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"*Candidatus* Phytoplasma balanitae" Associated with Witches' Broom Disease of Jackal Jujube (*Zizyphus oenoplia* L.) in South India

Güney Hindistan'da Çakal Hünnabı (*Zizyphus oenoplia* L.)'nın Cadı Süpürgesi Hastalığı "*Candidatus* Phytoplasma balanitae" ile İlişkisi

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ABSTRACT

During survey, 12 Jackal jujube plant samples showing the symptoms of witches' broom disease were collected from the Shivamogga district of Karnataka, India, between 2017 and 2018. The causal agent associated with Jackal jujube witches' broom disease was identified through polymerase chain reaction using phytoplasma 16S rRNA-encoding and *Sec*A gene-specific universal primers. All 12 Jackal jujube plant samples gave positive amplification for the phytoplasma-specific primers. The amplified polymerase chain reaction products (16S rRNA-encoding gene and SecA gene) were cloned and sequenced. The nucleotide sequence 16S rRNA-encoding and SecA genes comparisons were made with the available phytoplasmas from an NCBI database. The Jackal jujube witches' broom phytoplasma isolates shared the highest nucleotide identity of 97.8–98.2% (16S rRNA gene) with *Candidatus* Phytoplasma balanitae group (16SrV) and 89.4–99% (SecA gene) nucleotide identity with Jujube witches' broom phytoplasma. This was well supported by the close clustering of Jackal jujube witches' broom phytoplasma isolates in the current study with *Candidatus* Phytoplasma balanitae (16S rRNA gene) and Jujube witches' broom-phytoplasma (SecA gene) in phylogenetic analysis. The virtual Restriction fragment length polymorphism (RFLP) pattern generated by Jackal jujube witches' broom isolates was different (similarity coefficient of 0.91) to the reference pattern of *Candidatus* Phytoplasma balanitae (16SrV) group with respect to three enzymes (*Alu* I, *Hae* III, and *Mse* I). Based on the threshold similarity coefficient for the new subgroup, delineation should be set at 0.98. The significance of the research is discussed.

Keywords: Candidatus Phytoplasma balanitae, jackal jujube, PCR, phyllody, phylogenetic analysis

ÖZ

Araştırma sürecinde (2017-18), Hindistan'ın Karnataka ilçesinde fitoplazmalara özgü cadı süpürgesi (WB: Witches Broom) simptomu gösteren on iki (12) Çakal hünnap (*Jackal jujube*) bitkisinden örnek toplanmıştır. Örnekler fitoplazmaların 16S rRNA ve SecA genlerine sipesifik primerler kullanılarak Polymerase Chain Reaction (PCR) ile testlenmiş, amplifikasyon bölgeleri klonlanarak sekans dizilimleri çıkarılmış ve takiben NCBI veri tabanında yer alan fitoplazmaların sekans dizilimleri ile mukayese edilmiştir. Yapılan karşılaştırmada JJWB fitoplazma izolatlarının 16S rRNA gen bölgesinin *Candidatus* Phytoplasma balanitae gurubu (16SrV) ile %97,8-%98,2, SecA gen bölgesinin ise %89,4-%99 oranında Jujube witches broom (JWB) phytoplasma ile benzer nükleotit dizilimlerine sahip oldukları tespit edilmiş ve sonuç filogenetik analiz ile desteklenmiştir. Diğer taraftan, JJWB izolatları *Alu* I, *Hae* III ve *Mse* I enzimleri ile Restriction Fragmenth Length Polymorphism (RFLP) analizine tabi tutulduğunda kontrol olarak kullanılan *Ca*. P. balanitae (16SrV) gurubu ile 0,91 oranında benzerlik gösterdiği saptanmıştır. Bu oran yeni alt guruplar için eşik benzerlik oranını olan 0,98'den düşük bulunmuş ve bulgular bu veriler çerçevesinde tartışılmıştır.

Anahtar Kelimeler: *Candidatus* Phytoplasma balanitae, çakal hünhabı, PCR, fillodi, filogenetik analiz

Introduction

Ziziphus oenoplia (L.) Mill. (synonym: *Rhamnus oenoplia* L.) belongs to the family Rhamnaceae commonly known as the jackal jujube, small-fruited jujube or wild jujube in English, makora in Hindi, harasurali, karisurimullu, barige, and pargi in Kannada. It is a perennial, flowering, scandent, and thorny shrub that grows to a height of 1.5 m with spreading character as well. It is distributed in tropical and subtropical Asia and Australasia (Anonymous, 2003) and grows in moist and dry deciduous forests and also in plains along the roadsides. In India, it is distributed throughout except Jammu and Kashmir with more frequency in Karnataka, Kerala, and Maharashtra and specifically Western Ghats (https://indiabiodiversity.org/species/show/32385). Fruits of *Z. oenoplia* are edible, eating the fruit aids in the secretion of saliva and are very much liked by children, birds, and animals.

The various parts of *Z. oenoplia* plant are widely used in Ayurveda for treatments. The plant produces cyclopeptide alkaloids known as ziziphines and has a long history of using them as herbal medicine. In India, the roots are used in Ayurvedic medicine (Kuvar & Bapat, 2010). The Konkani people of Maharashtra use the chewed leaves as a dressing material for wounds. The extracts of ziziphine from *Z. oenoplia* var. *brunoniana* showed antiplasmodial activity in vitro against the malarial parasite *Plasmodium falciparum* (Suksamrarn et al., 2005). There is also a mention of the plant parts used in the treatment of ulcer, stomach ache, obesity, asthma, digestive ailments, and diuretic conditions apart from using for a stringent, antiseptic, hepatoprotective, and wound-healing properties (Suryakant et al., 2011). Further, pharmacognostical and physico-chemical standardization on the leaves of *Z. oenoplia* was carried out, particularly on those that are supplied in the form of powder (Eswari et al., 2014). Even though it is not grown commercially, it is an important plant species because of its medicinal properties and the ecological prospect in providing food and shelter for birds. Despite its many medicinal importance, the species is highly susceptible to witches' broom disease caused by phytoplasma.

Phytoplasmas are cell wall-less, non-helical prokaryotic microbes inhabiting in the plant phloem tissues. They are poorly understood plant pathogens, since these cannot be cultured under in vitro easily and are designated as "Candidatus" status based on 16S rDNA gene sequence analysis (Lee et al., 2000). Phytopathogenic phytoplasmas were first identified by Doi et al. (1967) and named as mycoplasma-like organisms (MLOs) which belong to the class Mollicutes. These are known to be transmitted by the phloem-feeding insect vectors (leafhoppers and psyllids), dodder, and grafting (Weintraub & Beanland, 2006). Phytoplasmas severely infect more than 700 important plant species belonging to cereals, fruits, vegetables, ornamentals, forage, and forest plants worldwide. The plants affected with phytoplasma displayed diverse kinds of typical symptoms on many crops (Bertaccini et al., 2014; Omar, 2017; Omar & Foissac, 2012). In most of the crops, the phytoplasmas are diagnosed through the application of both conventional and nested polymerase chain reaction (PCR) assays (Lee et al., 1994) targeting the amplification of 16Sr RNA-encoding genes, which are also being used for the classification (Lee et al., 2012). Based on the 16S rRNA-encoding gene sequence analysis, 34 groups and 100 subgroups of diverse phytoplasmas were identified across the world (Bertaccini & Lee, 2018). Of these, 11 groups are reported from India including 16SrI, II, VI, and XI groups as the major strains (Rao et al., 2017). *Candidatus* Phytoplasma balanitae (Ca. P. balanitae) group (16SrV) has been well documented on wild *Balanites trifloral* in Myanmar (Win et al., 2013) and Chinese jujube (*Ziziphus jujuba* Mill.) in China (Wang et al., 2018).

