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ORIGINAL ARTICLE

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Molecular detection and characterization of a stolbur phytoplasma associated with *Narcissus tazetta* phyllody in Iran

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Abstract

During field surveys in 2015, a phytoplasma-associated disease was identified in *Narcissus tazetta* plants in Behbahan, Iran. The characteristic symptoms were phyllody and virescence. The presence of phytoplasma in symptomatic plants was confirmed using PCR amplification and sequencing of *16S rRNA*, *tuf*, *sec*Y and *vmp1* genes. Based on the BLASTN results, the sequences of 16S rRNA, *tuf*, *sec*Y and *vmp1* genes shared, respectively, 99%, 100%, 99% and 99% sequence identity with phytoplasma strains in 16SrXII-A subgroup. RFLP and phylogenetic analyses using the sequences of 16S rRNA, *tuf* and *sec*Y genes confirmed the assortment of studied strains to 16SrXII-A phytoplasma subgroup. Sequence comparison of these four genes revealed that all the sequences of 28 strains studied were identical. To the best of our knowledge, the association of "*Candidatus* Phytoplasma solani" with *N. tazetta* was demonstrated for the first time in the world.

KEYWORDS
Narcissus, phyllody, phytoplasma, RFLP, virescence

1 | INTRODUCTION

Narcissus tazetta L. (family Amaryllidaceae) is a perennial ornamental and medicinal plant that is cultivated commercially for its essential oils. Several alkaloids isolated from this species have potential antitumor and antiviral activities (Evidente et al., 1994; Kornienko & Evidente, 2008; Ramanathan, Furusawa, Kroposki, Furusawa, & Cutting, 1968). The origin of *N. tazetta* is Mediterranean region across the Middle East (Kornienko & Evidente, 2008). The most important growing regions of *N. tazetta* in Iran are Khuzestan (Behbahan) and Shiraz provinces.

During field surveys in 2015, phyllody and virescence symptoms were observed in Behbahan, Iran. Although the phyllody and virescence may be of the non-infectious type (e.g., environmental condition changes, herbicides applications or physiological changes), the biotic factors including phytoplasmas, viruses or other pathogens can also cause these symptoms. However, the phytoplasma pathogens were reported as the most important infectious causal agent of phyllody in several plant species (Bertaccini, Duduk, Paltrinieri, & Contaldo, 2014; Weintraub & Jones, 2010).

Phytoplasmas are cell wall-less and obligate intracellular parasites inhabit phloem sieve elements of infected plants. These microorganisms can easily transmitted by sap-sucking insects, vegetative propagation and grafting (Bertaccini et al., 2014; Weintraub & Jones, 2010). Once the phytoplasma established in a plant, it causes physiological disorders and interferes with the plant defence system causing the losses up to 50%–100% (Bertaccini et al., 2014). Many plant species (including vegetables, cereals, ornamentals, fruits, forest plants) of 300 genera were globally recorded as the host of phytoplasma microorganisms (Weintraub & Jones, 2010). Various phytoplasmas have been reported to cause phyllody and virescence symptoms in plants (Akhtar, Dickinson, Asghar, Abbas, & Sarwar, 2016; Hemmati, Nikooei, Bagheri, & Faghihi, 2017; Ikten et al., 2014; Pamei & Makandar, 2016; Reddy et al., 2014; Win, Back, & Jung, 2010). To the best of our knowledge, there is only one report of phytoplasma disease in narcissus plants (Bellardi, Pisi, & Vicchi, 1990).

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In the study carried out by Bellardi et al. (1990), the symptoms of flower phyllody, green petals, degenerated flowers and chlorotic yellow leaves were observed in *Narcissus* sp. plants in Emilia-Romagna, Italy. They confirmed the association of these symptoms with phytoplasma infection using electron and optical fluorescent microscope. This study was carried out to ascertain the presence of phytoplasma in *N. tazetta* plants showing phyllody and virescence symptoms in Iran.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Thirty-two symptomatic narcissus flowers showing high proliferation of petals, virescence and phyllody and 10 symptomless plants were collected in 2015 from narcissus growing region in Behbahan known as "Nargeszar" with an area of 30 ha. The samples were frozen in liquid nitrogen until processed. The DNA of PW30 clone (KY398726.1) from potato (preserved in the Plant Protection Lab, Isfahan University of Technology, Iran) was used as a representative member of 16SrXII-A subgroup to perform real RFLP analysis.

