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RESEARCH PAPERS

Apricot yellows associated with *'Candidatus* Phytoplasma phoenicium' in Iran

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Summary. Almond witches' broom associated with '*Candidatus* Phytoplasma phoenicium' is an economically important disease of almond in Iran and Lebanon. During surveys of almond witches' broom in 2012–2015, an apricot yellows disease was observed in Fars Province of Iran. The characteristic symptoms of the disease were leaf yellowing, inward leaf curl, scorch of leaf margins, shortened internodes, production of rosettes at the tips of the branches, and decline, stunting, and death of affected trees. Healthy bitter almond and apricot seedlings, grafted with shoots from symptomatic trees, exhibited phytoplasma-type symptoms. A 16S rDNA fragment of 1,250 bp was amplified by nested-PCR from affected trees and grafted seedlings. Nucleotide sequence identity, presence of species-specific signature sequences, and phylogenetic analysis of 16S rDNA allowed the assignation of the phytoplasma strains identified to the '*Ca*. P. phoenicium'. *In vitro* and *in silico* RFLP analyses of the amplified fragment allowed affiliation of the apricot yellows phytoplasma to a molecular variant in the subgroup 16SrIX-B. Within the population strains identified in this and previous studies, 16 genetic lineages were determined within 16S rDNA nucleotide sequences by the combination of 19 single nucleotide polymorphisms. The apricot yellows phytoplasma strains belong to a unique genetic lineage distinguished by the presence of three lineage-specific SNPs. This first report of '*Ca*. P. phoenicium' in association with apricot yellows in Iran opens new perspectives on the epidemiology of almond witches' broom, suggesting possible adaptation of the phytoplasma to other fruit tree species.

Key words: almond witches' broom, 16S rDNA, pigeon pea witches' broom (16SrIX) group, emerging disease, phytoplasma.

Introduction

Phytoplasmas are cell wall-less plant pathogenic bacteria of the class Mollicutes, associated with diseases affecting economically important crops. These plant pathogens are restricted to the phloem sieve tubes of infected plants and are transmitted from plant to plant by phloem-sap-feeding insects (Weintraub and Beanland, 2006). Phytoplasmas can also be transmitted by dodder (*Cuscuta* spp.) and grafting, and can be spread by vegetative propagation of infected plant parts (Bertaccini *et al*., 2014; Marcone *et al*., 2014). Characteristic host symptoms associated with phytoplasma presence include abnormal development of flowers and shoot proliferation (i.e. virescence, phyllody, witches' broom), foliar yellowing and reddening, reduced leaf and fruit size, phloem necrosis, and overall decline and stunting. Some plant species hosts may also be asymptomatic or exhibit mild symptoms. Based on unique molecular and biological features, phytoplasmas have been classified into 43 '*Candidatus* Phytoplasma' species (IRPCM, 2004). Ribosomal groupings have also been delineated, according to similarity coefficients derived from the comparisons of collective restriction

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profiles of the 16S rRNA gene (Lee *et al.*, 1998; Wei *et al.*, 2007; Zhao *et al.*, 2009).

Stone fruit trees can be infected by phytoplasmas belonging to at least eight ribosomal groups including: aster yellows (16SrI), peanut witches' broom (16SrII), X-disease (16SrIII), elm yellows (16SrV), ash yellows (16SrVII), pigeon pea witches' broom (16SrIX), apple proliferation (16SrX), and "stolbur" (16SrXII) (Cieślińska, 2011). Phytoplasmas in the 16SrIX group are associated with numerous diseases affecting crops and wild plants in different geographic areas worldwide (Lee *et al.*, 2012). Almond witches' broom (AlmWB), associated with the presence of '*Candidatus* Phytoplasma phoenicium' subgroup 16SrIX-B and its variants (Abou-Jawdah *et al.*, 2002; Verdin *et al.*, 2003; Molino Lova *et al.*, 2011; Lee *et al.*, 2012; Quaglino *et al.*, 2015), is an economically important disease in Lebanon and Iran (Abou-Jawdah *et al.*, 2002; Salehi *et al.*, 2006a). In Iran, a 16SrIX-C phytoplasma was reported as associated with AlmWB (Salehi *et al.*, 2006b). Previous studies demonstrated the capability of the leafhopper *Asymmetrasca decedens* Paoli and the cixiid *Tachycixius* spp. to transmit '*Ca.* P. phoenicium' in Lebanon (Abou-Jawdah *et al.*, 2014; Tedeschi *et al.*, 2015). Peach (*Prunus persica*), nectarine (*P. persica* var. *nucipersica*) (Salehi *et al.*, 2006b; Abou-Jawdah *et al.*, 2009), GF-677 (*P. amygdalus* × *P. persica*) (Salehi *et al.*, 2011), wild almond (*P. scoparia*) (Salehi *et al.*, 2015), *Anthemis* spp. and *Smilax aspera* (Tedeschi *et al.*, 2015) are other natural plant hosts of '*Ca.* P. phoenicium' in these countries. Grafting experiments and molecular analyses revealed that '*Ca.* P. phoenicium' does not infect plum (*P. domestica*), apricot (*P. armeniaca*) and cherry (*P. avium*) trees (Abou-Jawdah *et al.*, 2003).

Apricot trees have been cultivated in Iran since antiquity, and apricots are important fruit in modern-day Iran. This country is the second in world apricot production, with annual production of more than 400,000 MT (http://www.faostat3.fao.org). During surveys for AlmWB disease from 2012 to 2015 in many areas of Fars Province (Iran), including Khafr and Estahban where AlmWB is reported, a disease, tentatively named apricot yellows (AprY), was observed in apricot trees of the local varieties Talkh and Asephi. The aim of the present research was to identify and characterize the agent associated with this disease.

Materials and methods

Apricot trees

During field surveys for AlmWB, carried out from 2012 to 2015 in three locations (Breijan, Aliabad, and Kheer) in the Fars Province of Iran, apricot trees (local cultivars Asefi and Talkh), grown on bitter apricot rootstock and affected by a disease inducing phytoplasma-like symptoms, were selected for collection of symptomatic shoots for disease transmission trials and molecular studies. Symptomless apricot trees were also collected as controls.

