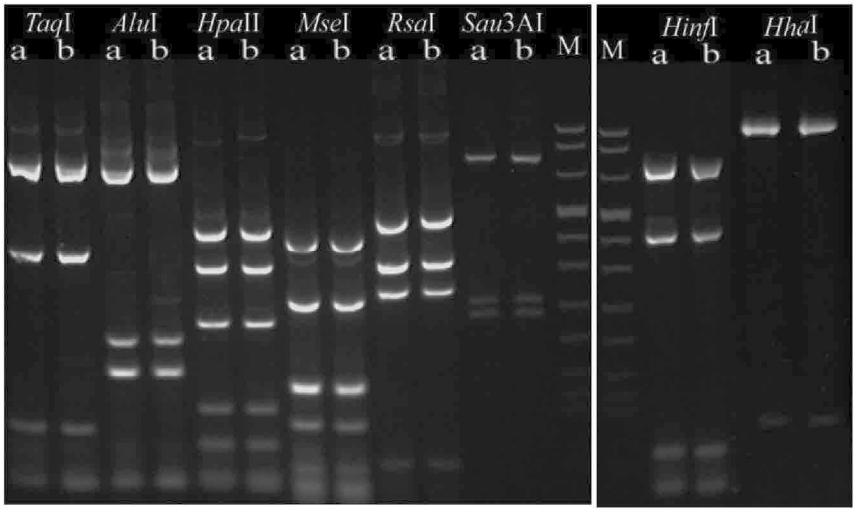

Fig. 2. RFLP profiles of 16S rDNA amplicons obtained in nested PCR primed by P1/P7 followed by R16F2n/R16R2 from Khafr (a) and Neyriz (b) samples of pomegranate little leaf phytoplasmas. Lane M, DNA ladder. DNA products were digested with the enzymes listed at the top of the figure.

onto pTZ57R/T vector and cloned into *Escherichia coli* DH5a cells using InsT/A clone^{M} PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania) according to manufacturer instructions. Presence of the correct insert was confirmed by restriction endonuclease analysis using *Eco*R1 and *Pst*1 enzymes. Plasmid DNA from three recombinant colonies per strain was purified using High Pure Isolation kit (Roche, Germany). Sequencing was performed by Macrogen (South Korea) on both strands by using M13 (-21) forward (5′-TGTAAAACGACGGCCAGT-3′) and M13 (-29) reverse (5′-CAGGAAACAGCTATGACC-3′) primers. The whole length of R16F2n/R16R2-primed rDNA product of KPLL and NPLL phytoplasmas were selected for further analyses. A database search of homologous sequences was performed by BLAST analyses at the National Center for Biotechnology Information (NCBI) to determine the closest phytoplasma relatives to KPLL and NPLL phytoplasmas. R16F2n/R16R2 primed 16S rDNA sequences of KPLL and NPLL phytoplasmas were compared with 21 phytoplasma sequences from GenBank. Sequences were aligned using Clustal W and phylogenetic tree was constructed using the neighbour-joining method with the program MEGA version 5 (Tamura et al., 2011) with 1,000 replicates for bootstrap analysis.

The 16S rDNA homology was evaluated after alignments using homology matrix distance option of DNAMAN program version 4.02 (Lynon Corporation, QC, Canada). Virtual RFLP analysis using *i*PhyClassifier on 1,250 bp sequences (R16F2n/R16R2 region of 16S rRNA gene) was used to compare profiles of KPLL and NPLL to each other and to those of described subgroups of related phytoplasma groups. Each aligned DNA fragment was digested *in silico* with 17 distinct restriction enzymes: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I (*Sau3A*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I used for the phytoplasma 16S rDNA RFLP analysis (Zhao et al., 2009).

Characteristic symptoms of PLL disease were yellowing, little leaf, internode shortening, witches' broom and tree decline. Small and misshaped fruits bearing small, dried and discolored seeds were other disease symptoms (Fig. 1).