2.2 | DNA extraction and PCR assays

Total DNA of all collected samples was extracted according to the protocol described by Murray and Thompson (1980). Extracted DNA was used as a template for PCR. DNA of two symptomless specimens and ddH_2O were employed as a negative control. Universal phytoplasma primers P1/P7 were used in direct PCR to amplify the 16S rRNA region (Deng & Hiruki, 1991; Schneider, Seemüller, Smart, & Kirkpatrick, 1995). PCR parameters were performed as previously described (Lee, Gundersen-Rindal, Davis, & Bartoszyk, 1998). Then, PCR products were diluted 1:100 with sterile distilled water and 3 µl was used in nested PCR with P1A/R16R2 (1,359 bp) and R16F2n/R16R2 (1,239 bp) primer pairs (Gundersen & Lee, 1996). All PCR amplifications were performed in an automated thermocycler Techne TC-512 (Bibby Scientific Ltd.). The PCR products were separated in 1% agarose gel stained with ethidium bromide and visualized via UV transilluminator. The marker used for the estimation of the molecular weight of PCR products was 100-bp DNA ladder (Fermentas, Vilnius, Lithuania).

All phytoplasma strains obtained were also subjected to genotyping on three non-ribosomal genes: *vmp*1, *sec*Y and *tuf*. The *sec*Y gene was amplified using POSecR1/POSecF1 (1,450 bp) primers, and the *vmp1* gene was amplified with primer pair TYPH10F/R (998 bp) (Fialová et al., 2009). Based on the protocol described by Schneider and Gibb (1997), the *tuf* gene was amplified with primer pair fTufu/rTufu (842 bp).

2.3 | DNA sequencing

The amplified nested PCR products (1,239 bp) using R16F2n/R16R2 primers from five strains studied were purified using GENECLEAN®III kit (Qbiogene, Cambridge, UK) and ligated into the pGEM®-T Easy vector (Promega, Madison, WI, USA) based on the manufacturer instructions. The competent *Escherichia coli* MC1061 was transformed using an aliquot of ligation mixture. Transformants were screened by digestion with *Eco*RI restriction enzyme (Fermentas), and finally, the recombinant colonies were sequenced in MacroGen (Seoul, Korea). For genes *vmp*1, *sec*Y and *tuf*, the amplicons from five strains for each gene were also sequenced. The fragments were sequenced on both strands and employed for phylogenetic analysis. The sequences of each gene were assembled by MEGA6 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Blast searches were carried out using the nucleotide BLAST program (BLASTN) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 | RFLP assays and phylogenetic analysis

P1A/R16R2 primed amplicons from NAR-1 strain and PW30 clone (KY398726.1) were digested using *Rsa*I, *Hin*P1I and *Taq*I restriction enzymes according to the manufacturer instructions (Fermentas). The restricted fragments were visualized via 2% agarose gel stained with ethidium bromide. Computer-simulated RFLP analysis of 16S rRNA, *tuf* and *sec*Y gene sequences of the phytoplasma strain NAR-1 and the sequences of described 16SrXII subgroups was also carried out using PDRAW32 software (ACACLONE Software). The sequence of each gene was digested in silico with 17 restriction enzymes.

For analysis of nucleotide sequences, multiple sequence alignments were performed by CLUSTALW embedded in MEGA6 software (Tamura et al., 2013). All the changes such as insertions, deletions and nucleotide substitutions were determined in comparison with the STOL11 reference strain (Quaglino et al., 2013).