There are few reports of diseases caused by fungal pathogens (Misra et al., 2013; Yuan et al., 2009) and witches' broom phytoplasma (Jamadar et al., 2009; Khan et al., 2008; Pandey et al., 1976) on its closely related species *Z. jujube* and *Z. mauritiana* grown commercially in several parts of the country. In the present study, attempt was made to characterize phytoplasmas based on 16Sr RNA and *Sec*A gene associated with witches' broom disease of Jackal jujube (*Z. oenoplia*) in India.

Methods

Collection of Disease Samples

Between 2017 and 2018, exploration was carried out for the collection of the leaf samples from the naturally grown Jackal jujube plants showing characteristic phytoplasma disease symptoms *viz.* witches' broom, yellowing, and reduction in leaf size from different locations of Shivamogga, Chikkamagaluru, Sirsi (toward the Western Ghats), and Bengaluru rural district (plain region) of Karnataka, India. Three locations (Shivamogga: 14.1670° N latitude, 75.0403° E longitude; Chikkamagaluru: 13.2650° N latitude, 75.3420° E longitude; and Sirsi: 14.6155° N latitude, 74.8347° E longitude) are mainly located in Western Ghats, which receives

Figure 1.
Jackal Jujube Plant Showing Little Leaf (A) and Witches' Broom (B) Disease Symptoms Under Natural Conditions

Figure 2.
Phylogenetic Tree Based on the Sequences of 16S rRNA (A) and secA Gene (B) of Jackal Jujube Witches' Broom (JJWB) Phytoplasm (in Red) with the Phytoplasma Strains Listed in Tables 4 and 5 Using the Neighbor-Joining Algorithm. Horizontal Distances Are Proportional to Sequence Distances, and Vertical Distances Are Arbitrary. The Trees Were Rooted with Acholeplasma laidlawii (GenBank accession number U14905) and Bacillus subtilis (GenBank accession number D10279) Respectively. A Bootstrap Analysis with 1000 Replicates Was Performed and the Bootstrap Percent Values Above 50 Are Showed Along the Branches.

more than 1500 mm annual rainfall over a period of 100 days (July–September) except Bengaluru rural district (plain region, 13.0767° N latitude, 77.5776° E longitude). In most of Western Ghats area, Jackal jujube plants are grown naturally in the forest area. The disease incidence assessed by observing 100 plants in each location and the percent of incidence was calculated by the number of infected plants divided by the number of plants observed multiplied by 100. The collected samples were brought to the plant pathology laboratory, CHES, Chettalli, Madikeri, Karnataka, India, and molecular characterization was carried out. Totally, 16 phytoplasma-infected Jackal jujube leaf samples were collected and designated as JJWB1 to JJWB10 (Shivamogga), JJWB11 and JJWB12 (Chikkamagaluru), JJWB-BLR1 and JJWB-BLR2 (Bengaluru), and JJWB-S1 and JJWB- S2 (Sirsi) (Table 1, Figure 1). Three leaf samples (JJWB13, JJWB-BLR, and JJWB-S) without any symptoms were also collected from the respective places and were used as a negative control.

DNA Isolation and Polymerase Chain Reaction Amplification

Total genomic DNA from each sample was extracted separately following the Cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1990). Total genomic DNA of previously characterized phytoplasma (sesame and brinjal) was used as positive control (Venkataravanappa et al., 2017, 2019). The total

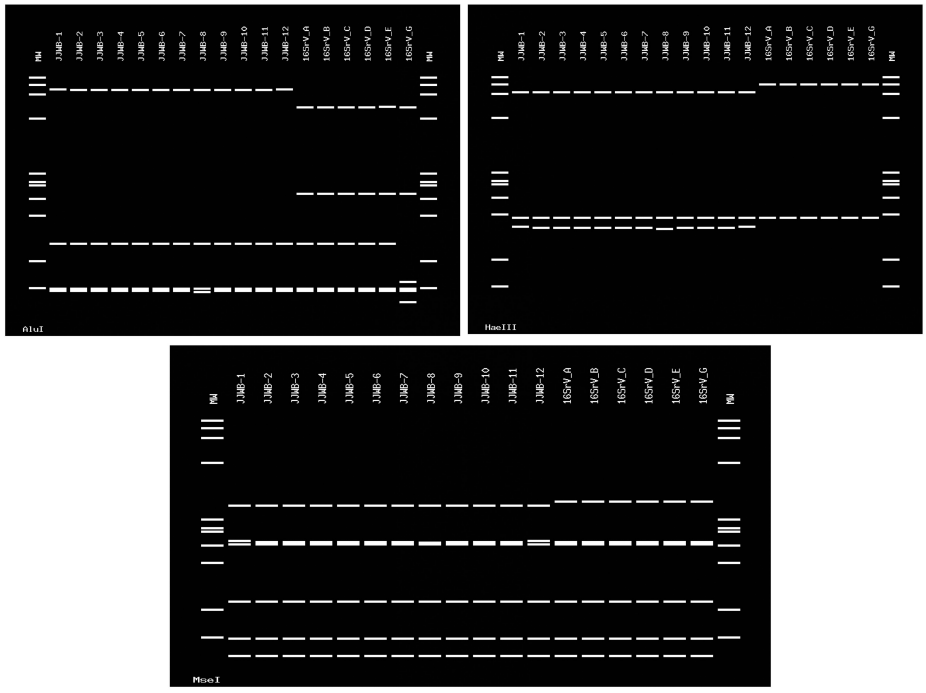

Figure 3.
Virtual RFLP Patterns Derived from in silico Digestions, using iPhyClassifier for R16F2n/R16R2 fragment of 16S rRNA Gene from Jackal Jujube Witches' Broom (JJWB1) Phytoplasm. The Virtual RFLP Patterns of JJWB1 (AluI, HaeIII, and Mse I) Distinguish the Strain from Those in a Number of Subgroups in Group 16SrV. The Restriction Fragments Were Resolved Through 3% Virtual Agarose Gel. M, molecular Weight Ladder phiX174 DNA HaeIII Digest.

DNA of 16 symptomatic and 3 asymptomatic plants along with the positive controls were subjected to the PCR amplification of 16S rRNA-encoding gene of phytoplasma using universal primer pair P1/P7 in the first reaction with the expected amplicon size of 1.8 kb (Makarova et al., 2013). This was followed by nested PCR with R16F2n/R2, R16mF2/16mR1, and fU5/rU3 primers specific to amplify genome segments within 16S rRNA-encoding gene of phytoplasma (Gundersen & Lee, 1996; Lee et al., 1998; Lorenz et al., 1995) in the second reaction with the expected amplicons size of 1.2 kb, 1.4 kb, and 0.8 kb, respectively.

Table 1.
Plant Samples Used for PCR-based Molecular Detection of Phytoplasma-Induced Witches' Broom Disease in Jackal Jujube

Sample No.	Sample	Place	Symptom Details
1	JJWB1	Sagara, Shivamogga	Typical witches' broom symptoms
2	JJWB2	Sagara, Shivamogga	Shoot proliferation symptoms
3	JJWB3	Sagara, Shivamogga	Typical witches' broom symptoms
4	JJWB4	Sagara, Shivamogga	Typical witches' broom symptoms
5	JJWB5	Sagara, Shivamogga	Shoot proliferation symptoms
6	JJWB6	Sagara, Shivamogga	Typical witches' broom symptoms
7	JJWB7	Sagara, Shivamogga	Shoot proliferation symptoms
8	JJWB8	Shikaripura, Shivamogga	Typical witches' broom symptoms
9	JJWB9	Shikaripura, Shivamogga	Typical witches' broom symptoms
10	JJWB10	Shikaripura, Shivamogga	Typical witches' broom symptoms
11	JJWB11	Cadure, Chikkamagaluru	Shoot proliferation symptoms
12	JJWB12	Cadure, Chikkamagaluru	Typical witches' broom symptoms
13	JJWB13 Healthy	Sagara, Shivamogga	Healthy plant as negative control
14	JJWB-BLR1	Bengaluru, UAS, GKVK	Typical witches' broom symptoms
15	JJWB-BLR2	Bengaluru (IIHR)	Typical witches' broom symptoms
16	JJWB-BLR	Bengaluru	Healthy plant as negative control
17	JJWB-S1	Sirsi	Typical witches' broom symptoms
18	JJWB-S2	Sirsi	Typical witches' broom symptoms
19	JJWB-S	Sirsi	Healthy plant as negative control

Note: PCR, polymerase chain reaction.