By direct amplification with P1/P7 primers a fragment of approximately 1.8 kbp was obtained only from the periwinkle plant infected with lime witches' broom phytoplasma (positive control). No products could be detected when little leaf pomegranate samples and negative controls were used. However, nested PCR by primer pair R16F2n/R2 yielded fragments of approximately 1.2 kbp from all 10 symptomatic pomegranate samples collected from Khafr and Neyriz areas, but not from negative controls (data not shown).

Phytoplasma sequences of KPLL and NPLL strains yielded the same RFLP patterns after restriction with *Alu*I, *Hha*I *Hin*fI, *Hpa*II, *Mse*I, *Rsa*I, *Sau*3AI and *Taq*I enzymes (Fig. 2). Collectively, RFLP patterns analyzed with these enzymes were similar to those of peanut witches' broom phytoplasma (16SrII) group (Lee et al., 1998).

R16F2n/R2 sequences of KPLL and NPLL strains were submitted to the GenBank database under the accession numbers KT265695 and KT265696, respectively. BLAST search showed that these two strains had 99% identity with the sequence of *Corchorus olitorius* phytoplasma strain JPHY2 (Genbank accession number:

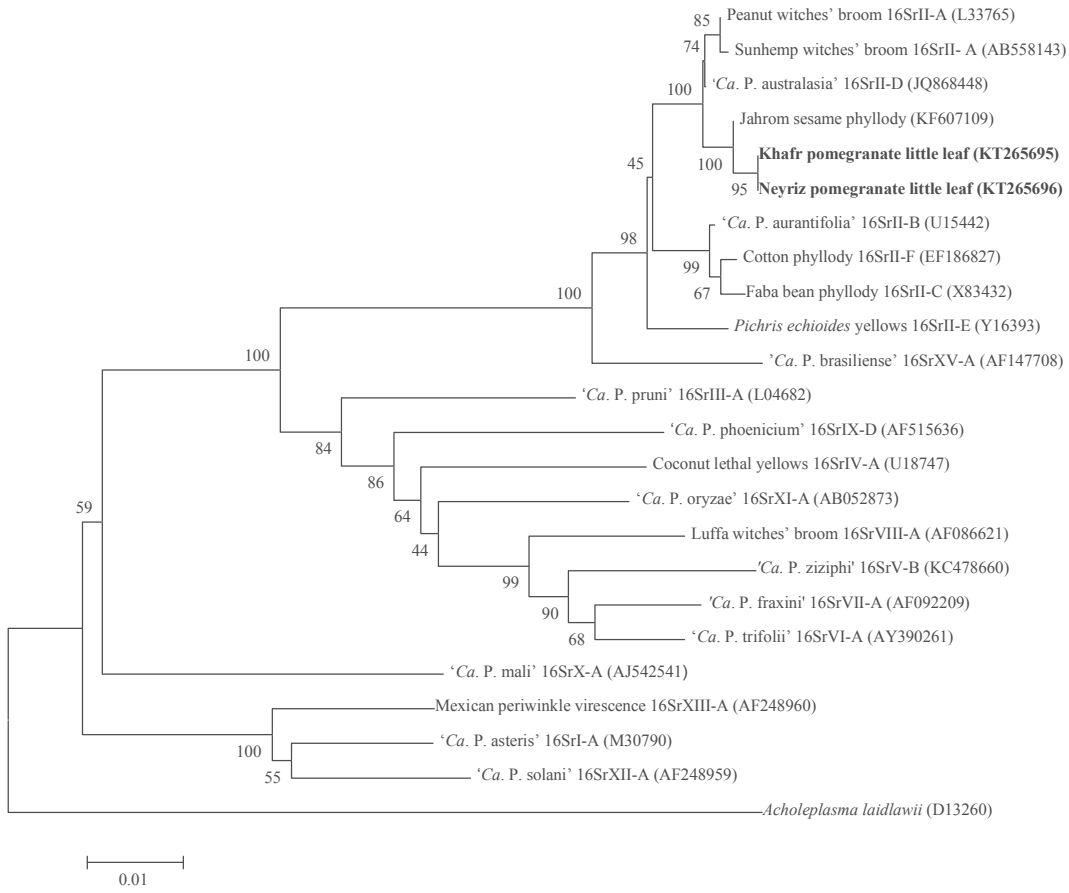

Fig. 3. Phylogenetic tree constructed by neighbour-joining method of 1,250 bp of 16S rRNA gene sequences from 23 phytoplasmas including Khafr and Neyriz pomegranate little leaf phytoplasmas (in bold) and *Acholeplasma laidlawii* as an outgroup. GenBank accession numbers are in parentheses to the right of phytoplasma names followed by the phytoplasma ribosomal subgroups. The numbers on the branches are bootstrap (confidence) values.

KM103729) classified as belonging to 16SrII-D subgroup (Özdemir and Cagirgan, 2015) and with a phytoplasma associated with sesame phyllody disease (Genbank accession number: KF607109) in Jahrom (Fars province of Iran) adjacent to Khafr area. Computer-assisted restriction analyses were carried out on R16F2n/R16R2 sequences of KPLL and NPLL phytoplasmas and the virtual RFLP patterns from both strains were identical to each other and assigned these phytoplasmas to 16SrII-D subgroup (Zhao et al., 2009) (data not shown).

Phylogenetic analysis also clustered KPLL and NPLL strains with 16SrII phytoplasma group closer to a Jahrom sesame phyllody (JSP) phytoplasma and '*Ca.* P. australasia' strain (Genbank accession number: JQ868448) (Fig. 3). Percentage of DNA homology showed that among selected members of 16SrII group ('*Ca.* P. aurantifolia' and '*Ca.* P. australasia'- related strains), KPLL and NPLL phytoplasmas had 100% homology with each other. For both strains, maximum homology percentage (99.45%) was found with '*Ca.* P. australasia' and JSP phytoplasmas.