Three partial sequenced genomic loci of 16S rRNA, *tuf* and *sec*Y genes were used for phylogenetic analysis. In this respect, the analysis was conducted with MEGA6 software (Tamura et al., 2013) using the Neighbor-Joining method and a bootstrap test with 1,000 replicates.

3 | RESULTS

3.1 | Symptoms description

The symptoms were a high proliferation of petals, virescence and phyllody during the flowering stage (Figure 1). The flower durability of symptomatic plants was lower than healthy ones. In "Nargeszar," near 2% of plants indicated such symptoms.

3.2 | Molecular detection of phytoplasma

Phytoplasma detection was performed by PCR amplification of 16S rRNA, *tuf*, *sec*Y and *vmp1* gene sequences. Twenty-eight of 32 symptomatic samples resulted positive by nested PCR amplification with P1A/R16R2 and R16F2n/R16R2 primers (data not shown). The presence of phytoplasma was not detected in other four samples

FIGURE 1 The symptoms of narcissus plants infected with *Candidatus* Phytoplasma solani; (a) infected (high proliferation of petals and light virescence) and healthy plants; (b) virescence and phyllody symptom

showing phyllody symptoms. Similarly, no PCR fragments were amplified from negative control and asymptomatic samples. The expected amplification was also positive for other three mentioned genes in identical 28 symptomatic samples.

3.3 | BLASTN search and sequence homology

The identity of obtained sequences with those phytoplasma from GenBank was assessed through BLASTN search. The results revealed that the sequences of 16S rRNA, *tuf*, *sec*Y and *vmp1* genes amplified from 28 symptomatic samples shared, respectively, 99%, 100%, 99% and 99% sequence identity with phytoplasma strains belonged to 16SrXII-A subgroup. The obtained sequences of 16S rRNA, *sec*Y, *vmp1* and *tuf* genes were 1,160, 925, 1,703 and 803 bp long, respectively. All nucleotides of the genes were compared in BLASTN search, while 1,159, 924, 1,071 and 803 bp were identical to reference accessions for 16S rRNA, *sec*Y, *vmp1* and *tuf* genes, respectively. All amplified and sequenced fragments from each gene were completely identical, and no diversity was observed within the region. In this respect, one sequence for each gene from NAR-1 strain was deposited in GenBank under accessions KR066493.2 (1,160 bp) for the 16S rRNA gene, KY315179 (803 bp) for the *tuf* gene, KY315180 (925 bp) for the *sec*Y gene and KY315181 (1,073 bp) for the *vmp1* gene.

3.4 | RFLP profiles

The amplified 16S rRNA fragments from NAR-1 strain and PW30 clone (KY398726.1) using P1A/R16R2 primers were digested by *Rsa*I, *Hin*P1I and *Taq*I restriction enzymes (Figure 2). Virtual RFLP analysis was also performed based on the sequences of the 16S rRNA, *sec*Y and *tuf* genes. The sequences of the same genes from STOL11 reference strain were restricted in silico and compared with NAR-1 strain restriction patterns. RFLP patterns of NAR-1 strain showed the highest identity with the members of the 16SrXII-A subgroup and were completely identical to RFLP patterns

