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Short communication

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

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ABSTRACT

During 2012–2014 surveys for the presence of phytoplasma diseases in Fars province (Iran), pomegranate 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 R16F2n/R16R2 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.

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

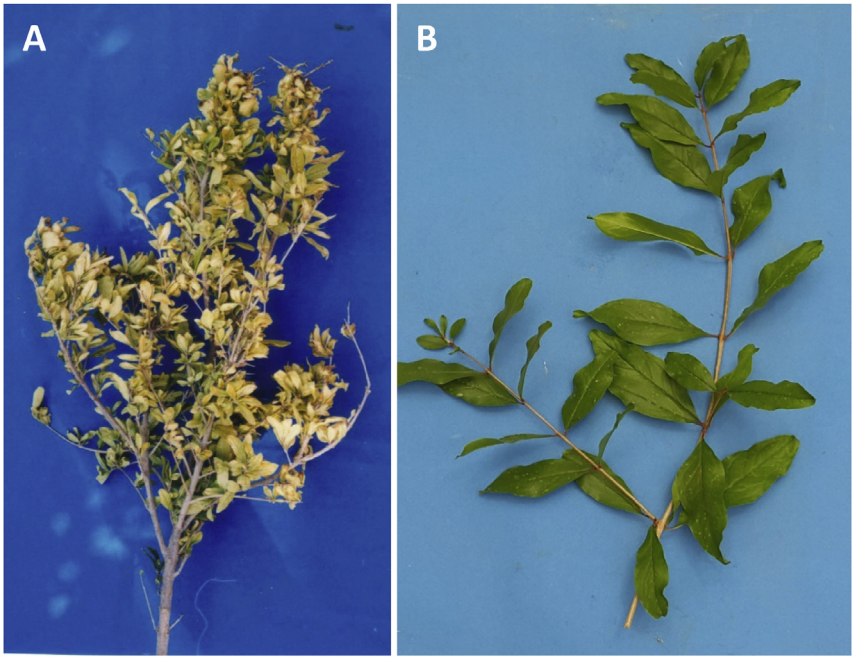

Fig. 1. Yellowing, little leaf, internode shortening and witches' broom in a little leaf affected pomegranate tree from Khafr area (A) compared to a healthy one (B).

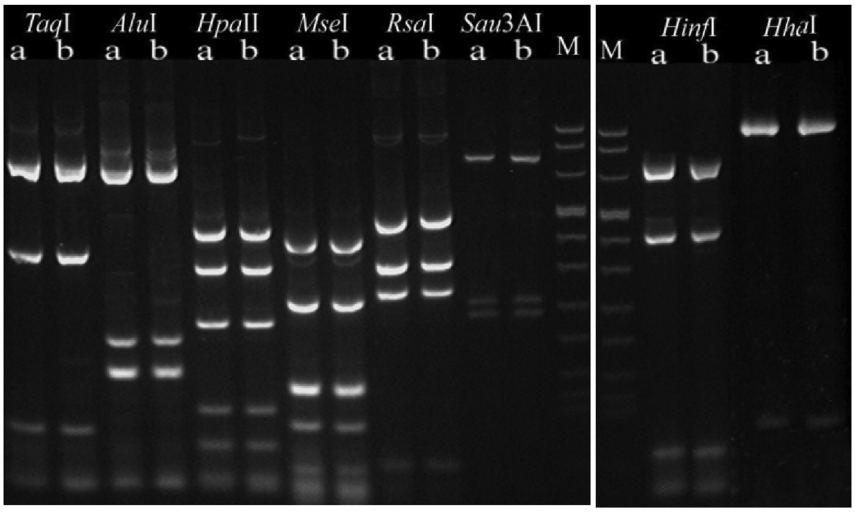

Fig. 2. RFLP profiles of 16S rDNA amplicons obtained in nested PCR primed by P1/P7 followed by R16F2n/R16R2 from Khafr (a) and Neyriz (b) samples of pomegranate little leaf phytoplasmas. Lane M, DNA ladder. DNA products were digested with the enzymes listed at the top of the figure.