Further, the *Sec*A gene of three representative Jackal jujube phytoplasma isolates (JJWB1, JWB-BLR, JWB-S1, and JWB-S2) was amplified by primer pair SecAfor1 and SecArev2 as described by Dickinson & Hodgetts (2013). The amplified products for the primer pair P1/P7 (1.8 kb size) and SecA gene (0.8 kb size) were purified by gel extraction kit (Qiagen, Germany) and ligated into the pTZ57R/T vector (Fermentas, Germany) according to the manufacturer's instructions. The ligated vector was transformed into *Escherichia coli* DH5α competent cells (Invitrogen Bioservices, India Pvt. Ltd., Bengaluru, Karnataka, India) by following the standard molecular biology procedures (Sambrook & Russell, 2001). The recombinant clones were confirmed by the restriction endonuclease digestion with EcoRI and PstI restriction endonucleases. Three colonies were selected from each transformation reaction of 16S rRNA-encoding and *Sec*A genes and were sequenced in both orientations with automated sequencing ABI PRISM 3730 (Applied Biosystems) from Eurofins Genomics India Pvt. Ltd (Karnataka, India).

Sequence Analysis

The 16S rRNA-encoding and *Sec*A gene sequences generated from the present study and reference phytoplasma strain sequences retrieved from GenBank were aligned using MUSCLE method implemented in the SEAVIEW program (Galtier et al., 1996). The nucleotide (nt) identity matrixes for the Jackal jujube witches' broom (JJWB) phytoplasma isolates were generated using Bioedit Sequence Alignment Editor (version 5.0.9) (Hall, 1999). The phylogenetic tree was constructed by the neighbor-joining method using MEGA 7 version software (Kumar et al., 2016) with 1000 bootstrap replications to estimate evolutionary distances between all pairs of sequences simultaneously.

RFLP analysis

In silico restriction enzyme digestions of R16F2n/R16R2 region of the 16S rRNA-encoding gene and virtual gel plotting was done by using 17 restriction enzymes [*Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (ThaI), *Dra*I, *EcoR*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (MboI), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I] for the classification of phytoplasma. Virtual gel plotting was generated using an iPhyclassifier online tool (Zhao et al., 2009). The virtual RFLP plotting of R16F2n/R16R2 fragment of JJWB phytoplasma isolates was carried out using pDRAW32 software (Lee et al., 1998)

Results

Symptomatology

The phytoplasma-infected Jackal jujube plants were weak, tall, and produced leaves, which are small and yellow. Axillary branches proliferate and produce many secondary branches covered with small yellow leaves giving a bushy appearance of the diseased plants (Figure 1). Phyllody is the characteristic symptom if the plants are infected 1 year before the flowering season. If the phytoplasma infection is before flowering, plants may produce few and/or small fruits and do not flower in the subsequent years. The leaves and shoots of the infected plant may become distorted, dwarfned, and discolored finally leading to witches' broom symptoms. The disease incidence varied from 50% to 60% and yield loss was 100% due to complete witches' broom symptoms in Jackal jujube plants in different places of Shivamogga, Chikkamagaluru, Sirsi (toward the Western Ghats), and Bengaluru rural district.

Detection and Characterization of Phytoplasma Infecting Jackal Jujube

Attempt to amplify 16S rRNA-encoding and SecA genes by PCR using specific primers resulted in the expected amplicons of 1.8 kb and ~1.2 kb, respectively, in the all 16 phytoplasma-infected Jackal jujube samples collected during the survey (Table 2). This suggested that all these samples were infected with phytoplasma. Further, in the nested PCR, 7 samples gave positive amplification to primer pair R16mF2/R16mR1, 15 samples gave positive amplification to primer pair R16F2n/R16R2, and 3 samples gave positive amplification to primer pair fU5/rU3 (Table 3). No amplification was observed with any of the phytoplasma primer pairs in a healthy plant sample. The results indicated that there might be some variation in the sequences at the primers binding regions among the isolates. The sequence information has revealed the minor change in the sequences of targeted regions using the above primers (Table 3). All samples in the study and the positive controls gave amplification in the PCR for *Sec*A gene-specific primers but not from the healthy samples.

The nt sequence specific to 1.8 kb of 16S rRNA-encoding gene and 0.8 kb SecA gene were obtained and used for sequence analysis. The alignment of nt sequences (16S rRNA-encoding and *Sec*A genes) of JJWB phytoplasma isolates collected from different locations of Karnataka revealed that they shared the nt identity of 99.5–100% in 16S rRNA-encoding gene and 99.1–99.9% in SecA gene among themselves. The sequence of 16S rRNA-encoding gene and *Sec*A gene of JJWB phytoplasma isolates (JJWB1 to 12) was deposited in NCBI GenBank (MH819290 and MT441821–MT441831), and MH816938 (SecA gene) was used for further analysis.

Analysis of 16S rRNA Sequence of Phytoplasma Infecting Jackal Jujube

Pairwise sequence comparison of the partial 16S rRNA-encoding gene sequence of JJWB phytoplasma isolates (16 JJWB) with 52 published members of phytoplasma retrieved from the database.

Table 2.
Sequence of Universal Primers P1/P7 and Nested Primer Pairs Used for Phytoplasma Detection in Jackal Jujube

Sl.No.	Primer	Nucleotide Sequences (5′–3′)	Amplicon Size	References
1	P1	AAGAGTTTGATCCTGGCTCAGGATT	1.8 kb	Makarova et al., 2013
	P7	CGTCCTTCATCGGCTCTT		
2	R16mF2	CATGCAAGTCGAACGA	1.4 kb	Gundersen & Lee, 1996
	R16mR1	CTTAACCCCAATCATCGAC		
3	R16F2n	GAAACGACTGCTAAGACTGG	1.2 kb	Lee et al. 1998
	R16R2	TGACGGGCGGTGTGTACAAACCCCG		
4	fU5	CGGCAATGGAGGAAACT	0.8 kb	Lorenz et al., 1995
	rU3	TTCAGCTACTCTTTGTAACA		

Table 3.
Amplification of Phytoplasma-Specific Polymerase Chain Reaction Products by Nested Primers

Lane No.	PCR	SecA	Sample Nested PCR Results with Different Primers		
			R16F2n/ R16R2	R16mF2/ 16mR1	fU5/ rU3
JJWB1	+	+	+	+	+
JJWB2	+	+	+	–	–
JJWB3	+	+	+	–	–
JJWB4	+	+	+	+	–
JJWB5	+	+	+	–	–
JJWB6	+	+	+	+	–
JJWB7	+	+	+	–	–
JJWB8	+	+	–	+	+
JJWB9	+	+	+	–	–
JJWB10	+	+	+	–	–
JJWB11	+	+	+	+	–
JJWB12	+	+	+	+	+
JJWB13 healthy	–	–	–	–	–
JJWB-BLR1	+	+	+	–	–
JJWB-BLR2	+	+	+	+	–
JJWB-BLR	–	–	–	–	–
JJWB-S1	+	+	+	–	
JJWB-S2	+	+	+	–	–
JJWB-S	–	–	–	–	–
Sesame (16SrII)	+	+	+	–	–
Brinjal (16SrVI)	+	+	+	–	–

Note: PCR, polymerase chain reaction.