On the basis of disease symptoms and positive reaction in PCR using phytoplasma universal primer pairs followed by RFLP and sequence analyses, the pomegranate little leaf disease in the two Fars areas was associated with phytoplasma presence. In Fars province, the greatest incidence of PLL disease was found in Khafr where in 2014 an incidence rate of up to 6% was found in some pomegranate orchards. Pomegranate little leaf and yellowing symptoms were observed in other parts of Fars and other provinces of Iran; however plants were not tested for phytoplasma presence. To our knowledge, this work represents the first report of 16SrII-D phytoplasmas in pomegranate.

PLL phytoplasmas were detected in pomegranate only after nested PCR. This may be related to the low concentration of phytoplasmas in plants or to the presence of PCR inhibitors. It has already been reported that polysaccharides may act as PCR inhibitors in pathogen detection (Demeke and Adams, 1992; Heinrich et al., 2001; Fránová, 2011). On the basis of sequence homology, and phylogenetic and RFLP analyses of R16F2n/R2 amplicons, the detected phytoplasmas in pomegranate plants from Khafr and Neyriz are molecularly classified as members of the 16SrII (peanut witches' broom group), subgroup 16SrII-D the same of Jahrom sesame phyllody phytoplasma. Alfalfa (Salehi et al., 1995, 2005) and lime witches' broom (Salehi et al., 2002) are two economically important diseases in Fars province. Phytoplasmas associated with these diseases are members of subgroups 16SrII-C and 16SrII-B, respectively (Salehi et al., 2014), and then probably are not source of PLL phytoplasma. The KPLL and NPLL phytoplasma strains from pomegranate had more than 99% homology with a phytoplasma associated with sesame phyllody in Jahrom sesame fields. Khafr is a central District in Jahrom county and Jahrom sesame phyllody phytoplasma is therefore a possible source of PLL phytoplasma. In Fars province sesame phyllody phytoplasma is vectored by *Circulifer haematoceps* leafhopper (Salehi and Izadpanah, 1992). It is yet to be determined whether Jahrom sesame phyllody phytoplasma and *C. haematoceps* have any role in epidemiology of PLL disease. Studies are in progress to verify this epidemiologic feature in order to define the best management in preventing dangerous epidemic outbreak of the disease in pomegranate.

