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Molecular identification of phytoplasmas associated with some weeds in West Azarbaijan province, Iran

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ABSTRACT

During field surveys in 2013 and 2014, about 14 weed plants showing phytoplasma diseases symptoms including yellowing and witches' broom were collected and tested by polymerase chain reaction (PCR) using universal primers for 16SrRNA starting by primer pairs P1/P7 in first round PCR followed by primer pair R16F2n/R16R2 in nested PCR. The detected phytoplasmas were characterized and differentiated through sequence analysis of PCR-amplified rDNA and virtual restriction fragment length polymorphism (RFLP). The phytoplasmas detected in symptomatic horseweed (*Erigeron canadensis* L.), common madder (*Rubia tinctorum* L.), Johnson grass (*Sorghum halepense* [L.] Pers.) and Sophora root (*Sophora alopecuroides* L.) were identified as members of the clover proliferation group (16SrVI group) by construction of phylogenetic trees. Further analysis by virtual RFLP classified the phytoplasmas of *Erigeron canadensis* L. and *Sorghum halepense* L. in subgroup 16SrVI-A and phytoplasmas of *Rubia tinctorum* L. and *Sophora alpecuriodes* L. in subgroup 16SrVI-D. This is the first report on the occurrence of phytoplasma diseases of weeds in west Azarbaijan, Iran.

Key words: common madder, Sophora root, Johnson grass, horseweed, nested-PCR, Urmia

IZVLEČEK

MOLEKULSKA DOLOČITEV FITOPLAZEM NAJDENIH V NEKATERIH PLEVELIH V PROVINCI ZAHODNI AZARBEJDŽAN, IRAN

Med pregledom polj, v letih 2013 in 2014, je 14 plevelnih vrst kazalo simptome okužbe s fitoplazmami kot so rumenenje in čarovniške metle. Nabrane vzorce smo analizirali z verižno reakcijo s polimerazo (PCR) z uporabo splošnih začetnih oligonukleotidov za 16SrRNA, začenši s pari začetnih oligonukleotidov P1/P7 v prvem krogu PCR analize, ki ji je sledila vgnezdena PCR anliza s parom začetnih oligonukleotidov R16F2n/R16R2. Odkrite fitoplazme so bile okarakterizirane in razlikovane s sekvenčno analizo PCR pomnožene rDNA in virtualnim polimorfizmom dolžin restrikcijskih fragmentov (RFLP). Fitoplazme, ki smo jih odkrili v simptomatični kanadski suholetnici (*Conyza canadensis* (L.) Cronq.), pravem brošču (*Rubia tinctorum* L.), divjem sirku (*Sorghum halepense* (L.) Pers.) in korenasti sofori (*Sophora alopecuroides* L.) so bile z izdelavo filogenetskega drevesa določene kot pripadnice skupine, ki povzroča proliferacijo detelje (16SrVI skupina). Nadaljnja analiza z virtualno klasifikacijo RFLP je fitoplazmi v kandski suholetnici in divjem sirku uvrstila v podskupino 16SrVI-A in fitoplazmi v pravem brošču in korenasti sofori v podskupino 16SrVI-D. To je prvo poročilo o pojavljanju od fitoplazem povzročenih bolezni na plevelih v zahodnem Azarbeidžanu, Iranu.

Ključne besede: pravi brošč, korenasta sophora, divji sirek, kanadska suholetnica, vgnezdena-PCR, Urmia