The result revealed that the 16S rRNA-encoding gene sequence of JJWB phytoplasma isolates showed maximum nt identity of 99.4–99.9% with *Ca*. P. balanitae (HG937644, MN902086, MN902087) from India (Table 4). The result phylogenetic analysis showed that three JJWB phytoplasma isolates (JJWB1, JJWBS1, and JJWBS2) are closely clustering with *Ca.* P. balanitae (HG937644, MN902086, MN902087, LT558785, MK975463, and MK975462) infection, whereas other 12 JJWB phytoplasma isolates (JJWB2, JJWB3, JJWB4, JJWB5, JJWB6, JJWB7, JJWB8, JJWB9, JJWB10, JJWB11 JJWB12, and JJWB-BLR) (Figure 2A) are formed as a separate claude. Since the 16S rRNA-encoding gene nt sequence similarity of JJWB phytoplasma with members of 16SrV (*Ca.* P. balanitae) was above the threshold level of 94%, it is proposed that JJWB phytoplasma isolates should be regarded as a member of *Ca*. P. balanitae (16SrV group) (Win et al., 2013). A similar oligonucleotide sequences of unique regions of the 16S rRNA-encoding gene 5′-TTGGAAACGG-3′ and 5′-CGGCC-3′ were identified in the phytoplasma isolates associated with JJWB.

*Sec*A Gene Sequence Analysis of Phytoplasma Infecting Jackal Jujube

For the analysis of the *Sec*A gene, representative sequence among the four JJWB phytoplasma isolates (JJWB1, JWB-BLR, JWB-S1, and JWB-S2) was compared with the corresponding region of 20 different *Ca*. Phytoplasmas (Table 5). The analysis showed that the JJWB phytoplasma isolates showed maximum of nt identity 99.1% with Jujube witches' broom (JWB) phytoplasma infecting Jujube in China (GU471771 and GU968588) and less than 99% nt identity with JWB phytoplasma (GU471769, GU471766, GU471770, GU471767, GU471764, GU471765, and GU968586) and *Sophora* sp. witches' broom (JF792183). In contrast, JJWB phytoplasma showed less than 89% identity with the other members of different *Ca*. Phytoplasma (Table 5). This result is also well supported by a phylogenetic analysis, which shows *Sec*A gene of JJWB1, JWB-BLR, JWB-S1, and JWB-S2 phytoplasma closely clustering with JWB phytoplasma (GU968588, GU471769, GU471766, GU471771, GU471770, GU471767, GU471764, and GU471765) and *Sophora* sp. witches' broom (JF792183) (Figure 2B).

Virtual RFLP Analysis

Computer-simulated, virtual RFLP analyses were carried out for R16F2n/R16R2 primers amplified sequence from JJWB phytoplasma isolates using an iPhyClassifier (Zhao et al., 2009). The virtual RFLP patterns (Figure 3) derived from the query 16SrDNA F2nR2 fragment of JJWB phytoplasma isolates had a similarity coefficient of 0.91 to the reference pattern of *Ca*. P. ziziphi that belongs to 16Sr group V, subgroup B (GenBank accession: AB052876). Based on *Alu*I, *Hae*III, and *Mse*I restriction enzymes, JJWB phytoplasma isolates were clearly differentiated from 16SrV phytoplasma subgroups, 16SrV-A (AY197655), 16SrV-B (AB052876), 16SrV-C (AY197642), 16SrV-D (AY197644), and 16SrV-E (AY197650) (Figure 3). Similarly, pDRAW32 (AcaClone Software; http://www.acaclone.com) analysis with *Alu*I, Hae*III*, and *Mse*I also showed that restriction map of JJWB phytoplasma isolates are having significant differences with closely related *Ca*. P. ziziphi-16SrV-B (AB052876) and other five representatives of subgroups (16SrV-A, B, C, D, and E) belongs to 16SrV (data not shown). The similarity coefficient values for the JJWB phytoplasma isolates are less than 0.97, which is the threshold similarity coefficient for delineation of a new subgroup. RFLP pattern type within a given group (Wei et al., 2007) supporting the designation of the JJWB phytoplasma as a member of a new 16SrV subgroup.

Discussion

Jackal jujube is a scandent shrub distributed throughout India in the forest thickets and along the forest roadside. Despite mentioning of its uses in Ayurvedic medicine, the reports of biotic stresses affecting this plant species are absent may be because this plant is not grown for commercial production. However, looking into the medicinal value and ecological perspective in providing food and nutritional source to the birds, small animals, and the tribal populations in the forest, it deserves the attention with respect to biotic stresses imposed on it. However, there are few reports about the biotic factors affecting the production of commercialized species of *Ziziphus*, an Indian ber affected by witches' broom caused by phytoplasma-like organisms. The witches' broom disease on *Z. jujube* and *Z. nummularia* was first reported from India during 1970s (Pandey et al., 1976), recently, from Bahraich district of Uttar Pradesh, and the causal agent of this disease was identified as *Ca*. Phytoplasma ziziphi (Khan et al., 2008). Further, the JWB disease caused by *Ca*. Phytoplasma ziziphi was also recorded on common jujube (*Z. jujube*), which is a widely grown important fruit crop in China, Korea, and Japan, causing a serious problem for the industry due to 30–80% reduction in the yield (Jung et al., 2003; Ohashi et al., 1996).

In the present study, JJWB samples collected from different places of the Karnataka state, India, were confirmed with the presence of phytoplasma. The universal nested PCR primer sets used in the study viz. R16F2n/R16R2, R16mF2/R16mR1, and fU5/

Table 4.
Pair-wise Nucleotide Identity of 16S rRNA Gene of Jackal Jujube Isolates with Corresponding Region of Different Phytoplasma Available in NCBI Database