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References

Anonymous, 2013. Agricultural Statistics. Center for Information, Technology and Communication, Iranian Ministry of Agriculture, p. 138.

Ashkan, S.M., 2006. A Textbook of Fruit Crops Diseases in Iran. Aeeizh, Tehran, Iran, p. 403.

Bardas, G.A., Tzelepis, G.D., Lotos, L., Karaoglanidis, G.S., 2009a. First report of *Botrytis cinerea* causing gray mold of pomegranate (*Punica granatum*) in Greece. Plant Dis. 93, 1346.

Bardas, G.A., Tzelepis, G.D., Lotos, L., Karaoglanidis, G.S., 2009b. First report of *Penicillium glabrum* causing fruit rot of pomegranate (*Punica granatum*) in Greece. Plant Dis. 93, 1347.

Benagi, V.I., Ravikumar, M.R., Nargund, V.B., 2012. Threat of bacterial blight on pomegranate, in India—Mitigation by an integrated approach. In: Melgarejo, P., Valero, D. (Eds.), Options Mediterraneennes Series a: Mediterranean Seminars, II International Symposium on the Pomegranate, 103, pp. 113—116.

Demeke, T., Adams, R.P., 1992. The effect of plant polysaccharides and additives on PCR. BioTechniques 12, 332—334.

Deng, S.J., Hiruki, C., 1991. Amplification of 16S ribosomal-RNA genes from culturable and non culturable mollicutes. J. Microbiol. Meth. 14, 53—61.

Elyatem, S.M., Kader, A., 1984. Postharvest physiology and storage behaviour of pomegranate fruits. Sci. Hortic. 24, 287—298.

Fránová, J., 2011. Difficulties with conventional phytoplasma diagnostic using DP analyses. Bull. Insectol. 64 (Suppl.), S287—S288.

Gazel, M., Caglayan, K., Baspınar, H., Mejia, J.F., Paltrinieri, S., Bertaccini, A., Contaldo, N., 2015. Detection and identification of phytoplasmas in pomegranate trees with yellows symptoms. J. Phytopathol. 164 (2), 136—140.

Gundersen, D.E., Lee, I.-M., 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathol. Mediterr. 35, 144—151.

Hasni Sayyed, H.Y., Patel, M.R., Patil, J.K., 2012. Pharmacognostical and phytochemical study of fruit peel of *Punica granatum* L. J. Pharm. Sci. 3, 3047—3057.

Hebert, T.T., Clayton, C.N., 1963. Pomegranate fruit rot caused by *Coniella granati*. Plant Dis. Rep. 47, 222—223.

Heinrich, M., Botti, S., Caprara, L., Arthofer, W., Strommer, S., Hanzer, V., Katinger, H., Bertaccini, A., Laimer da Camara Machado, M., 2001. Improved detection methods for fruit tree phytoplasmas. Plant Mol. Biol. Rep. 19, 169—179.

Jamadar, M.M., Sataraddi, A.R., Patil, P.V., Jawadagi, R.S., Patil, D.R., 2011. Status of pomegranate diseases of Northen Karnataca in India. Acta Hort. 890, 501—507.

Lee, I.-M., Gundersen-Rindal, D.E., Davis, R.E., Bartoszyk, I.M., 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int. J. Syst. Bacteriol. 48, 1153—1169.