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1 INTRODUCTION

Phytoplasmas are a group of wall-less phloem-limited plant pathogenic bacteria belonging to Mollicutes which have been described relative recently (Lee *et al*., 1998). They are associated with diseases in several hundreds of plant species, including weeds (Marcone *et al*., 1997). Typical symptoms include virescence/phyllody, sterility of flowers, proliferation of axillary buds resulting in witches broom growth, abnormal internodes elongation and generalized stunting (Bertaccini *et al*., 1996; 2014). Phytoplasmas are introduced by insect vectors (mostly leafhoppers) during feeding activity into plant sieve tube elements, from which they spread systemically through the plants (Bertaccini *et al*., 2014). Currently, characterization and classification of phytoplasmas are based mainly on restriction fragment length polymorphism (RFLP) and sequence analysis of 16SrDNA or other less-conserved genes, whereas detection is done mainly by polymerase chain reaction (PCR) assay (Bertaccini *et al*., 2014; Lee *et al*., 2000; Seemuller *et al*., 1998). Weeds serve as both a reservoir for phytoplasma infection and as reproductive hosts for the vectors (Singh and Upadhyaya, 2013). Phytoplasmas are known to cause diseases in weeds including field bindweed (*Convolvulus arvensis* L.), prickly lettuce (*Lactuca serriola* L.), Johnson grass (*Sorghum halepense* (L.) Pers.), bermuda grass (*Cynodon dactylon* (L.) Pers.), horseweed (*Conyza canadensis* (L.) Cronq.) and some others were reported worldwide (Arocha-Rosete and Jones, 2010; Babaie *et al*., 2007; Chen *et al*., 2003; Li *et al*., 2013; Marcone *et al*., 1997; Salehi *et al*., 2006; Shubhrata, 2004; Thereza and Baross, 2002; Vali Sichani *et al*., 2014). However little is known about phytoplasma diseases of weeds in west Azarbaijan province of Iran. The aim of this study was to verify the presence of phytoplasma diseases in symptomatic weeds in West Azarbaijan province using PCR assay. The detected phytoplasmas were characterized and classified using sequence analysis of PCR-amplified 16SrDNA and virtual gel RFLP.

2 MATERIALS AND METHODS

2.1 Plant materials

Fourteen weed plants related to 4 plant species including Johnson grass (*Sorghum halepense* (L.) Pers.), Canadian horseweed (*Conyza canadensis*. (L.) Cronquist), Sophora root (*Sophora alopecuroides* L.) and common madder (*Rubia tinctorum* L.) showing symptoms typical of phytoplasmal infection were collected during 2013 and 2014 growing seasons (Table 1) from different regions of West Azarbaijan province including Urmia, Salmas, Khoy and Mahabad cities. Asymptomatic plants were also collected and used in molecular analysis as negative controls.

2.2 DNA Extraction

Total DNA was extracted from 0.25g of leaves and midribs according to the method described by Zhang *et al* (1998). Total DNA of healthy plants were extracted and used as negative controls.

2.3 PCR Analysis and Primer Pairs

The universal primer pair P1/P7 (Schneider *et al*., 1995) was employed in first round PCR to amplify a 1.8kbp fragment of 16SrDNA. A 30-fold dilution of the first round PCR product used as template for nested PCR using primer pair R16f2n/R16R2 which amplified an internal fragment of 1.2kbp from the 16SrDNA (Lee *et al*., 1993). The total volume of 20µl PCR reaction mixtures contained 20ng DNA, 0.2mM of each dNTP (Cinnagen, Iran), 1.6mM $MgCl_2$, 1U of *Taq* DNA polymerase (Cinnagen, Iran), 0.5µl of each primer pair (20pmol/µl) and 1X polymerase buffer. The reaction mixtures were subjected to 35 cycles at the following conditions: First round PCR (35 cycles): 1 min (3 min for the first cycle) for denaturation step at 94ºC, 1 min for annealing at 57ºC and 1.5 min (10 min for the last cycle) for primer extension step at 72ºC. Second round nested PCR (35 cycles): 2 min (5 min for the first cycle) for denaturation step at 94ºC, 1 min for annealing at 57ºC and 2 min (10 min for the last cycle) for primer extension step at 72ºC. The PCR

products were analyzed by electrophoresis in a 1 % agarose gel and stained with ethidium bromide. An ultraviolet (UV) transilluminator was used to visualize DNA band.

2.4 Sequencing and Phylogenetic Analysis

PCR products of nested PCR were sequenced directly. Sequencing was performed by Macrogen (South Korea) on both strands. Nucleotide sequence similarity, multiple alignment and phylogenetic tree construction using the neighbor-joining (NJ) method were done with MEGA5 software (Tamura *et al*., 2011) and subjected to bootstrap analysis using 500 replicates. The *Acholeplasma laidlawii* isolate was used as outgroup.