Phytoplasma Species	Group/ SubGroup	Accession No.	Jackal Jujube Isolates														
			JJWB1	JJWB2	JJWB3	JJWB4	JJWB5	JJWB6	JJWB7	JJWB8	JJWB9	JJWB10	JJWB11	JJWB12	JJWB-BLR	JJWB-S1	JJWB-S2
Ca. Phytoplasma balanitae	16SrV	HG937644	99.9	99.7	99.7	99.7	99.7	997	99.7	99.4	99.7	99.7	99.7	99.5	99.7	99.9	99.9
Jujube witches' broom	16SrV	MK975463	98.0	97.9	97.9	97.9	97.9	97.9	97.9	97.9	97.9	97.9	97.9	97.6	97.9	98.0	98.0
Jujube witches' broom	16SrV	MK975462	97.1	96.9	96.9	96.9	96.9	96.9	96.9	96.9	96.9	96.9	96.9	96.7	96.9	97.1	97.1
Ca. Phytoplasma balanitae	16SrV	LT558785	99.8	99.6	99.6	99.6	99.6	99.6	99.6	99.3	99.6	99.6	99.6	99.4	99.6	99.8	99.8
*Ca.*Phytoplasma balanitae	16SrV	MN902086	99.9	99.7	99.7	99.7	99.7	99.7	99.7	99.4	99.7	99.7	99.7	99.5	99.7	99.9	99.9
Ca. Phytoplasma balanitae	16SrV	MN902087	99.9	99.7	99.7	99.7	99.7	99.7	99.7	99.4	99.7	99.7	99.7	99.5	99.7	99.9	99.9
Japanese raisin witches' broom	16SrV	AB442218	98.4	98.3	98.3	98.3	98.3	98.3	98.3	97.9	98.3	98.3	98.3	98.2	98.3	98.4	98.4
Prunus avium virescence	16SrV	MF848960	98.4	98.3	98.3	98.3	98.3	98.3	98.3	97.9	98.3	98.3	98.3	98.2	98.3	98.4	98.4
Amaranthus retroflexus	16SrV	HQ660580	98.3	98.2	98.2	98.2	98.2	98.2	98.2	97.9	98.2	98.2	98.2	98.1	98.2	98.3	98.3
Jujube witches' broom	16SrV	HQ199312	98.4	98.3	98.3	98.3	98.3	98.3	98.3	97.9	98.3	98.3	98.3	98.2	98.3	98.4	98.4
Black locust witches' broom	16SrV	KC527061	98.4	98.3	98.3	98.3	98.3	98.3	98.3	97.9	98.3	98.3	98.3	98.2	98.3	98.4	98.4
Ca. Phytoplasma ziziphi	16SrV	KC478660	98.3	98.2	98.2	98.2	98.2	98.2	98.2	97.9	98.2	98.2	98.2	98.1	98.2	98.3	98.3
Jujube witches' broom	16SrV	GU184184	98.3	98.2	98.2	98.2	98.2	98.2	98.2	97.9	98.2	98.2	98.2	98.1	98.2	98.3	98.3
Jujube witches' broom	16SrV	GU184181	98.3	98.2	98.2	98.2	98.2	98.2	98.2	97.9	98.2	98.2	98.2	98.1	98.2	98.3	98.3
Jujube witches' broom	16SrV	GU184180	98.4	98.3	98.3	98.3	98.3	98.3	98.3	97.9	98.3	98.3	98.3	98.2	98.3	98.4	98.4
Ziziphus jujube witches' broom	16SrV	AY072722	98.1	97.9	97.9	97.9	97.9	97.9	97.9	97.6	97.9	97.9	97.9	97.9	97.9	98.1	98.1
Blackberry phytoplasma	16SrV-I	.KR233473	98.2	98.0	98.0	98.0	98.0	98.0	98.0	97.7	98.0	98.0	98.0	98.0	98.0	98.2	98.2
Alder yellows	16SrV-C	AY197642	98.1	97.9	97.9	97.9	97.9	97.9	97.9	97.6	97.9	97.9	97.9	97.9	97.9	98.1	98.1
Flavescence dorée	16SrV-D	AY197644	97.9	97.7	97.7	97.7	97.7	97.7	97.7	97.4	97.7	97.7	97.7	97.6	97.7	97.9	97.9
Rubus stunt	16SrV-E	AY197650	98.2	98.0	98.0	98.0	98.0	98.0	98.0	97.7	98.0	98.0	98.0	98.0	98.0	98.2	98.2
Ca. Phytoplasma balanitae	16SrV-F	AB689678	99.8	99.6	99.6	99.6	99.6	99.6	99.6	99.3	99.6	99.6	99.6	99.4	99.6	99.8	99.8
Jujube witches' broom	16SrV-G	AB052879	87.1	87.0	87.0	87.0	87.0	87.0	87.0	86.9	87.0	87.0	87.0	86.9	87.0	87.1	87.1
Bischofia polycarpa witches' broom	16SrV-H	KJ452547	98.3	98.2	98.2	98.2	98.2	98.2	98.2	97.9	98.2	98.2	98.2	98.1	98.2	98.3	98.3
Ca. Phytoplasma trifolii	16VI	EU573925	89.0	89.0	89.0	89.0	89.0	89.0	89.0	89.0	89.0	89.0	89.0	88.9	89.0	89.0	89.0
Ca. Phytoplasma aurantifolia	16SrII-B	U15442	62.5	62.4	62.4	62.4	62.4	62.4	62.4	62.2	62.4	62.4	62.4	62.4	62.4	62.5	62.5
Ca. Phytoplasma australasia	16SrII-D	Y10097	90.0	89.8	89.8	89.8	89.8	89.8	89.8	89.5	89.8	89.8	89.8	89.7	89.8	90.0	90.0
Ca. Phytoplasma pruni	16SrIII-A	L04682	93.1	93.0	93.0	93.0	93.0	93.0	93.0	92.7	93.0	93.0	93.0	92.9	93.0	93.1	93.1
Ca. Phytoplasma palmae	16SrIV-A	U18747	94.9	94.8	94.8	94.8	94.8	94.8	94.8	94.4	94.8	94.8	94.8	94.7	94.8	94.9	94.9
Ca. Phytoplasma ulmi	16SrV-A	AY197655	98.1	97.9	97.9	97.9	97.9	97.9	97.9	97.6	97.9	97.9	97.9	97.9	97.9	98.1	98.1
Ca. Phytoplasma ziziphi	16SrV-B	AB052876	98.0	98.2	98.2	98.2	98.2	98.2	98.2	98.2	98.2	98.2	98.2	98.1	98.2	98.0	98.0
Ca. Phytoplasma vitis	16SrV-C	AF176319	98.2	98.0	98.0	98.0	98.0	98.0	98.0	97.7	98.0	98.0	98.0	98.0	98.0	98.2	98.2
Ca. Phytoplasma trifolii	16SrVI-A	AY390261	96.2	96.0	96.0	96.0	96.0	96.0	96.0	95.7	96.0	96.0	96.0	96.0	96.0	96.2	96.2
Ca. Phytoplasma fraxini	16SrVII-A	AF092209	95.7	95.6	95.6	95.6	95.6	95.6	95.6	95.2	95.6	95.6	95.6	95.5	95.6	95.7	95.7
Ca. Phytoplasma luffae	16SrVIII-A	AF086621	94.9	94.8	94.8	94.8	94.8	94.8	94.8	94.5	94.8	94.8	94.8	94.8	94.8	94.9	94.9

(*Continued*)

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Table 4.
*Pair-wise Nucleotide Identity of 16S rRNA Gene of Jackal Jujube Isolates with Corresponding Region of Different Phytoplasma Available in NCBI Database (**Continued**)*