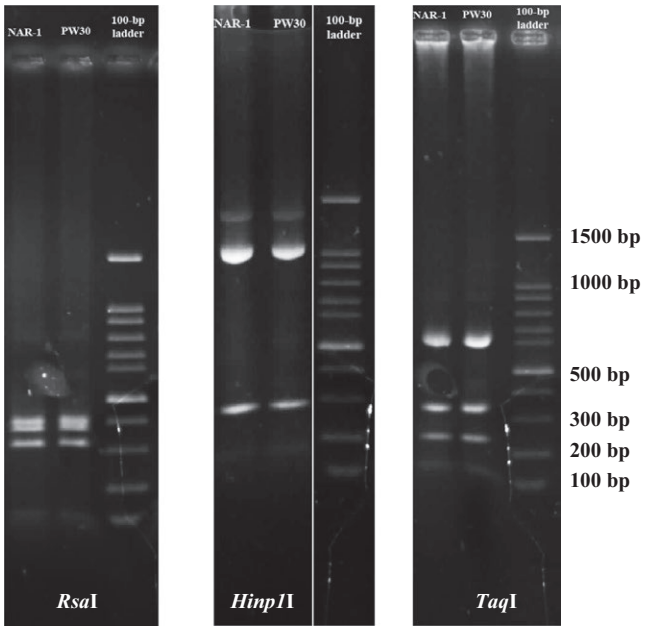

FIGURE 2 RFLP patterns obtained from P1A/R16R2 primed fragments from NAR-1 strain compared with PW30 clone (KY398726.1) belonged to 16SrXII-A subgroup. PCR products were digested by three *Rsa*I, *Taq*I and *Hin*P1I restriction enzymes

of STOL11 strain (Figure S1). The only difference was found in *sec*Y gene digested by *Hinf*I restriction enzyme. With H*inf*I digestion of *sec*Y gene, there were three bands for NAR-1 strain, while the STOL11 strain had four bands.

3.5 | Phylogenetic analysis

Nucleotide sequence alignments revealed that the gene sequences of NAR-1 strain had some nucleotide substitutions as compared to STOL11 reference strain (Quaglino et al., 2013). In the case of 16S rRNA gene, there were nucleotide substitutions including T-A, A-G, C-A and T-C at positions 628, 719, 1,012 and 1,208, respectively.