Grafting experiments

Two-year-old seedlings of bitter almond (*Prunus amygdalus*) and apricot (*P. armeniaca*: cultivars Nouri, Talkh, Asefii, Tokhme morghee, and Shekarpareh) were purchased from a local nursery in Eghleed, an AlmWB free area in Fars Province, and verified as phytoplasma-free by nested PCR reactions using the protocol described below. Four seedlings of each cultivar were side grafted (three scions per seedling) with symptomatic shoots of yellows affected apricot trees from Breijan, Aliabad and Kheer. For each cultivar, seedlings grafted with scions prepared from symptomless apricot trees and ungrafted seedlings were used as controls. For comparison, bitter almond seedlings (four per phytoplasma strain) were graft inoculated with the phytoplasmas Neyriz AlmWB (NAlmWB) [16SrIX-B, GenBank Accession Number (Acc. No.) JN565014] and Khafr AlmWB (KAlmWB) (16SrIX-C, GenBank Acc. No. DQ195209), that had been maintained in almond seedlings. Grafted and ungrafted seedlings were maintained in an insect-proof screenhouse until the end of the trial.

Phytoplasma detection

Total nucleic acids were extracted from fresh leaf midrib tissues of field-collected symptomatic and symptomless apricot trees, and bitter almond and apricot seedlings employed in grafting transmission trials, using the method of Zhang *et al.* (1998) with minor modifications described by Abou-Jawdah *et al.* (2002). Extracted total nucleic acids were used as templates in nested PCR reactions, using the universal phytoplasma primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996) in, respectively, direct and nest-

ed PCR assays. PCR conditions and reagents were as outlined by Salehi *et al.* (2011). PCR products were electrophoresed through 1% agarose gel in 1 × TBE buffer (67 mM Tris–HCl, 22 mM boric acid, 10 mM EDTA, pH 8.0), stained with ethidium bromide and visualized by a UV transilluminator. Total nucleic acids extracted from a periwinkle plant infected with the phytoplasma strain that causes witches' broom disease of lime (WBDL) (Faghihi *et al*., 2011), and from a healthy almond seedling, were subjected to PCR as, respectively, positive and negative controls. PCR mixture devoid of DNA was also employed as a further negative control.

Molecular characterization of phytoplasmas

R16F2n/R16R2 primed PCR products, amplified from AprY-affected plant samples from Aliabad, Breijan and Kheer, and from bitter almond and apricot seedlings grafted with AprY-affected apricot scions, were ligated into pTZ57R/T vector and cloned into *Escherichia coli* DH5α cells using the InsT/A clone™ PCR Product Cloning Kit (Fermentas) according to manufacturer's instructions. For each PCR product, plasmid DNA from three recombinant colonies was purified using the GF-1 PCR Clean-Up Kit (Vivantis), sequenced on both strands by Macrogen, and assembled by the Contig Assembling program of the software BioEdit version 7.0.5 (Hall, 1999).

For ribosomal group/subgroup attribution, a virtual RFLP analysis was carried out using the *i*PhyClassifier tool, (Wei *et al*., 2007; Zhao *et al*., 2009). Results were verified by RFLP of R16F2n/R16R2-primed PCR products from AprY-associated phytoplasma strains and from strains Neyriz and Khafr AlmWB with the restriction enzymes *Alu*I, *Dra*I, *Hae*III, *Hha*I, *Hin*fI, *Hpa*II, *Mse*I, *Tha*I, *Rsa*I, *Sau*3AI, and *Taq*I (Fermentas). Restriction fragments were electrophoresed in 2% agarose gel in TBE buffer, stained with ethidium bromide and visualized by UV light.

BlastN analyses and comparison with 16S rDNA nucleotide sequences of selected '*Ca*. Phytoplasma' strains, retrieved from NCBI GenBank, were carried out to classify the AprY phytoplasma strains. The nucleotide sequences were aligned using the "ClustalW Multiple Alignment" application and analyzed for sequence similarity determinations using the "Sequence Identity Matrix" application of the BioEdit software. The alignment of 16S rDNA nucleotide sequences from this and previous studies (Table 1) was utilized, firstly, to confirm the '*Ca*. Phytoplasma' species attribution by the presence of species-specific unique signature sequences and determining the sequence similarity values, and secondly, to evaluate the diversity among the phytoplasmas strictly related to AprY phytoplasma, and to detect AprY-specific single nucleotide polymorphisms (SNPs). Nucleotide sequences of 16S rRNA gene of AprY phytoplasma strains, 16SrIX group phytoplasmas (Table 1) and reference strains of '*Ca*. Phytoplasma' species were employed for phylogenetic analyses. The Minimum-Evolution method was employed using the Jukes-Cantor model and bootstrap replicated 1,000 times with the software MEGA6 to obtain a phylogenetic tree (Tamura *et al*., 2013).

Results

Apricot yellows symptoms

AprY symptoms were observed in Talkh and Asefi cultivars of apricot. On each affected plant, the symptoms first appeared on one branch or a section of branches. The main symptoms were leaf yellowing, inward leaf curl, scorch of leaf margins, shortened internodes, production of rosettes at the tips of branches, die back decline, and plant death (Figure 1). Affected branches either bore no fruit or the fruit were small and were abnormal in shape and taste. AprY-affected trees were mainly found in almond witches' broom affected orchards.

Disease transmission

On apricot and bitter almond seedlings, infected scions remained alive and produced witches' broom symptoms. The agent of AprY was graft transmitted from affected apricot trees (cultivars Talkh and Asefi from Breijan, Aliabad, and Kheer locations) to all inoculated seedlings of bitter almond and apricot. The disease symptoms observed in apricot plants were shortened internodes, upward growth of rows of spindly shoots on main branches, mild yellowing and upward rolling of leaves, and (rarely) witches' broom. Symptoms observed in bitter almond plants were little leaf, internode shortening, mild witches' broom, yellowing, and stunting. The minimum time between seedling inoculation and symptom expression in graft inoculated seedlings was 11 months for bitter almond and 17 months for apricot. NAlmWB

Table 1. Phytoplasma strains in ribosomal group 16SrIX employed for 16S rDNA nucleotide sequence analyses.