Phytoplasma Species	Group/ SubGroup	Accession No.	Jackal Jujube Isolates														
			JJWB1	JJWB2	JJWB3	JJWB4	JJWB5	JJWB6	JJWB7	JJWB8	JJWB9	JJWB10	JJWB11	JJWB12	JJWB-BLR	JJWB-S1	JJWB-S2
Ca. Phytoplasma phoenicium	16SrIX-D	AF515636	93.7	93.6	93.6	93.6	93.6	93.6	93.6	93.2	93.6	93.6	93.6	93.5	93.6	93.7	93.7
Ca. Phytoplasma mali	16SrX-A	AJ542541	91.2	91.4	91.4	91.4	91.4	91.4	91.4	91.1	91.4	91.4	91.4	91.3	91.4	91.2	91.2
Ca. Phytoplasma pyri	16SrX-C	AJ542543	91.3	91.5	91.5	91.5	91.5	91.5	91.5	91.1	91.5	91.5	91.5	91.4	91.5	91.3	91.3
Ca. Phytoplasma spartii		X92869	90.4	90.5	90.5	90.5	90.5	90.5	90.5	90.2	90.5	90.5	90.5	90.4	90.5	90.4	90.4
Ca. Phytoplasma prunorum	16SrX-F	AJ542544	91.2	91.4	91.4	91.4	91.4	91.4	91.4	91.1	91.4	91.4	91.4	91.3	91.4	91.2	91.2
Ca. Phytoplasma oryzae	16SrXI-A	AB052873	94.3	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.4	94.5	94.3	94.3
Ca. Phytoplasma solani	16SrXII-A	AF248959	88.2	88.3	88.3	88.3	88.3	88.3	88.3	88.0	88.3	88.3	88.3	88.3	88.3	88.2	88.2
Ca. Phytoplasma australiense	16SrII	L76865	87.9	87.9	87.9	87.9	87.9	87.9	87.9	87.6	87.9	87.9	87.9	87.8	87.9	87.9	87.9
Ca. Phytoplasma japonicum	16SrXII-D	AB010425	89.1	89.1	89.1	89.1	89.1	89.1	89.1	88.8	89.1	89.1	89.1	89.0	89.1	89.1	89.1
Ca. Phytoplasma fragariae	16SrXII-E	DQ086423	89.1	89.2	89.2	89.2	89.2	89.2	89.2	88.9	89.2	89.2	89.2	89.2	89.2	89.1	89.1
Mexican periwinkle virulence	16SrXIII-A	AF248960	89.2	89.4	89.4	89.4	89.4	89.4	89.4	89.1	89.4	89.4	89.4	89.3	89.4	89.2	89.2
Ca. Phytoplasma cynodontis	16SrXIV	AJ550984	94.7	94.5	94.5	94.5	94.5	94.5	94.5	94.2	94.5	94.5	94.5	94.4	94.5	94.7	94.7
Ca. Phytoplasma brasiliense	16SrXV	AF147708	90.1	90.1	90.1	90.1	90.1	90.1	90.1	89.8	90.1	90.1	90.1	90.1	90.1	90.1	90.1
Ca. Phytoplasma graminis	16SrXVI	AY725228	85.6	85.8	85.8	85.8	85.8	85.8	85.8	85.5	85.8	85.8	85.8	85.7	85.8	85.6	85.6
Ca. Phytoplasma caricae	16SrXVII	AY725234	84.5	84.7	84.7	84.7	84.7	84.7	84.7	84.4	84.7	84.7	84.7	84.6	84.7	84.5	84.5
Ca. Phytoplasma americanum	16SrXVIII	DQ174122	88.7	88.8	88.8	88.8	88.8	88.8	88.8	88.5	88.8	88.8	88.8	88.8	88.8	88.7	88.7
Ca. Phytoplasma castaneae	16SrXIX	AB054986	93.0	92.8	92.8	92.8	92.8	92.8	92.8	92.5	92.8	92.8	92.8	92.8	92.8	93.0	93.0
Ca. Phytoplasma rhamni	16SrXX	X76431	91.2	91.2	91.2	91.2	91.2	91.2	91.2	90.8	91.2	91.2	91.2	91.1	91.2	91.2	91.2
Ca. Phytoplasma pini	16SrXXI	AJ632155	93.8	93.6	93.6	93.6	93.6	93.6	93.6	93.3	93.6	93.6	93.6	93.6	93.6	93.8	93.8
Ca. Phytoplasma cocosnigeriae	16SrXXII-A	Y14175	93.0	92.8	92.8	92.8	92.8	92.8	92.8	92.5	92.8	92.8	92.8	92.8	92.8	93.0	93.0
Buckland Valley graph yellows	16SrXXIII-A	AY083605	89.4	89.5	89.5	89.5	89.5	89.5	89.5	89.2	89.5	89.5	89.5	89.5	89.5	89.4	89.4
Sorghum bunchy shoot	16SrXXIV-A	AF509322	94.2	94.2	94.2	94.2	94.2	94.2	94.2	93.9	94.2	94.2	94.2	94.1	94.2	94.2	94.2
Weeping tea witches' broom	16SrXXV-A	AF521672	89.5	89.5	89.5	89.5	89.5	89.5	89.5	89.2	89.5	89.5	89.5	89.5	89.5	89.5	89.5
Sugarcane yellows	16SrXXVI-A	AJ539179	93.0	93.2	93.2	93.2	93.2	93.2	93.2	92.8	93.2	93.2	93.2	93.1	93.2	93.0	93.0
Sugarcane yellows	16SrXXVII-A	AJ539180	93.1	93.2	93.2	93.2	93.2	93.2	93.2	92.9	93.2	93.2	93.2	93.2	93.2	93.1	93.1
Derbid phytoplasma	16SrXXVIII-A	AY744945	71.0	71.1	71.1	71.1	71.1	71.1	71.1	70.9	71.1	71.1	71.1	71.1	71.1	71.0	71.0
Ca. Phytoplasma omanense	16SrXXIX	EF666051	92.8	92.7	92.7	92.7	92.7	92.7	92.7	92.4	92.7	92.7	92.7	92.6	92.7	92.8	92.8
Ca. Phytoplasma tamaricis	16SrXXX	FJ432664	90.6	90.7	90.7	90.7	90.7	90.7	90.7	90.4	90.7	90.7	90.7	90.7	90.7	90.6	90.6
Ca. Phytoplasma allocasuarinae		AY135523	67.8	67.8	67.8	67.8	67.8	67.8	67.8	67.6	67.8	67.8	67.8	67.8	67.8	67.8	67.8
Ca. Phytoplasma lycopersici		EF199549	71.4	71.5	71.5	71.5	71.5	71.5	71.5	71.3	71.5	71.5	71.5	71.5	71.5	71.4	71.4
Ca. Phytoplasma cocostanzaniae		X80117	77.7	77.5	77.5	77.5	77.5	77.5	77.5	77.3	77.5	77.5	77.5	77.5	77.5	77.7	77.7
Chinaberry yellows		AF495882	63.2	63.3	63.3	63.3	63.3	63.3	633	63.1	63.3	63.3	63.3	63.3	63.3	63.2	63.2

Note: For each column, the highest value is underlined.

Table 5.
SecA Gene Sequences of Phytoplasmas Employed in Analysis

Phytoplasma/Disease Common Name	Gene Bank Accession No.	Crop	Country	Pair-wise Identity of Jackal Jujube Isolates			
				JJWB1	JJWB-BLR	JJWB-S1	JJWB-S2
Jujube witches' broom phytoplasma	GU471771	Jujube	China	99.1	99.2	99.1	99.1
Jujube witches'broom phytoplasma	GU471769	Jujube	China	99.0	98.9	98.8	98.8
Jujube witches' broom phytoplasma	GU471766	Jujube	China	99.1	99.0	98.9	98.9
Jujube witches' broom phytoplasma	GU968588	Jujube	China	99.0	99.1	99.0	99.0
Sophora sp. witches' broom	JF792183	*Sophora* sp.	China	98.9	98.8	98.6	98.6
Jujube witches'broom phytoplasma	GU471770	Jujube	China	98.9	99.0	98.9	98.9
Jujube witches' broom phytoplasma	GU471767	Jujube	China	98.9	98.8	98.6	98.6
Jujube witches' broom phytoplasma	GU471764	Jujube	China	98.9	99.0	98.9	98.9
Jujube witches' broom phytoplasma	GU471765	Jujube	China	98.8	98.9	98.8	98.8
Jujube witches' broom phytoplasma	GU968586	Jujube	China	98.6	98.8	98.6	98.6
Crotalaria witches' broom	KY872722	Crotalaria	Oman	47.7	47.8	47.7	47.7
Crotalaria witches' broom	KY872721	Crotalaria	Oman	47.7	47.8	47.7	47.7
Crotalaria witches' broom	KY872720	Crotalaria	Oman	47.7	47.8	47.7	47.7
Crotalaria witches' broom	KY872719	Crotalaria	Oman	50.7	50.8	50.7	50.7
Tanzanian lethal decline	KJ462071	*Cocos nucifera*	Tanzania	61.5	61.4	61.5	61.5
Ca. Phytoplasma fraxini	EU168745	–	United Kingdom	48.8	48.8	48.6	48.6
Chrysanthemum yellows	KJ462009	Chrysanthemum	Germany	45.7	45.7	45.7	45.7
New Jersey aster yellows	KJ462010	*Callistephus chinensis*	USA	48.7	48.6	48.7	48.7
Maryland aster yellows	KJ462011	*Callistephus chinensis*	USA	45.8	45.8	45.8	45.8
Aster yellows witches' broom	KJ462012	Aster	France	46.8	46.7	46.8	46.8
Clover phyllody	KJ462014	Clover	United Kingdom	47.9	47.8	47.9	47.9
Carrot yellows	KJ462015	*Daucus carota*	Italy	48.5	48.4	48.5	48.5
Ca. Phytoplasma aurantifolia	KJ462017	Citrus	Oman	51.6	51.5	51.6	51.6
Primula blue yellows	KJ462018	Primula	United Kingdom	51.2	51.1	51.2	51.2
Tomato big bud	KJ462021	Tomato	Australia	53.0	52.9	53.0	53.0
Helminthotheca echioides yellows	KJ462023	*Helminthotheca echioides*	Italy	53.0	52.9	53.0	53.0
Canadian peach X disease	KJ462024	Peach	Canada	50.6	50.5	50.6	50.6
Plum leptonecrosis	KJ462027	Plum	Italy	51.7	51.6	51.7	51.7
Goldenrod yellows	KJ462028	*Cornus racemosa*	USA	51.4	51.4	51.4	51.4
Spirea stunt phytoplasma	KJ462029	Spirea	USA	51.2	51.1	51.2	51.2
Vaccinium myrtillus	KJ462030	*Vaccinium myrtillus*	USA	51.3	51.3	51.3	51.3
Ca. Phytoplasma ulmi	KJ462034	Elm	USA	64.4	64.3	64.4	64.4
Jujube witches' broom	KJ462036	Jujube	China	66.7	66.6	66.7	66.7
Rubus stunt	KJ462043	Rubus	Italy	67.5	67.4	67.5	67.5
Ca. Phytoplasma trifolii	KJ462045	Clover	Canada	57.8	57.7	57.8	57.8
Ca. Phytoplasma mali	KJ462047	Apple	Germany	52.0	52.0	52.0	52.0
Plum leptonecrosis	KJ462048	Plum	Italy	52.6	52.6	52.6	52.6
Napier grass stunt	KJ462053	*Pennisetum purpureum*	Uganda	51.2	51.1	51.2	51.2
Ca. Phytoplasma australiense	KJ462054	*Vitis* sp.	Australia	48.3	48.3	48.3	48.3