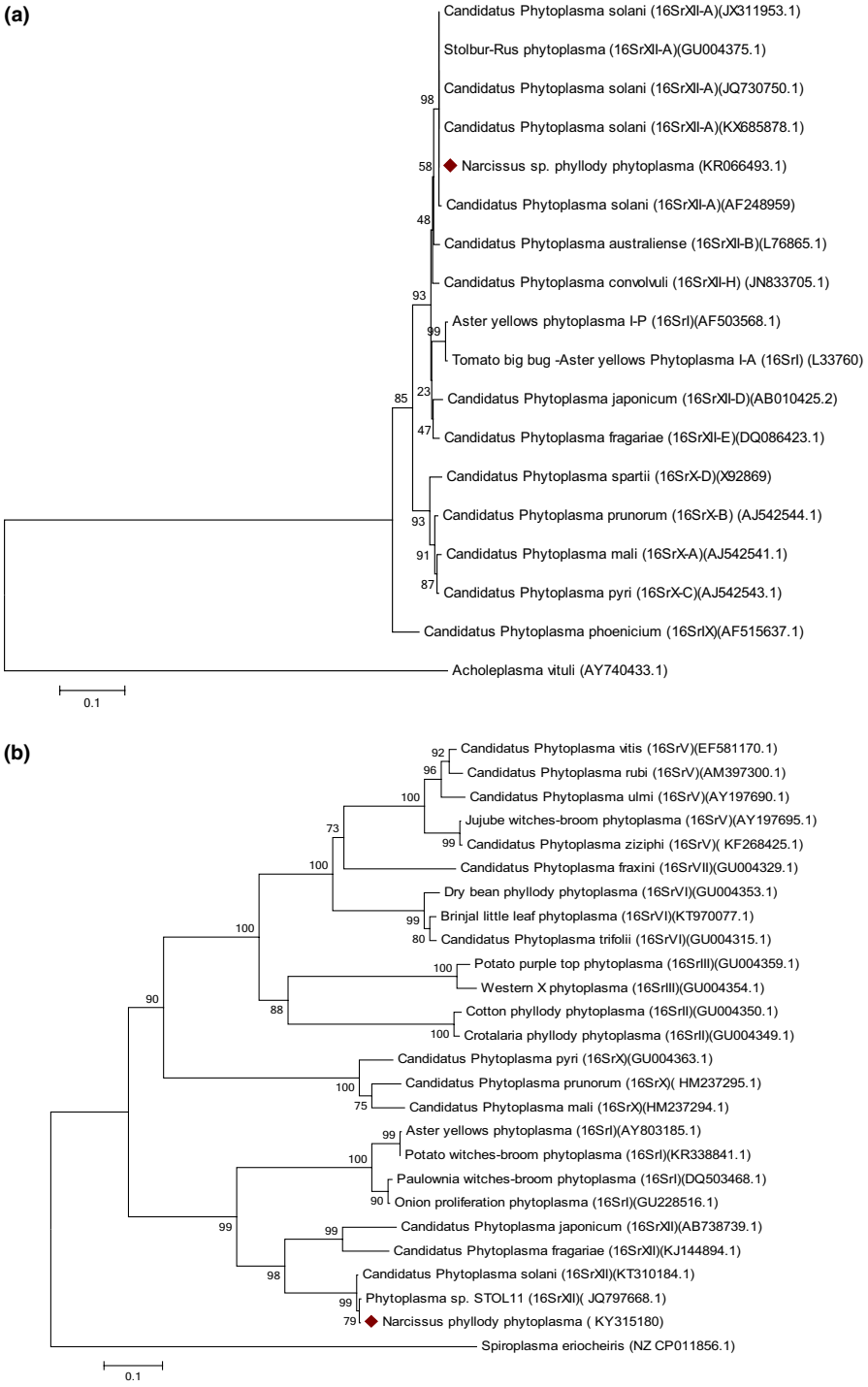

FIGURE 3 The evolutionary history was inferred using the Neighbor-Joining method. Phylogenetic trees constructed on the basis of (a) 16S rRNA, (b) secY and (c) tuf genes. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. (a) The analysis involved 18 nucleotide sequences. The optimal tree with the sum of branch length = 1.53673265 is shown; (b) the analysis involved 26 nucleotide sequences. The optimal tree with the sum of branch length = 4.15146671 is shown and (c) the analysis involved 19 nucleotide sequences. The optimal tree with the sum of branch length = 3.41409623 is shown. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6

For *sec*Y gene, there were four nucleotide substitutions containing A-C, G-A, C-G and C-T at positions 356, 367, 795 and 797, respectively. The nucleotide positions were calculated from the beginning of STOL11 reference strain sequences (Acc. No. AF248959 for 16S rRNA gene and Acc. No. JQ797668 for *sec*Y gene). The G-A nucleotide substitution at position 367 in *sec*Y gene removed the recognition site for *Hinf*I restriction enzyme in NAR-1 strain. Although nucleotide substitutions were confirmed in the 16S rRNA and *sec*Y gene sequences from NAR-1 strain compared to STOL11 reference strain, these differences had no significant impacts on recognition sites (except for G-A substitution at position 367 in *sec*Y gene) for any of 17 restriction enzymes employed for virtual RFLP analysis of phytoplasmas.

The phylogenetic trees were constructed based on the 16S rRNA, *sec*Y and *tuf* genes using the Neighbor-Joining method. A bootstrap test with 1,000 replicates was applied to validate the trees. All trees indicated that the detected phytoplasma in *N. tazetta* clustered with phytoplasmas in the 16SrXII group and shared a common clade with 16SrXII-A subgroup members (Figure 3).