16SrIX Subgroup	Strain	Host	Location	Acc. No.
IX-A	PPWB	Pigeon pea	USA, Florida	AF248957
	RLL-FL	Least snout-bean	USA, Florida	AF361019
	Pigeon pea (JD) 33	Pigeon pea	Puerto Rico	KJ817866
	Orange (JD) 32	Orange	Puerto Rico	KJ817867
	Orange (Is) 40	Orange	Puerto Rico	KJ817868
	Periwinkle (Ma) 5	Periwinkle	Puerto Rico	KJ817869
	Tabebuia (Ma) 2	Tabebuia	Puerto Rico	KJ817870
	Pigeon pea (Is) 43	Pigeon pea	Puerto Rico	KJ817871
	Pigeon pea (Is) 44	Pigeon pea	Puerto Rico	KJ817872
	Pigeon pea (Is) 45	pPgeon pea	Puerto Rico	KJ817873
	Tabebuia (Ma) 3	Tabebuia	Puerto Rico	KJ817874
	Periwinkle little leaf (Ma) 6	Periwinkle	Puerto Rico	KJ817875
	Colpoptera (Ad)	*Colpoptera maculifrons*	Puerto Rico	KJ817878
	Coffee (Ad) 22	Coffee	Puerto Rico	KJ817880
	PW1	Colombian periwinkle	Colombia	EU816776
	BrazHLB	Orange	Brazil	HQ423159
	PwK-AR1	Periwinkle	Brazil	JN792515
	PwK-CP3	Periwinkle	Brazil	JN792516
	Cu205	Soybean	Cuba	KU749595
	Cu185	Soybean	Cuba	KU749596
IX-B	CaPphoe reference strain A4	Almond	Lebanon	AF515636
	Khafr	Almond	Iran	JN565016
	Sanandaj	Almond	Iran	JN565015
	Kerman I	Almond	Iran	JN565012
	A21	Almond	Iran	AF515637
	BT18	Salix	Iran	KX500119
	Breijan (clone 12)	Apricot	Iran	KY014991
	Aliabad (clone 2)	Apricot	Iran	KY014992
	Kheer (clone 24)	Apricot	Iran	KY014993
	Kavar	Wild almond	Iran	KM235725
	Bidzard	Wild almond	Iran	JX445141
	Estahban	Wild almond	Iran	JX445142
	Meymand	Wild almond	Iran	KM235727
	Neyriz	Almond	Iran	JN565014
	Moshkan	Almond	Iran	JN565017
	Kerman II	Almond	Iran	JN565013

(Continued)

Table 1. (Continued).

16SrIX Subgroup	Strain	Host	Location	Acc. No.
	AlmWB2	Almond	Lebanon	AF390137
	AlmWB1(IX-B)	Almond	Lebanon	AF390136
	AlmWB-N1	Nectarine	Lebanon	AF455041
	AlmWB-P1	Peach	Lebanon	AF455040
	AlmWB4	Almond	Lebanon	AF455039
	AlmWB3	Almond	Lebanon	AF455038
	N13-1	Nectarine	Lebanon	HQ407535
	P10(297)	Peach	Lebanon	HQ407534
	PL3-1	Almond	Lebanon	HQ407533
	A16-4	Almond	Lebanon	HQ407531
	N18-1	Nectarine	Lebanon	HQ407530
	N9-7	Nectarine	Lebanon	HQ407528
	P1-2	Peach	Lebanon	HQ407527
	N8-1	Nectarine	Lebanon	HQ407525
	N10-8	Nectarine	Lebanon	HQ407524
	A11-4	Almond	Lebanon	HQ407523
	N19-1	Nectarine	Lebanon	HQ407520
	P2-6	Peach	Lebanon	HQ407517
	N14-1	Nectarine	Lebanon	HQ407513
	Smilax10	*Smilax aspera*	Lebanon	KF583754
	Smilax9	*Smilax aspera*	Lebanon	KF583755
	Na202-1	Almond	Lebanon	KF583758
	Na203-1	Almond	Lebanon	KF583759
	Na208-1	Almond	Lebanon	KF583760
	Na235-1	Almond	Lebanon	KF583761
	SN205	Nectarine	Lebanon	KF583762
	SN206	Nectarine	Lebanon	KF583763
	SN209	Nectarine	Lebanon	KF583764
	R0_221	*Cixius* sp.	Lebanon	KF583767
	R11_34	*Cixius* sp.	Lebanon	KF583768
	R12_29	*Cixius* sp.	Lebanon	KF583769
	R12_45	*Cixius* sp.	Lebanon	KF583770
	R12_139	*Eumecurus* sp.	Lebanon	KF583771
	R12 _266	*Tachycixius* sp.	Lebanon	KF583772
	R13 _130	*Tachycixius viperinus*	Lebanon	KF583773
	R12 _254	*Tachycixius* cf. *bidentifer*	Lebanon	KF583774

(Continued)

Table 1. (Continued).

16SrIX Subgroup	Strain	Host	Location	Acc. No.
	R12 _351	*Tachycixius* cf. *creticus*	Lebanon	KF583775
	SA213	Almond	Lebanon	KM275491
	AlmWB4_1	Peach	Lebanon	KF500030
	AlmWB4_2	Nectarine	Lebanon	KF500029
	AlmWB4_3	*Asymmetrasca decedens*	Lebanon	KF488577
	AlmWB4_4	*Asymmetrasca decedens*	Lebanon	KF359551
	N27-2	Nectarine	Lebanon	HQ407532
	N5	Nectarine	Lebanon	HQ407522
	N28-1	Nectarine	Lebanon	HQ407526
	N29-1	Nectarine	Lebanon	HQ407529
	A14	Almond	Lebanon	HQ407521
	A13	Almond	Lebanon	HQ407519
	A18-1	Almond	Lebanon	HQ407516
	A13-1	Almond	Lebanon	HQ407515
	A1-1	Almond	Lebanon	HQ407514
	Smasp	*Smilax aspera*	Lebanon	KP851773
	N1-2	Nectarine	Lebanon	HQ407512
	P3-1	Peach	Lebanon	HQ407518
	Smilax12	*Smilax aspera*	Lebanon	KF583756
	Anth1	*Anthemis* sp.	Lebanon	KF583765
	Smilax13	*Smilax aspera*	Lebanon	KF583757
	Anth2	*Anthemis* sp.	Lebanon	KF583766
IX-C	PEY phytoplasma	*Picris echioides*	Italy	Y16389
	PEY-Cal	*Picris echioides*	Italy	JQ181546
	PEY	*Picris echioides*	Italy	JQ868441
	PEYc2	*Picris echioides*	Italy	JX857827
	NaxYc4	Periwinkle	Italy	JN791265
	NAXOS	Periwinkle	Italy	HQ589191
	NaxYc3	Periwinkle	Italy	JN791266
	KAP	*Knautia arvensis*	Italy	EF186823
	Khafr AlmWB phytoplasma	Almond	Iran	DQ195209
	Iranian AlmWB	Almond	Iran	FJ160959
	Zarghan1	Sesame	Iran	KT265702
	Fasa1	Sesame	Iran	KT265703
	Sarvestan1	Sesame	Iran	KT265704
	Seph1	Sesame	Iran	JF508515