rU3 represent the primer sets used previously for the detection of phytoplasma in different crops like citrus, coconut, tomato, and acid lime in India (Manimekalai et al., 2011). The difference in the amplification to these primer was seen in the current phytoplasma isolates reflecting minor variations in the sequence of template, which indicates diversity in the pathogen population. Consistent and reproducible amplifications observed with R16mF2/R16mR1 and fU5/rU3 primer sets suggest that these primer sets can be routinely used for the diagnosis of phytoplasma in different crops in India.

The phytoplasma associated with witches' broom disease of Jackal jujube was identified on the basis of 16S rRNA-encoding gene and *Sec*A gene sequences and in silico restriction analysis (Wei et al., 2007) as the phytoplasma isolates causing witches' broom disease on Jackal jujube in Karnataka, India, as a member of *Ca.* P. balanitae reported from India and Myanmar belong to 16Sr group V, subgroup B.

According to the proposed 16SrDNA sequence-based phytoplasma classification scheme by applying computer-simulated RFLP analysis and automated similarity coefficient calculation (Wei et al., 2007), similarity coefficients between representative strains of any two subgroups within a given 16Sr group should be equal to or lower than 0.97. As the value of similarity coefficient between JJWB isolates and *Ca.* P. ziziphi (16SrV-B, AB052876) is 0.91, the phytoplasma under investigation is a strain of 16Sr group V. The restriction patterns of JJWB isolates subjected to the enzymes *Alu*I, *Hae*III, and *Mse*I have differed from those exhibited by the 16SrV phytoplasma subgroups viz elm yellows (16SrV-A, AY197655) from USA, JWB (16SrV-B, AB052876) from Japan, alder yellows (16SrV-C, AY197642) from southern Italy, "flavescence doree" (16SrV-D, AY197644) from northern Italy, rubus stunt strain Rus971 (16SrV-E, AY197650) from Switzerland, balanites witches' broom (16SrV-F, AB689678) from Myanmar, JWB (16SrV-G, AB052879) from Korea, and *Bischofia polycarpa* witches' broom (16SrV-H, KJ452547) phytoplasma from China.

The finer classification and description of the biology and ecology of phytoplasmas that are closely related but distinct strains cannot be easily resolved by the highly conserved 16S rRNA-encoding gene alone (Duduk & Bertaccini, 2011). Therefore, less conserved markers including *Sec*A, imp, tuf, ribosomal protein (rp), *Sec*Y, and SAP11 genes have been utilized for finer classification of closely related phytoplasmas within or between the existing 16S group or subgroup (Makarova et al., 2013; Marcone et al., 2000). The findings from our study showed that the sequence analysis of *Sec*A genes confirmed the JJWB phytoplasma isolates having closer relationships to the JWB. Computer-simulated RFLP analysis of the 16S rRNA-encoding gene and calculation of similarity coefficients for delineation of new subgroups(Wei et al., 2007, 2008) revealed that witches' broom phytoplasma associated with Jackal jujube in the present study was identified as a typical member of *Ca.* P. balanitae 16Sr V group.

Jackal jujube is a perennial flowering scandent and thorny shrub distributed in tropical and subtropical Asia and Australasia. It will grow in moist and dry deciduous forests and also in plains along the roadsides. Fruits of Jackal jujube are edible consumed by birds and animals. The various parts of the plant are widely used in Ayurvedic medicine for the treatment of various diseases. Besides its many health benefits, the crop is highly susceptible to witches' broom disease caused by phytoplasma. The witches' broom phytoplasma associated with Jackal jujube was identified based on the 16S rRNA-encoding gene and *Sec*A gene, and in silico restriction analysis showed that the phytoplasma is a typical member of *Ca.* P. balanitae which belongs to the 16Sr V group. Therefore, the outcomes of the study described here will provide basic and valuable knowledge for future research studies.

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References

Anonymous (2003). *The wealth of India: A dictionary of Indian raw materials and industrial productsraw materials series, Publication and Information Directorate* (pp. 49–51). Council of Scientific and Industrial Research.

Bertaccini, A., Duduk, B., Paltrinieri, S., & Contaldo, N. (2014). Phytoplasmas and Phytoplasma diseases: A severe threat to agriculture. *American Journal of Plant Sciences*, *05*(12), 1763–1788. [CrossRef]

Bertaccini, A., & Lee, I.-M. (2018). Phytoplasmas: An update. In *Phytoplasmas: Plant Pathogenic Bacteria-I, in Characterization and Epidemiology of Phytoplasma-Associated Diseases* G. P. Rao, A. Bertaccini, N. Fiore & L. Liefting (Eds.). Springer. [CrossRef]

Dickinson, M., & Hodgetts, J. (2013). PCR Analysis of phytoplasmas based on the SecA Gene. In *Phytoplasma: Methods and protocols*. (pp. 205–215). Humana Press. [CrossRef]

Doi, Y., Teranaka, M., Yora, K., & Asuyama, H. (1967). Mycoplasma or PLT grouplike microrganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows or Pawlownia Witches' Broom. *Japanese Journal of Phytopathology*, *33*(4), 259–266. [CrossRef]

Doyle, J. J., & Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. *Focus*, *12*, 13–15. [CrossRef]

Duduk, B., & Bertaccini, A. (2011). Phytoplasma classification: Taxonomy based on 16S ribosomal gene, is it enough? *Phytopathogenic Mollicutes*, *1*(1), 3–13. [CrossRef]

Eswari, M. L., Bharathi, R. V., & Jayshree, N. (2014). Pharmacognostical and physico-chemical standardization on the leaves of *Ziziphus oenoplia*. *International Journal of Green Pharmacy*, *8*(4), 424. [CrossRef]

Galtier, N., Gouy, M., & Gautier, C. (1996). SEA-VIEW and PHYLO WIN: Two graphic tools for sequence alignment and molecular phylogeny. *Computer Applications in the Biosciences: CABIOS*, *12*(6), 543–548. [CrossRef]