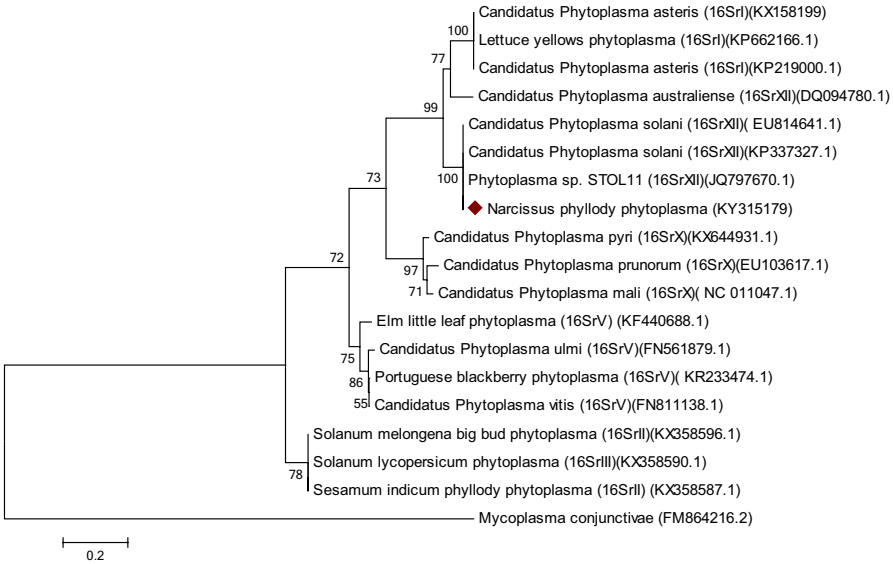

FIGURE 3 (Continued)

4 | DISCUSSION

The main purpose of this study was to determine the association of symptoms observed in *N. tazetta* in Behbahan with phytoplasma infection. PCR amplification of four genes revealed positive in 28 of 32 symptomatic plants. The symptoms in other four PCR-negative symptomatic plants may be due to the environmental factors that result in an imbalance in plant hormone. On the other hand, there is a form of *N. tazetta* that has double petals which is called Double Roman. This trait (double petal formation) may have a genetic origin (e.g., genetic change). The flower form of Double Roman plants is very similar to infected plants with phytoplasma showing proliferation of petals. Thus, it is likely that the four non-positive symptomatic plants are Double Roman plants, which are called traditionally "PorPar" in Iran. No other distinct symptoms or pest damage except the leaf tip burn in some cases were observed on collected samples.

On the basis of sequencing, real and virtual RFLP and phylogenetic analyses, it was turned out that the detected phytoplasma is a member of 16SrXII-A subgroup. Some nucleotide differences were observed between sequences of 16S rRNA and *sec*Y genes compared to the sequences of STOL11 reference strain, but these alternations had no significant effects on recognition sites for any of 17 restriction enzymes employed for virtual RFLP analysis (except for G-A substitution at position 367 in *sec*Y gene). Therefore, these nucleotide alternations could not play important role in the assignment of detected phytoplasma to new or other subgroups.

The phytoplasma of 16SrXII-A subgroup "*Candidatus* Phytoplasma solani" infects a broad range of plant species worldwide and also has a wide distribution in Iran. "*Ca*. P. solani" has been previously reported in different parts of Iran infecting several plants such as almond (Zirak, Bahar, & Ahoonmanesh, 2009a), plum (Zirak, Bahar, & Ahoonmanesh, 2009b), peach (Zirak, Bahar, & Ahoonmanesh, 2010), potato (Hosseini, Bahar, Madani, & Zirak, 2011), cannabis (Sichani, Bahar, & Zirak, 2011) and grapevine (Mirchenari, Massah, & Zirak, 2015). Phytoplasmas usually can colonize different parts of a plant and move from one part to another (Crosslin, Hamlin, Buchman, & Munyaneza, 2011; Khadhair, Duplessis McAlister, Ampong-Nyarko, & Bains, 2002; Munyaneza & Crosslin, 2006; Weintraub & Jones, 2010). Thus, the phytoplasma can move from narcissus plant to its bulb and bulbous and infect future plants. In this respect, detection and eradicating the infected plants with their bulbs is essential to reduce the further spread of the infection.

Altogether, the results of this study indicated that the "*Ca*. P. solani" is present in the narcissus plants showing phyllody and virescence in Behbahan, Iran. To the best of our knowledge, there is only one report for phytoplasma disease in narcissus plants (Bellardi et al., 1990). This study revealed for the first time the association of "*Ca*. P. solani" with narcissus phyllody and virescence disease in the world.

Further investigations are in progress to determine the disease status in other narcissus growing regions in Iran and to specify its vector and alternate host plants to adopt appropriate containment measures.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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