(Continued)

Table 1. (Continued).

16SrIX Subgroup	Strain	Host	Location	Acc. No.
	Sabzevar	Sesame	Iran	KF774193
	STBB	Tomato	Iran	JF508510
	GY phytoplasma 'Shiraz'	Grapevine	Iran	KX011516
	Brmul	*Bryonia multiflora*	Lebanon	KP851762
	Gepur	*Geranium purpureum*	Lebanon	KP851763
	Invis	*Inula viscosa*	Lebanon	KP851764
	Laser	*Lactuca serriola*	Lebanon	KP851765
	Madom	Apple tree	Lebanon	KP851766
	Masyl	*Malva sylvestris*	Lebanon	KP851767
	Osalb	*Osyris alba*	Lebanon	KP851768
	Pipal	*Pistacia palaestina*	Lebanon	KP851769
	Rhpun	*Rhamnus punctata*	Lebanon	KP851770
	Scmac	*Scolymus maculatus*	Lebanon	KP851771
	Siarv	*Sinapis arvensis*	Lebanon	KP851772
	Sonig	*Solanum nigrum*	Lebanon	KP851774
	BraR	*Brassica rapa*	India	GU111554
	ANT1	*Sesamum indicum*	Turkey	KC139791
IX-D	EchinWB	*Echinops spinosissimus*	Oman	GU902973
	CHMA	Chrysanthemum	Iran	KC176800
IX-E	JunWB-2C IX-E	Western juniper	USA	GQ925918
	JunWB-2A	Western juniper	USA	GQ925919
	BBS3NJ	Blueberry	USA	JN791268
	BBS41NJ	Blueberry	USA	JN791267
	BBS40-NJ	Blueberry	USA	JX857823
	Osb2	*Osbornellus horvathi*	Italy	KU758969
	OsbA	*Osbornellus horvathi*	Italy	KU896983
	OsbC	*Osbornellus horvathi*	Italy	KU896985
	OsbD	*Osbornellus horvathi*	Italy	KU896986
	OsbE	*Osbornellus horvathi*	Italy	KU896987
	OsbF	*Osbornellus horvathi*	Italy	KU896988
	OsbH	*Osbornellus horvathi*	Italy	KU896990
IX-F	GLL-Hon	*Gliricidia sepium*	Honduras	AF361017
IX-G	JTBB	Tomato	Iran	JF508513
IX-H	SAR2	*Brassica campestris*	Pakistan	KU892213
IX-I	SSY	*Onobrychis vicifolia*	Iran	KX461906
IX-J	ChicBS	*Cichorium intybus*	Saudi Arabia	KY986922

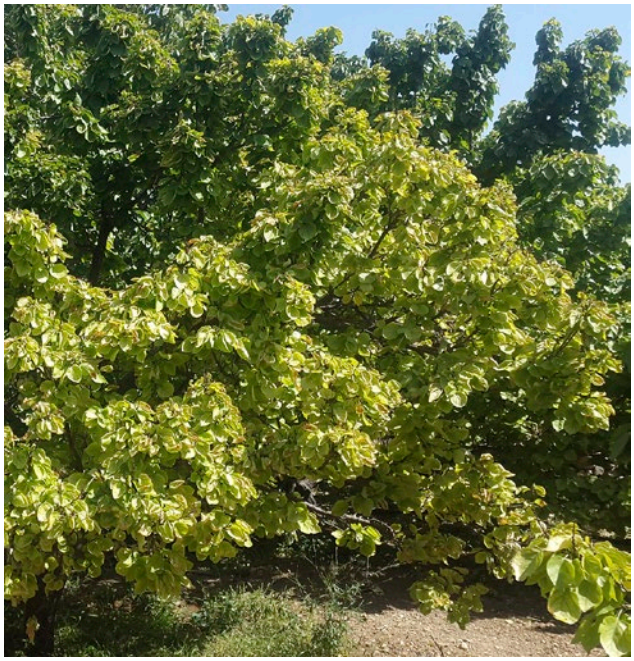

Figure 1. Apricot yellows symptoms (leaf yellowing, size reduction and inward curling, rosette and die back) observed on apricot trees at MeshKan (Neyriz, Fars Province, Iran).

and KAlmWB phytoplasmas were also graft transmitted to all bitter almond seedlings, inducing severe yellowing, little leaf and internode shortening, and the minimum time between inoculation and symptom expression was almost 10 months. Collectively, symptoms of AprY phytoplasma infection in bitter almond were milder than those caused by NAlmWB and KAlmWB strains.

All inoculated plant samples showed positive results in nested PCR assays. Control plants (not grafted with AprY-affected scions) were negative for the phytoplasma-type symptoms, and analyses by nested PCR gave negative results.

Phytoplasma identification

A fragment of approximately 1.8 kbp was obtained by direct amplification with P1/P7 primers, only from the periwinkle plant infected with WBDL phytoplasma (positive control). Bands of approx. 1,250 bp were amplified from all DNA samples tested, including symptomatic apricot trees, graft inoculated bitter almond and apricot seedlings, and positive controls (data not shown). No PCR products were obtained from symptomless apricot trees, control (not grafted) bitter almond or apricot seedlings, or from healthy almond seedling or PCR mixture devoid of DNA (negative control).