Gundersen, D. E., & Lee, I. M. (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea*, *35*, 114–151

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids*, *41*, 95–98

Jamadar, M. M., Balikai, R. A., & Sataraddi, A. R. (2009). Status of diseases on ber (*Ziziphus mauritiana* L.) in India and their management options. *Acta Horticulturae*, (840), 383–390. [CrossRef]

Jung, H. Y., Sawayanagi, T., Kakizawa, S., Nishigawa, H., Wei, W., Oshima, K., Miyata, S. I., Ugaki, M., Hibi, T., & Namba, S. (2003). 'Candidatus phytoplasma ziziphi', a novel phytoplasma taxon associated with jujube witches'-broom disease. *International Journal of Systematic and Evolutionary Microbiology*, *53*(4), 1037–1041. [CrossRef]

Khan, M. S., Raj, S. K., & Snehi, S. K. (2008). Natural occurrence of '*Candidatus* Phytoplasma ziziphi' isolates in two species of jujube trees (*Ziziphus* spp.) in India. *Plant Pathology*, *57*(6), 1173. [CrossRef]

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. [CrossRef]

Kuvar, S. D., & Bapat, U. C. (2010). Medicinal plants used by Kokani tribals of Nasik district Maharashtra to cure cuts and wounds. *Indian Journal of Traditional Knowledge*, *9*(1), 114–115

Lee, I. M., Bottner-Parker, K. D., Zhao, Y., Bertaccini, A., & Davis, R. E. (2012). Differentiation and classification of phytoplasmas in the pigeon pea witches' broom group (16SrIX): An update based on multiple gene sequence analysis. *International Journal of Systematic and Evolutionary Microbiology*, *62*(9), 2279–2285. [CrossRef]

Lee, I. M., Davis, R. E., & Gundersen-Rindal, D. E. (2000). Phytoplasma: Phytopathogenic Mollicutes. *Annual Review of Microbiology*, *54*, 221–255. [CrossRef]

Lee, I.-M., Gundersen, D. E., Hammond, R. W., & Davis, R. E. (1994). Use of Mycoplasmalike Organism (MLO) group specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology*, *84*(6), 559–566. [CrossRef]

Lee, I.-M., Gundersen-rindal, D. E., Davis, R. E., & Bartoszyk, I. M. (1998). Revised classification scheme of phytoplasmas based on RFLP analysis of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology*, *48*(4), 1153–1169. [CrossRef]

Lorenz, K.-H., Schneider, B., Ahrens, U., & Seemuller, E. (1995). Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. *Phytopathology*, *85*(7), 771–776. [CrossRef]

Makarova, O., Contaldo, N., Paltrinieri, S., Bertaccini, A., Nyskjold, H., & Nicolaisen, M. (2013). DNA bar-coding for Phytoplasma identification. *Methods in Molecular Biology*, *938*, 301–317. [CrossRef]

Manimekalai, R., Nair, S., Soumya, V. P., Roshna, O. M., & Thomas, G. V. (2011). Real-time PCR technique-based detection of coconut root (wilt) Phytoplasma. *Current Science*, *101*, 1209–1213

Marcone, C., Lee, I. M., Davis, R. E., Ragozzino, A., & Seemüller, E. (2000). Classification of aster yellows-group phytoplasmas based on combined analyses of ribosomal RNA and tuf gene sequences. *International Journal of Systematic and Evolutionary Microbiology*, *50*(5), 1703–1713. [CrossRef]

Misra, D. K., Saha, J., Devidas, P. V., & Bauri, F. K. (2013). Diseases of ber (*Zizyphus jujube*) in Eastern India. *Journal of Plant Protection Sciences*, *5*(1), 65–69

Ohashi, A., Nohira, T., Yamaguchi, K., Kusunoki, M., & Shiomi, T. (1996). Jujube (*Zizyphus jujuba*) witches broom caused by Phytoplasma in Gifu Prefecture. *Transactions of the Japan Society for Computational Engineering and Science*, *107*, 309–310

Omar, A. F. (2017). Detection and molecular characterization of phytoplasmas associated with vegetable and alfalfa crops in Qassim region. *Journal of Plant Interactions*, *12*(1), 58–66 . [CrossRef]

Omar, A. F., & Foissac, X. (2012). Occurrence and incidence of phytoplasmas of the 16SrII-D subgroup on solanaceous and cucurbit crop in Egypt. *European Journal of Plant Pathology*, *133*(2), 353–360. [CrossRef]

Pandey, P. K., Singh, A. B., Nimbalkar, M. R., & Marathe, T. S. (1976). A witches broom diseases of jujube from India. *Plant Disease Reporter*, *60*(4), 301–330.

Rao, G. P., Madhupriya, V., Thorat, V., Manimekalai, R., Tiwari, A. K., & Yadav, A. (2017). A century progress of research on Phytoplasma diseases in India. *Phytopathogenic Mollicutes*, *7*(1), 1–38. [CrossRef]

Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press.

Suksamrarn, S., Suwannapoch, N., Aunchai, N., Kuno, M., Ratananukul, P., Haritakun, R., Jansakul, C., & Ruchirawat, S. (2005). Ziziphine N, O, P and Q, new antiplasmodial cyclopeptide alkaloids from *Ziziphus oenoplia* var brunoniana. *Tetrahedron*, *61*(5), 1175–1180. [CrossRef]

Venkataravanappa, V., Reddy, C. N. L., Swarnalatha, P., Shankarappa, K. S., & Krishna Reddy, M. (2017). Detection and characterization of '*Candidatus* Phytoplasma asteris' associated with little leaf disease of bitter gourd from India by 16S rRNA phylogenetic and RFLP (in vitro and virtual) analysis. *Archives of Biological Sciences*, *4*, 707–714. [CrossRef]

Venkataravanappa, V., Swarnalatha, P., Saha, S., Lakshminarayana Reddy, C. N. L., & Krishna Reddy, M. (2019). Detection, characterization and in-silico analysis of '*Candidatus* Phytoplasma australasiae' associated with big bud disease of tomato in India. *Proceedings of the National Academy of Sciences, India Section B*, *89*(2), 493–503. [CrossRef]

Wang, J., Song, L., Jiao, Q., Yang, S., Gao, R., Lu, X., & Zhou, G. (2018). Comparative genome analysis of jujube witches'-broom Phytoplasma, an obligate pathogen that causes jujube witches'-broom disease. *BMC Genomics*, *19*(1), 689. [CrossRef]

Wei, W., Davis, R. E., Lee, I. M., & Zhao, Y. (2007). Computer simulated RFLP analysis of 16S rRNA genes: Identification of ten new Phytoplasma groups. *International Journal of Systematic and Evolutionary Microbiology*, *57*(8), 1855–1867. [CrossRef]

Weintraub, P. G., & Beanland, L. (2006). Insect vectors of phytoplasmas. *Annual Review of Entomology*, *51*, 91–111. [CrossRef]

Win, N. K. K., Lee, S. Y., Bertaccini, A., Namba, S., & Jung, H. Y. (2013). '*Candidatus* Phytoplasma balanitae' associated with witches broom disease of Balanites triflora. *International Journal of Systematic and Evolutionary Microbiology*, *63*(), 636–640. [CrossRef]

Yuan, G. Q., Li, Q. Q., Wei, J. G., & Lai, C. Y. (2009). Identification of the pathogens of different diseases of Yunnan jujube *Zizyphus mauritiana*. *South China Fruits*, *3*, 57–59

Zhao, Y., Wei, W., Lee, I. M., Shao, J., Suo, X., & Davis, R. E. (2009). Construction of an interactive online Phytoplasma classification tool, iPhyClassifier, and its application in analysis of the peach X-disease Phytoplasma group (16SrIII). *International Journal of Systematic and Evolutionary Microbiology*, *59*(10), 2582–2593. [CrossRef]