2.5 Virtual RFLP Analysis

Virtual RFLP analysis using iPhyclassifier (Zhao *et al*., 2009) was used to determine 16Sr group and subgroup affiliation of the detected phytoplasmas. RFLP profiles of detected phytoplasmas were compared to those of 16SrVI–subgroups A, B, C, D, E, F, H using *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I enzymes.

3 RESULTS AND DISCUSSION

During growing seasons of 2013 and 2014, fourteen weed samples with phytoplasma symptoms were collected from different parts of West Azarbaijan province, Iran. The symptoms varied with the host plant and the most characteristic symptoms were witches' broom, leaf malformation, little leaf and yellowing (the symptoms are summarized in Table 1 and some symptomatic plants were shown in Fig.1).

Table 1: Weeds showing phytoplasma-like symptoms

Common name	Scientific name	Plant family	symptoms	No of samples	Place of sampling	Date of collection	Latitude, Altitude and height above level sea of the city
Johnson grass	*Sorghum halepense* (L.) Pers.	Gramineae	Little leaf	2	Salmas	2 June 2014	38° N, 44°E, 1406 meters
Canadian horseweed	*Conyza canadensis* (L.)Cronq.	Asteraceae	Leaf malformation and witches' broom	6	Khoy	17 June 2013	38° N, 44°E, 1139 meters
Sophora root	*Sophora alopecuroides* L.	Fabaceae	Yellowing and little leaf	2	Abajaloo-Urmia	21 July 2014	37° N, 45°E, 1362 meters
Common madder	*Rubia tinctorum* L.	Rubiaceae	Little leaf	4	Mahabad	20 July 2013	36° N, 45°E, 1304 meters

Figure 1: Weeds with phytoplasma-like symptoms in West Azarbaijan province. A-Common madder showing little leaf, B- Johnson grass with little leaf symptoms, C- Sophora root with yellowing and little leaf symptoms, D-Canadian horseweed showing leaf malformation and witches' broom.

DNA fragments of approximately 1.8kbp and 1.25kbp were amplified using phytoplasma universal primer pairs, P1/P7 and R16f2n/R16R2 in first round and nested PCR, respectively from Johnson grass, Canadian horseweed, Sophora root and common madder. Neither by direct nor by nested PCR were DNA amplified form other weeds tested in this survey and from asymptomatic plants. The nucleotide sequences of the phytoplasmas detected in four plants (Johnson grass, Sophora root, Canadian horseweed and common madder) were deposited in GenBank (with accession numbers of KT807469, KT807470, KT807471 and KT807472, respectively). BLAST search showed that the 16SrDNA sequences of four detected phytoplasmas shared the highest homology (99 %) to members of the 16SrVI group '*Candidatus* Phytoplasma trifolii'. Computer-stimulated restriction analysis were carried out on R16f2n/R16R2 sequences from Johnson grass, Canadian horseweed, Sophora root and common madder together with 12 reference phytoplasmas and seven representative strains belonging to 16SrVI subgroups (A, B, C, D, E, F, H). Comparison of virtual gel plotted images revealed that RFLP patterns of common madder (Ruph) and Sophora root (Tph) were most similar to periwinkle little leaf, representative of 16SrVI-D subgroup and RFLP patterns of Canadian horseweed (Pph) and Johnson grass (Nph) were most similar to clover proliferation, representative of 16SrVI-A (Fig. 2). Phylogenetic analysis of sequences presented in this survey with 19 phytoplasmas from GenBank clustered phytoplasmas detected on Johnson grass, Canadian horseweed, Sophora root and common madder with phytoplasmas of 16SrVI group (Fig.3).