The obtained 16S rDNA nucleotide sequences (R16F2n/R2 fragment), amplified from field yellows-affected apricot trees and graft inoculated bitter almond and apricot seedlings, were identical. One sequence per location was therefore submitted to GenBank database, under the accession numbers KY014991 (from Breijan), KY014992 (from Aliabad) and KY014993 (from Kheer).

The *i*PhyClassifier analyses revealed that the virtual RFLP pattern derived from the AprY strain sequences (Figure 2a) was identical (similarity coefficient 1.00) to the pattern of the '*Ca*. P. phoenicium' strain A21, representing a variant of subgroup 16SrIX-B (previously classified as 16SrIX-D). AprY strain shared a similarity coefficient of 0.97 with the '*Ca*. P. phoenicium' strain A4, classified in subgroup 16SrIX-B. This difference is due to the restriction pattern produced using the enzyme *Taq*I (Figure 2a). RFLP analysis of three R16F2n/R2 nested PCR products, amplified from Breijan, Aliabad and Kheer AprY phytoplasma strains, produced undistinguishable restriction patterns with the enzymes *Alu*I, *Dra*I, *Hae*III, *Hha*I, *Hin*fI, *Hpa*II, *Mse*I, *Tha*I, *Rsa*I, *Sau*3AI and *Taq*I (Figure 2b). The AprY comprehensive pattern was different to that of NAlmWB phytoplasma strain (16SrIX-B subgroup) based on *Taq*I enzyme, and to those of KAlmWB (subgroup 16SrIX-C) based on the enzymes *Dra*I, *Hha*I, *Rsa*I, and *Taq*I (Figure 2b).

BlastN search showed that 16S rDNA nucleotide sequences of AprY phytoplasma strains shared closest homology (>99%) with members of the pigeon pea witches' broom (16SrIX) group, including phytoplasma strains associated with wild almond (*Prunus scoparia*) witches' broom from Kavar and Meymand (GenBank Acc. Nos, respectively, KM235725 and KM235727), GF-677 witches' broom from Estahban and Bidzard (GenBank Acc. Nos, respectively, JX445142 and JX445141) and almond witches' broom in Fars Province of Iran.

The AprY phytoplasma strain shares 99.6% sequence identity with the strain A4, (Verdin *et al.*, 2003) (Table 2). The strain also harbours the species-specific signature sequence (5'-CCTTTTTCGGAAGGTATG-3'; nt 58…75 from the annealing site of the primer F2n) of '*Ca*. P. phoenicium' (Verdin *et al.*, 2003), and two additional signatures (5'-TTGATAAGTC-