Miguel, G., Antunes, D., Neves, A., 2010. Pomegranate (*Punica granatum* L.): a medicinal plant with myriad biological properties. A short review. J. Med. Plants Res. 4, 2836—2847.

Mirabolfathy, M., Groenewald, J.Z., Crous, P.W., 2012. First report of *Pilidiella granati* causing dieback and fruit rot of pomegranate (*Punica granatum*) in Iran. Plant Dis. 96, 461.

Özdemir, Z., Cagirgan, M., 2015. Identification and characterization of a phytoplasma disease of jute (*Corchorus olitorius* L.) from south-western Turkey. Crop Prot. 74, 1—8.

Salehi, E., Salehi, M., Taghavi, S.M., Izadpanah, K., 2014. A 16SrII-D Phytoplasma strain associated with tomato witches' broom in Bushehr province, Iran. J. Crop Prot. 3, 377—388.

Salehi, M., Izadpanah, K., 1992. Etiology and transmission of sesame phyllody in Iran. J. Phytopathol. 135, 37—47.

Salehi, M., Izadpanah, K., Ebrahimnesbat, F., 1995. Etiology, transmission and host range of alfalfa witches' broom in Iran. Iran. J. Plant Pathol. 31, 1—9.

Salehi, M., Izadpanah, K., Taghizadeh, M., 2002. Witches' broom disease of lime in Iran: new distribution areas, experimental herbaceous hosts and transmission trials. Riverside, CA, USA. In: Proceedings 15th Conference of International Organisation of Citrus Virologists (IOCV), pp. 293—296.

Salehi, M., Heydarnejad, J., Izadpanah, K., 2005. Molecular characterization and grouping of 35 phytoplasmas from central and southern provinces of Iran. Iran. J. Plant Pathol. 41, 62—64.

Salehi, M., Izadpanah, K., Siampour, M., 2011. Occurrence, molecular characterization and vector transmission of a phytoplasma associated with rapeseed phyllody in Iran. J. Phytopathol. 159, 100—105.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual, 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schneider, B., Seemüller, E., Smart, C.D., Kirkpatrick, B.C., 1995. Phylogenetic classification of plant pathogenetic mycoplasmalike organisms or phytoplasmas. In: Razin, S., Tully, J.G. (Eds.), Molecular and Diagnostic Procedures in Mycoplasmology, 1. Academic Press, San Diego, California, USA, pp. 369—380.

Sharma, N.D., Jain, A.C., 1978. Two new fruit rot diseases of pomegranate (*Punica granatum* L.) caused by *Coniella* spp. Curr. Sci. 47, 908—909.

Singh, A., 1997. Fruit Physiology and Production, 4thEdition. Kalyani Publisher, Rajinder Nagar, Ludhiana, India, p. 564.

Snowdon, A.L., 1990. General introduction and fruits. A Color Atlas of Post-harvest Diseases and Disorders of Fruits and Vegetables, 1. CRC Press, Boca Raton, FL, USA, 320 pp.

Stover, E., Mercure, E.W., 2007. The pomegranate: a new look at the fruit of paradise. Sci. Hort. 42, 1088—1092.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731—2739.

Tehranifar, A., Zarei, M., Nematia, Z., Esfandiyaria, B., Vazifeshenas, M.R., 2010.

Investigation of physico-chemical properties and antioxidant activity of twenty Iranian pomegranate (*Punica granatum* L.) cultivars. Sci. Hort. 126, 180—185.

Zhang, Y.P., Uyemoto, J.K., Kirkpatrick, B.C., 1998. A small-scale procedure for extracting nucleic acids from woody plants infected with various phytoplasmas for PCR assay. J. Virol. Methods 71, 45—50.

Zhao, Y., Wei, W., Lee, I.-M., Shao, J., Suo, X., Davis, R.E., 2009. Construction of an interactive online phytoplasma classification tool, *i*PhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). Int. J. Syst. Evol. Microbiol. 59, 2582—2593.