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Multigene Analysis for Differentiation of *Candidatus* Phytoplasma australasiae-Related Strain Associated with Witches-Broom of *Daucus carota* in India

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Abstract

Leaf samples (seven) from carrot plants exhibiting witches' broom symptoms were collected from the farmers' fields of Bangalore Rural and Chikkamaglore Districts of Karnataka, India. The presence of the causal agent was identified through PCR using the 16SrRNA, Ribosomal protein (rp) and *SecY* gene-specific primers. The seven carrot samples gave positive amplification for the phytoplasma specific primers. The amplified products were cloned and sequenced. The sequence analysis showed that the *16SrRNA*, *rep* gene and *SecY* gene of seven CarWB phytoplasma isolates shared highest not identity of 94.5% to 97.0%, 94.2% to 98.8% and 99.1% to 99.5% with *16SrRNA*, *rep* and *SecY* gene of *Ca.* P. australasiae (16SrII) group isolates reported so far. This result is well supported by close clustering of CarWB phytoplasma isolates in the current study with *Ca.* P. australasiae (16SrII) group isolates in the phylogenetic analysis. The virtual RFLP pattern generated for the CarWB phytoplasma revealed seven out of six isolates infecting carrot were different (similarity coefficient is ranged from 0.87 to 0.93) with respect to the nine enzymes from the reference pattern of the *Ca.* P. australasiae (16SrII) subgroups reported so far. Based on the threshold similarity coefficient for the new subgroup, delineation is set at 0.97. Therefore, the six CarWB phytoplasmas may be considered as a new subgroup under *Ca.* P. australasiae 16SrII group. This is the first the report of a phytoplasma associated with the little leaf disease of carrot from India.

Keywords: *Ca.* P. australasiae; 16SrII group; PCR; Phylogenetic analysis; Witches broom disease

Introduction

Carrot (*Daucus carota* L.) is an important root vegetable crop originated from Asia and belongs to the family, Apiaceae. China is the leading producer of carrot followed by Russia, America, Uzbekistan, and Poland. In India, different coloured carrots are being cultivated commercially round the year in an area of 3.46 million hectares with the annual production of 5.32 metric tonnes [1]. Carrot is rich in carotene (pro-vitamin A) and is consumed either as a fresh salad or in cooked form. The crop is a natural host for many viruses and globally, more than 30 different viruses were known to affect carrot [2,3]. Apart from different viruses, the carrot is being known to be susceptible to many phytoplasmas, reported under different names *viz.* aster yellows, ash witches' broom, "stolbur" and yellows [4]. The important phytoplasma infecting carrot belongs to the 16SrI subgroups are C and B [5-10], 16SrII subgroup C [11,12], 16SrIII, 16SrV [13,14], 16SrVI subgroup A [15] and 16SrXII subgroup A [8]. Apart from these, aster yellows phytoplasma (16SrI group), major phytoplasma infecting carrot was reported from different parts of the world [16-18]. The discovery of new phytoplasmas in many crops revealing the diversity of these pathogens across the world. In India, eleven groups (16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXII, 16SrXIII and 16SrXIV) of phytoplasmas infecting different crops were identified [19]. In the present study, we have characterized another phytoplasma belonged to the *Candidatus* Phytoplasma australasiae (16SrII group) associated with carrot witches' broom disease of carrot in India.

Material and Methods

Collection of disease samples

During the year 2016-2017, leaf samples from the carrot plants showing symptoms typical to phytoplasma infection such as dense clusters of highly proliferating apical shoot region, malformed flowers growth (phyllody), witches broom and stunted growth (Figure 1) were collected from the fields located at Bangalore Rural and Chikkamanglore Districts, Karnataka State, India.

Five carrot samples were from Bangalore Rural District (one each from Hesseraghatta, Rajanukuta, Doddabalikerae, Shivakote, and Kudige) and two samples were from Chikkamanglore District. Two non-symptomatic plants were also collected to serve as negative controls. The samples were stored at -80°C for further studies.

The carrot isolates collected from different locations were designated as CarWB1, CarWB2, CarWB3, CarWB4, CarWB5 (Bangalore Rural District), CarWB6-CM and CarWB7-CM (Chikkamanglore).

Figure 1: Carrot sample showing the typical witches broom phtoplasma symptoms under natural conditions.