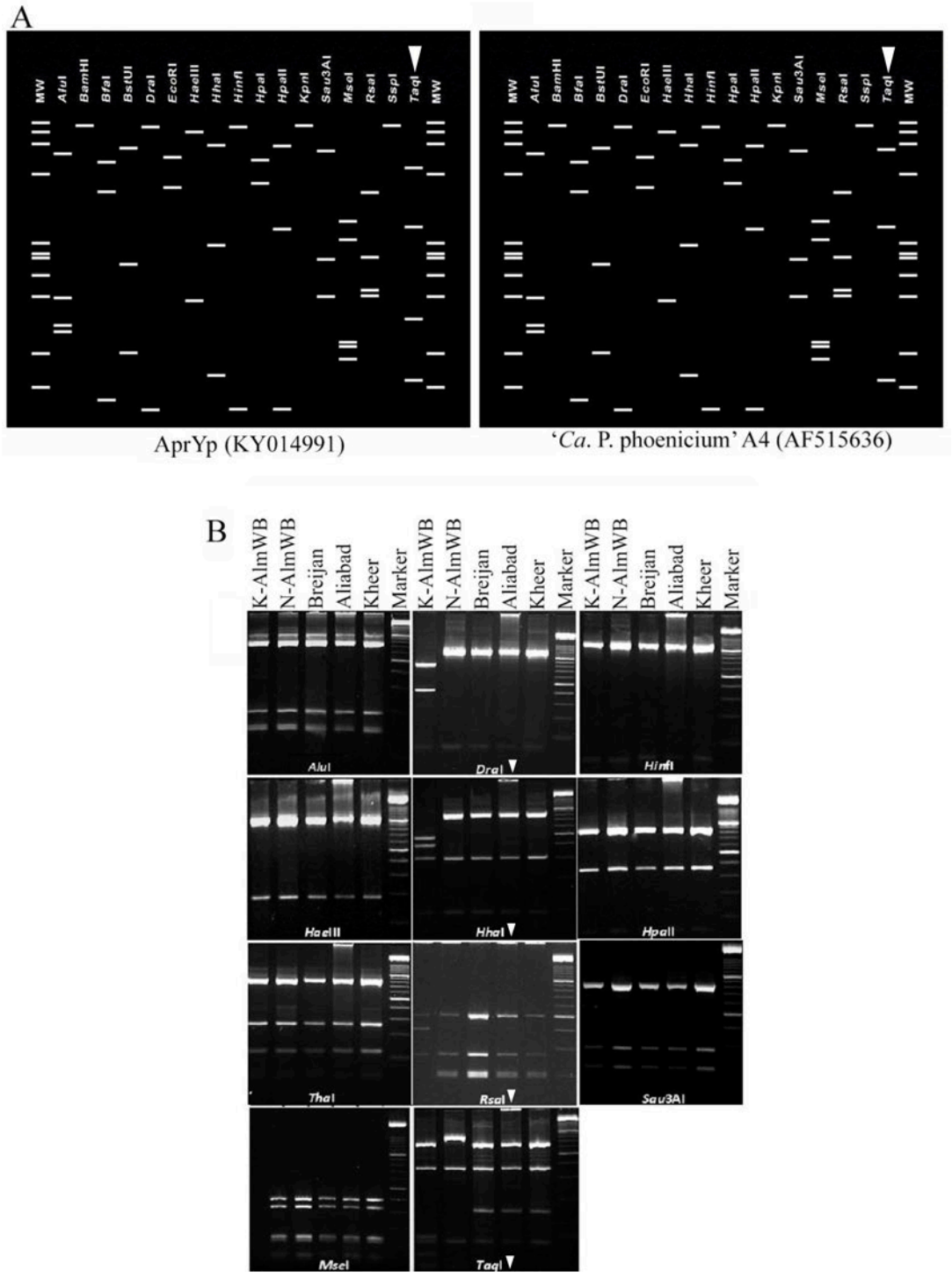

Figure 2. *In silico* and RFLP profiles. A, *in silico* RFLP profiles of Breijan AprY phytoplasma and '*Ca*. P. phoenicium' reference strain A4 (16SrIX-B), generated with *i*PhyClassifier; B, RFLP profiles derived from enzymatic digestions of R16F2n/R2 fragments amplified from AprY phytoplasma strains Breijan (clone 12), Aliabad (clone 2) and Kheer (clone 24), and strains N (Neyriz)-AlmWB (subgroup rIX-B) and K (Khafr)-AlmWB (subgroup rIX-C). Restriction fragments were electrophoresed in 2% agarose gel. Distinguishing enzymes are marked with white triangles.

Table 2. 16S rDNA nucleotide sequence identity (%) of AprY phytoplasma versus 16SrIX group reference strains.

Phytoplasma (16S rDNA GenBank Acc. No.)	Sequence Identity vs AprYp
'*Ca*. P. phoenicium' (AF515636) 16SrIX-B	99.6
SAR2 (KU892213) 16SrIX-H	99.4
PEY (Y16389) 16SrIX-C	99.3
EchinWB (GU902973) 16SrIX-D	99.2
SSY (KX461906) 16SrIX-I	99.2
BBS3NJ (JN791268) 16SrIX-E	98.9
PPWB (AF248957) 16SrIX-A	98.8
JTBB (JF508513) 16SrIX-G	98.8
ChicBS (KY986922) 16SrIX-J	98.8
GLL-Hon (AF361017) 16SrIX-F	98.4

TATAGTTTAAT-3' at nt 441…461, and 5'-TACCGC-TATAGAAACT-3' at nt 479…494 from the annealing site of the primer F2n) (Figure 3). Phylogenetic analysis positioned the AprY phytoplasma strains with high confidence values in a cluster including 16SrIX phytoplasmas in which the AprY strain belongs to a distinct subcluster including the '*Ca*. P. phoenicium' strain A4 (Figure 4). The detected AprY phytoplasma therefore belonged to '*Ca*. P. phoenicium', for which strain members (subgroup 16SrIX-B) are clearly distinct from phytoplasma strains of other 16SrIX subgroups. This was also confirmed by calculation of average sequence identity of 16SrIX subgroup strains and the reference strain A4 (Table 3).

Identification of SNP genetic lineages within '*Ca*. P. phoenicium' (subgroup 16SrIX-B)

Alignment of 16S rDNA nucleotide sequences of 74 '*Ca*. P. phoenicium' strains (subgroup 16SrIX-B and variants), available in GenBank or identified in the present study, revealed that 45 strains shared identical sequences with the reference strain A4 (Table 4). Within the remaining 28 strains (16 from Lebanon and 12 from Iran), it was possible to identify 19 SNPs in comparison with the sequence of the strain A4. In detail, 11 SNPs were present in '*Ca*. Phytoplasma' strains identified in Iran, and eight were in strains from Lebanon. The combination of such SNPs, mutually exclusive in the phytoplasma strain populations identified in the two Countries, allowed the recognition of nine SNP lineages in Lebanon and six in Iran.

Phytoplasma strain	'*Ca*. P. phoenicium' signature sequences		
	nt 58..75	nt 441..461	nt 479..494
A4 (rIX-B_lineage a)	CCTTTTTCGGAAGGTATG	TTGATAAGTCTATAGTTTAAT	TACCGCTATAGAAACT
Khafr (rIX-B_lineage a)	.....	.....	.....
N27-2 (rIX-B_lineage b1)	.....	.....	.....
N5 (rIX-B_lineage b2)	.....	.....	.....
N28-1 (rIX-B_lineage b3)	.....	.....	.....
N29-1 (rIX-B_lineage c1)	.....	.....	.....
Al4 (rIX-B_lineage c2)	.....	.....	.....
Smasp (rIX-B_lineage c3)	.....	.....	.....
P3-1 (rIX-B_lineage c4)	.....	.....	.....
Smilax12 (rIX-B_lineage d)	.....	.....	.....
Smilax13 (rIX-B_lineage d)	.....	.....	.....
A21 (rIX-B_lineage f1)	.....	.....	.....
Breijan (rIX-B_lineage f2)	.....	.....	.....
Kavar (rIX-B_lineage f3)	.....	.....	.....
Meymand (rIX-B_lineage g)	.....	.....	.....
Moshkan (rIX-B_lineage h1)	.....	.....	.....
Keman II (rIX-B_lineage h2)	.....	.....	.....
PPWB(rIX-A)	.....A.....A	.....A	..G.....
PEY (rIX-C)	.....A.....	.....A	..G.....
EchinWB (rIX-D)	.....N.....	.....A	..G.....
BBS3NJ (rIX-E)	.....-..A.....	.....A	..G.....
GLL-Hon (rIX-F)	.....A.....A	.....A	..G.....
JTBB (rIX-G)	.....A.....A	.....A	..G.....
SAR2 (rIX-H)	.....A	.....A	..G.....
SSY (rIX-I)	.....A.....	.....A	..G.....
ChicBS (rIX-J)	.....A.....	.....A	..G.....

Figure 3. '*Ca*. P. phoenicium'-specific signature sequences in phytoplasma strains of 16SrIX subgroups. Nucleotide position of each signature sequence is calculated from the annealing site of the primer R16F2n.