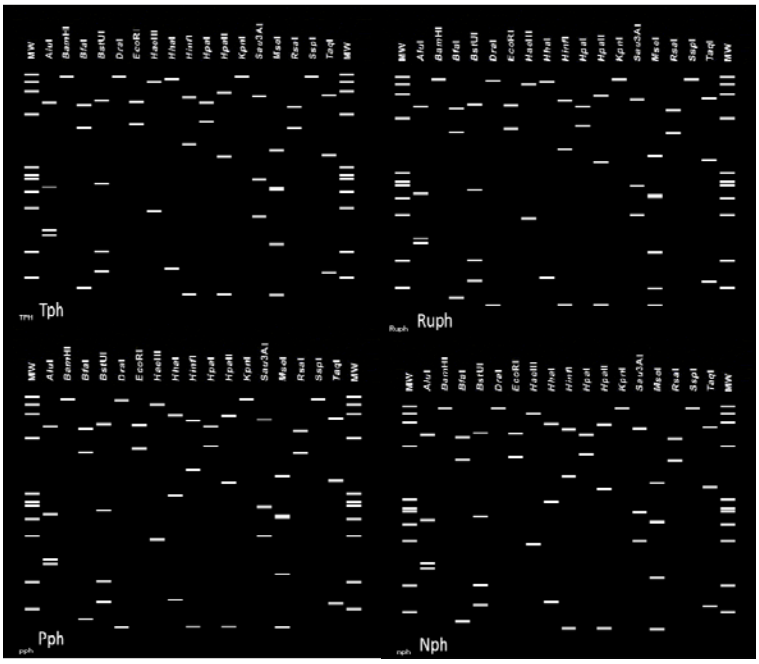

Figure 2: Virtual restriction fragment length polymorphism (RFLP) pattern of $R_{16}F_{2n}/R_{16}R_2$ PCR product sequence from Sophora root (Tph), common madder (Ruph), Canadian horseweed (Pph), and Johnson grass (Nph) phytoplasmas. Restriction sites for the 17 restriction enzymes were used in the simulated digestions: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I.

Two phytoplasmas which were previously reported from Canadian horseweed exhibiting yellowing and witches' broom symptoms were classified in 16SrI (SrI-A) (Lee *et al*., 2000) and 16SrVII groups (Thereza and Baross, 2002), respectively. This is the first report of little leaf symptom on Canadian horseweed that is classified in phytoplasma 16SrVI group. The phytoplasma which causes yellowing on Johnson grass, detected in our survey, was classified in 16SrVI group. Previously, on Johnson grass exhibiting yellowing Arocha-Rosete and Jones (2010) and Singh and Upadhyaya (2013) found phytoplasmas of 16SrXXIV-A and 16SrXII groups, respectively. Sophora root with little leaf and yellowing symptoms was classified in 16SrVI group in this study. Previous reports of phytoplasmas affecting *Sophora* species include a 16SrXII phytoplasma associated with *S. japonica* L (*Styphnolobium japonicum* (L.) Schott.) yellows in China (Duduk *et al*., 2010), '*Ca.* Phytoplasma ziziphi' in China associated with witches' broom and a 16SrI '*Ca.*Phytoplasma asteris' associated with Sophora root yellows (Yu *et al*., 2012; Chen *et al*., 2013). Recently Allahverdi *et al*. (2014) reported a 16SrXII phytoplasma association with *S. alopecuroides* from Firooz-koh (Tehran Province, Iran) with leaf yellowing, little leaf and stunting symptoms. The association of a phytoplasma belonging to 16SrVI was previously established in Sophora root (*Sophora alopecuroides* L.) exhibiting yellowing and witches' broom symptoms from China (Li *et al*., 2013). There is no data on common madder phytoplasma infection and to our knowledge it is the first report of phytoplasma (16SrVI group) infection of common madder.