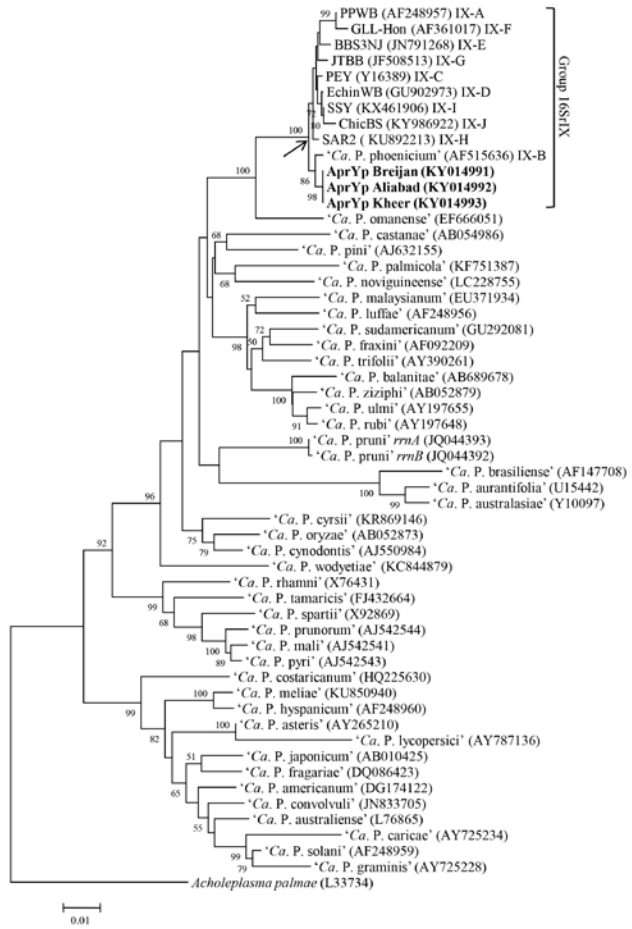

Figure 4. Phylogenetic tree inferred from 16S rDNA nucleotide sequences of phytoplasma strains of group 16SrIX (including AprY phytoplasmas, identified in this study) and reported '*Ca.* Phytoplasma' species. Minimum-Evolution analyses were carried out using the Jukes-Cantor model and bootstrap replicated 1,000 times. 16S rDNA nucleotide sequence of *Acholeplasma palmae* (Acc. No. L33734) was used for rooting the tree. GenBank accession number of each sequence is given in parentheses, and gene sequences obtained in the present study are indicated in bold font.

The lineage named f2, characterized by the presence of three unique SNPs at nucleotide positions 473, 691 and 692, included exclusively the AprY phytoplasma strains identified in the present study (Table 4).

Discussion

Almond witches' broom is an economically important disease in several provinces of Iran, including Fars, Chaharmahal-Bakhtiari, Kerman, Isfahan, Yazd, and Kurdistan (Salehi and Izadpanah, 1995; Salehi *et al.*, 2005; Salehi *et al.*, 2006a; Pourali and Salehi, 2012). In this country, AlmWB is associated with phytoplasmas belonging to subgroups 16SrIX-B and -C (Salehi *et al.*, 2006a, 2006b). Peach, nectarine, GF-677, and *P. scoparia* (wild almond) were reported as additional stone fruit plant hosts of 16SrIX group phytoplasmas in Iran (Salehi and Izadpanah, 2001; Salehi *et al.*, 2006b). A previous study (Abbasian and Salehi, 2010) reported the graft transmission of Iranian AlmWB phytoplasma strains to apricot trees under experimental conditions, suggesting that apricot can be a host of this phytoplasma. This hypothesis is not supported by evidence obtained in other studies carried out in Lebanon, where natural infection of apricot trees with 16SrIX phytoplasmas was never reported, and experimental trials for AlmWB phytoplasma transmission to different stone fruits always gave negative results for apricot (Abou-Jawdah *et al.*, 2003).

Apricot chlorotic leaf roll (ACLR), commonly associated with '*Ca.* P. prunorum' (Marcone *et al.*, 2010), is another economically important disease of apricot in several areas of the world. A '*Ca.* P. asteris' strain (16SrI-F) has also been reported as the cause of ACLR in Spain and the Czech Republic (Fialová *et al.*, 2004; Lee *et al.*, 2004), and '*Ca.* P. mali', cause of apple prolif-

Table 3. 16S rDNA nucleotide sequence identity (%) of 16SrIX subgroup phytoplasmas versus '*Ca.* P. phoenicium' reference strain A4.

16SrIX Subgroup	No. Strains	Sequence identity (%) vs strain A4	
		Average	Range
-A	20	98.3	95.7–98.7
-B	74	99.8	99.3–100
-C	32	99	98.2–99.3
-D	2	99.1	99–99.2
-E	12	98.8	98.4–99.3
-F	1	98.5	-
-G	1	98.6	-
-H	1	99.3	-
-I	1	99.3	-
-J	1	98.8	-

Table 4. SNPs-based genetic lineages identified among '*Ca.* P. phoenicium' (16SrIX-B) strains.

Ref. strain	Location[a]	Host (Plant or insect)[a]	SNPs in 16S rDNA (position from the annealing site of the primer R16F2n)																		Lineage
			159[b, d]	460[b]	473[b]	572[c]	639[c]	646[b]	691[b]	692[b]	715[c]	761[b]	783[c]	809[c]	905[b]	945[b]	988[c]	1095[b]	1113[b]	1158[c]	1177[c]	
A4	Lebanon (43)	almond (13); nectarine (12); peach (5); smilax (2); *Cixius* (4); *Tachycixius* (4); *Eumecurus* (1); *Asymmetrasca* (2)	T	T	G	G	G	-	T	G	A	A	A	C	-	C	G	T	A	T	T	a
	Iran (3)	almond (3)																				
N27-2	Lebanon (1)	nectarine (1)	T	T	G	C	G	-	T	G	A	A	A	C	-	C	G	T	A	T	T	b1
N5	Lebanon (1)	nectarine (1)	T	T	G	C	G	-	T	G	A	A	A	C	-	C	G	T	A	G	T	b2
N28-1	Lebanon (1)	nectarine (1)	T	T	G	G	G	-	T	G	A	A	A	C	-	C	G	T	A	G	T	b3
N29-1	Lebanon (1)	nectarine (1)	T	T	G	G	T	-	T	G	A	A	A	C	-	C	G	T	A	T	T	c1
A14	Lebanon (5)	almond (5)	T	T	G	G	G	-	T	G	A	A	A	T	-	C	G	T	A	T	T	c2
Smasp	Lebanon (2)	smilax (1); nectarine (1)	T	T	G	G	G	-	T	G	A	A	C	T	-	C	G	T	A	T	T	c3
P3-1	Lebanon (1)	peach (1)	T	T	G	G	T	-	T	G	A	A	C	T	-	C	G	T	A	T	T	c4
Smilax12	Lebanon (2)	smilax (1); anthemis (1)	T	T	G	G	G	-	T	G	A	A	A	C	-	C	A	T	A	T	T	d
Smilax13	Lebanon (2)	smilax (1); anthemis (1)	T	T	G	G	G	-	T	G	G	A	A	C	-	C	G	T	A	T	C	e
A21	Iran (2)	almond (1); salix (1)	C	T	G	G	G	C	T	G	A	A	A	C	-	C	G	G	A	T	T	f1
Breijan	Iran (3)	apricot (3)	C	T	**A**	G	G	-	**C**	**T**	A	A	A	C	-	C	G	G	A	T	T	f2
Kavar	Iran (3)	wild almond (3)	C	T	G	G	G	-	T	G	A	A	A	C	G	C	G	G	G	T	T	f3
Meymand	Iran (2)	wild almond (1); almond (1)	T	C	G	G	G	-	T	G	A	G	A	C	-	C	G	G	A	T	T	g
Moshkan	Iran (1)	almond (1)	T	T	G	G	G	-	T	G	A	A	A	C	-	A	G	G	A	T	T	h1
Kerman II	Iran (1)	almond (1)	T	T	G	G	G	-	T	G	A	A	A	C	-	A	G	T	A	T	T	h2

[a] Number of phytoplasma strains is noted in parentheses.
[b] SNPs identified exclusively in phytoplasma strains from Iran. SNPs at nucleotide 473, 691 and 692 (evidenced in bold) are specifically associated with the AprY phytoplasma strains.
[c] SNPs identified exclusively in phytoplasma strains from Lebanon.
[d] SNP (159T>C) *Taq*I restriction site (TCGA) allowing the distinction of AprYp and '*Ca.* P. phoenicium' reference strain A4.

eration, was reported in ACLR-affected apricot trees in Slovenia (Mehle *et al*., 2007). In apricot, AprY is more lethal than ACLR, since in addition to chlorotic leaf roll, it induces leaf scorch, severe rosette and stunting in affected plants. A recent study reported the association of '*Ca*. P. solani' (16SrXII-A), '*Ca*. P. asteris' (16SrI-B), and *Xylella fastidiosa* with apricot trees exhibiting decline and leaf scorch in Iran (Karimi *et al*., 2016).

In the present research, overall results of field surveys, experimental transmission trials and molecular analyses have proven that yellows symptoms, observed in apricot trees cultivated in almond orchards localized in Iranian provinces widely affected by AlmWB disease, are associated with an apricot infection by 16SrIX group phytoplasma strains. Nucleotide sequence similarity, presence of species-specific signature sequences, and phylogenetic analyses of 16S rRNA gene allowed the assignation of the 16SrIX phytoplasma strains identified in AprY-affected plants to '*Ca*. P. phoenicium'. This and previous sequence analyses (Lee *et al*., 2012; Quaglino *et al*., 2015; Casati *et al*., 2016) reinforced that, within the pigeon pea witches' broom (16SrIX) group, the '*Ca*. P. phoenicium' should exclusively include strains of subgroup 16SrIX-B. Such strains are clearly distinct from strains of other 16SrIX subgroups, both at molecular and biological levels. Within '*Ca*. P. phoenicium' (16SrIX-B) strain populations, identified here and in previous studies, 16 genetic lineages were identified based on the combination of 19 single nucleotide polymorphisms (SNPs) positioned within the 16S rDNA nucleotide sequences. This reinforces evidence indicating the usefulness of molecular markers within the conserved 16S rDNA to resolve the genetic complexity in phytoplasma populations (Cheng *et al*., 2015; Quaglino *et al*., 2017). Furthermore, the identification of distinct genetic lineages in Lebanon and in Iran suggests that, as reported in previous studies of phytoplasma strain populations (Cai *et al*., 2008; Quaglino *et al*., 2009, 2017; Cheng *et al*., 2015), climatic and geographic features in the ecosystems may be significant, directly or indirectly, in determining the strain composition of phytoplasma populations in different regions.

Phytoplasma strains causing AprY probably belong to a unique genetic lineage (here named f2), distinguished from others by the presence of three lineage-specific SNPs. Possible association of lineage f2 with AlmWB-affected almond trees in Iran, especially with those near yellows affected apricot trees, should be further investigated. This report of a specific '*Ca*. P. phoenicium' genetic lineage associated with AprY in Iran opens a new perspective on the epidemiology of AlmWB phytoplasma, suggesting the possible adaptation of this phytoplasma to other fruit tree species, as previously reported for peach and nectarine in Lebanon (Abou-Jawdah *et al*., 2009).

Based on detection of '*Ca*. P. phoenicium' in insect bodies and saliva and consistent insect finding and rearing on almond trees, the leafhopper (Cicadellidae, Typhlocybinae) *Frutioidea bisignata* was reported as a potential vector of this phytoplasma in Iran (Taghizadeh and Salehi, 2002; Siampour *et al*., 2004). In Lebanon, *F. bisignata*, collected on almond trees, was not positive for '*Ca*. P. phoenicium' (Dakhil *et al*., 2011). On the other hand, in Iran, despite the presence of high populations and rearing on almond trees, the leafhopper *Asymmetrasca decedens* Paoli, vector of '*Ca*. P. phoenicium' (Abou-Jawdah *et al*., 2014) in Lebanon, was not able to transmit '*Ca*. P. phoenicium' (Taghizadeh and Salehi, 2002). The apparently distinct association with leafhoppers supports the differences evidenced by molecular and phylogenetic analyses. The agents associated with Iranian and Lebanese AlmWB and related diseases seem, therefore, to represent at least two distinct genetic lineages of '*Ca*. P. phoenicium'. Further investigations are required to determine the insect vector(s) of '*Ca*. P. phoenicium' in Iran, and to obtain accurate information about the ecology of '*Ca*. P. phoenicium' strains and the epidemiology of the associated diseases.

Acknowledgments

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