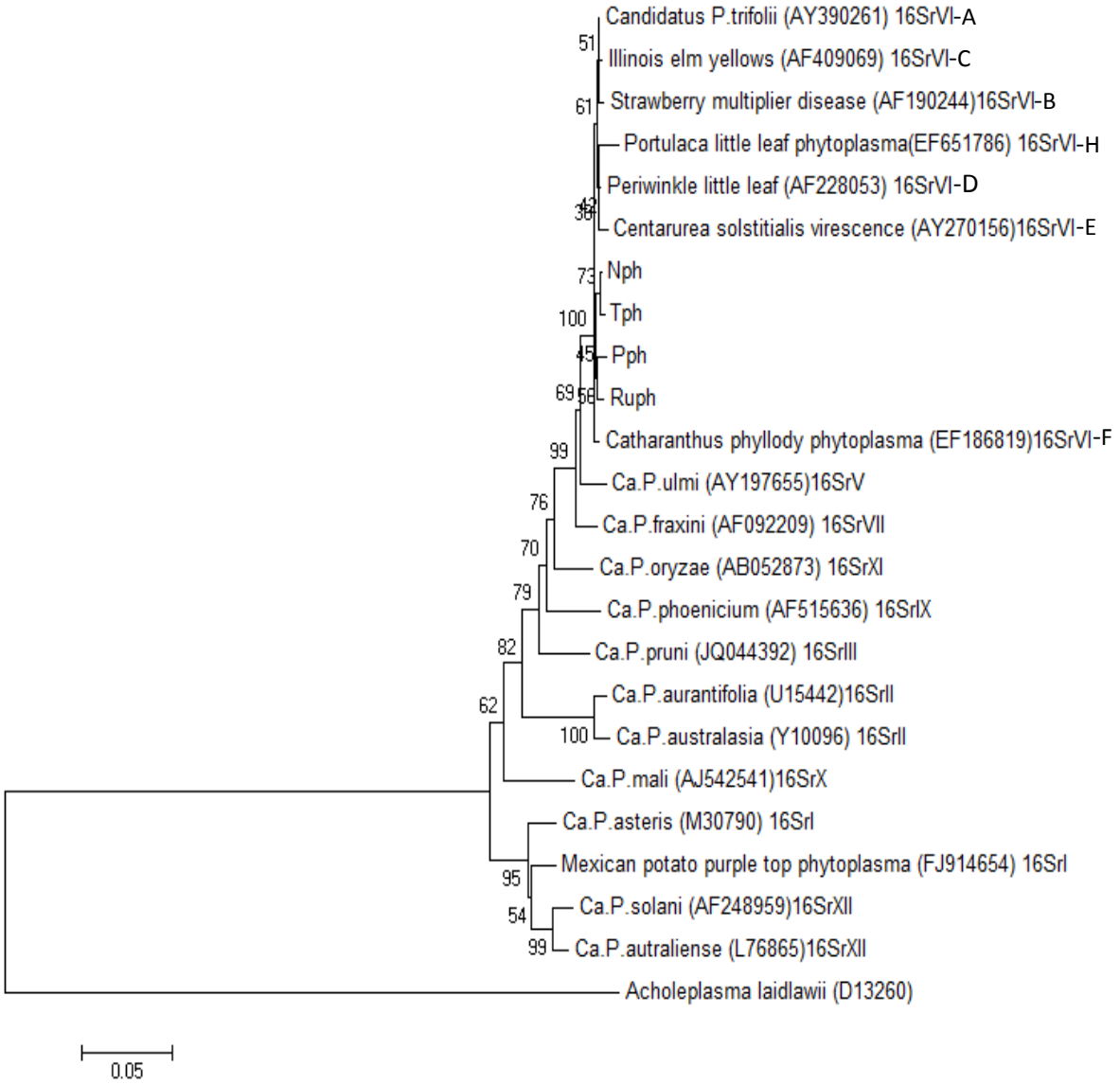

Figure 3: Phylogenetic tree constructed by the neighbor joining method of 16SrRNA gene sequences from 19 phytoplasma and phytoplasmas identified from Sophora root (Tph), common madder (Ruph), Canadian horseweed (Pph), and Johnson grass (Nph) and *Acholeplasma laidlawii* as outgroup. Numbers at the nodes are bootstrap values based on 500 repetitions. GenBank accession numbers for sequences are given in parentheses.

4 CONCLUSIONS

To date, phytoplasmas have been documented in more than 100 weed plant species. Phytoplasmas cause diseases on several weeds which result in serious threat as a source of alternative natural host for the spread of phytoplasma pathogen to other economically important plants, thereby creating a chance of causing severe losses. Detection of phytoplasma associated with diseases of weed crops is very important to check the possibility of further spread of their diseases to other plants. Results of this study can facilitate further works on ecology, epidemiology and diversity of phytoplasmas in Iran. This is the first report on the occurrence of phytoplasma diseases on weeds in West Azarbaijan province, Iran.

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