DNA extraction and PCR amplification of 16SrRNA, *SecY* gene, and *ribosomal protein* (rp) gene

To confirm the phytoplasma infection in carrot, total DNA was isolated from seven infected carrot leaf samples using Cetyl-trimethyl ammonium bromide (CTAB) method [20]. The DNA isolated (Sesame and brinjal) from a known phytoplasma was used as positive control and from samples without symptoms used as negative control. Both direct and nested PCR assays were performed for detection of the phytoplasma DNA in carrot samples using phytoplasma universal 16SrDNA primer pairs P1/P7 [21,22] and R16F2n/R16R2 [23], respectively. After general detection, the rp operon encompassed with *rps19*, *rpl22* and *rps3* genes, was amplified using *rp* gene specific *(II) F1A/rp (II) R1A* primer pair [24]. Similarly, the *SecY* gene was amplified by PCR using primers SecYF1/SecYR1 [25] of carrot phytoplasma, which has been proven to be useful for fine-scale differentiation of phytoplasma strains. The PCR reactions were carried out for DNA amplification as described by Venkataravanappa et al. [26]. PCR amplified products of 16SrRNA (1.8 kb), *rp* gene (1.2 kb) and *SecY* gene (1.5 kb) were purified and cloned into the pTZ57R/T vector (Fermentas, Germany) as per manufactures instructions. The transformed recombinant clones were identified by restriction endonuclease digestion and selected positive clones were sequenced.

Sequence analysis

The full-length 16SrRNA, Ribosomal protein *(rp)* gene, and *SecY* gene sequence of CarWB phytoplasma isolates obtained were subjected to BLAST search for finding the similar sequences in the database. The sequences showing more homology with *16SrRNA* (Table 1), Ribosomal protein *(rp)* gene (Table 2) and *SecY* gene (Table 3) of different phytoplasma infecting crops were retrieved from NCBI database and aligned using SEAVIEW program [27]. A phylogenetic tree was constructed using MEGA 7 software [28] using the Neighbour-Joining method with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously. The *in-silico* RFLP patterns of the 16SrRNA gene from CarWB phytoplasma isolates were generated using the gel plotting program pDRAW32 (http://www.acaclone.com/) and iPhyClassifier software [29].

Phytoplasma species	Sub-group	Accession No.	Country
Eggplant big bud	16SrII	JX483699	Iran
Faba bean phyllody	16SrII	KP869129	Iran
Eggplant big bud	16SrII	JX441321	Iran
Sweet potato little leaf	16SrII	JQ868446	Australia
Scaevola witches broom	16SrII	AB257291	Oman
Crotalaria witches-broom	16SrII	EU650181	China
Pear decline	16SrII	EF193157	Taiwan
Alfalfa phytoplasma	16SrII	KY449416	Sudan
Tomato witches-broom	16SrII-D	HM584815	Saudi Arabia
Ca. P. aurantifolia	16SrII-B	U15442	Oman
Peanut witches-broom phytoplasma	16SrII-A	L33765	Taiwan
Ca. P. austrlasia	16SrII-D	Y10097	Australia
Crotalaria phyllody phytoplasma	[16SrII-C	EF193355	Thailand
Cactus witches'-broom phytoplasma	16SrII-G	EU099568	China
Cactus witches'-broom phytoplasma	16SrII-F	EU099556	China
Cactus witches-broom phytoplasma	16SrII-H	EU099569	China

Cactus witches-broom phytoplasma	16SrII-I	EU099551	China
Cactus witches-broom phytoplasma	16SrII-J	EU099552	China
Cactus witches-broom phytoplasma	16SrII-K	EU099572	China
Cactus witches-broom phytoplasma	16SrII-L	EU099546	China
Picris echiodes phyllody phytoplasma	16Sr11-E	Y16393	Italy
Ca. P. fraxini	16SrVII	AF092209	USA
Ca. P. ulmi	16SrV	AY197655	USA
Ca. P. palmae	16SrVIII	U18747	USA
Ca. P. cynodontis	16SrXIV	AJ550984	Italy
Ca. P. phoenicium	16SrIX	AF515636	Lebanon
Ca. P. pruni	16SrIII	L04682	USA
Ca. P. mali	16SrX	AJ542541	Italy
Ca. P. asteris	16SrI	M30790	Michigan
Ca. P. australiense	16SrXIII	L76865	Australia
Pigeon pea witches'-broom	16SrIX	AF248957	USA
Ash yellows	16SrVIII	AF189215	USA: New York
Ca. P. braziliense	16SrXV	AF105315	USA
Apple proliferation	16SrX	AF248958	Italy
Chickpea phyllody	16SrII	FJ870549	Pakistan Faisalabad
Clover phyllody	16SrI	AF222065	Canada
Cactus witches'-broom	16SrII	AJ293216	China
Clover yellow edge	16SrIII	AF189288	USA: Oregon
Coconut lethal yellowing phytoplasma	16SrIV	AF498307	Jamaica
Ca. P. trifolii	16SrVI	AY390261	Canada
Fragaria multicipita phytoplasma	16VI-G	AF190225	Canada
Ca. P. luffae	16SrVIII	AF353090	Taiwan
Ca. P. oryzae	16SrXI	AB052873	Thailand
Ca. P. solani	16SrXII	AJ964960	Spain
Periwinkle virescence	16SrXIII	AF248960	Mexican
Ca. P. brasiliense	16SrXV	AF147708	USA
Ca. P. graminis	16SrXV1	AY725228	Cuba
Ca. P. caricae	16SrXVII	AY725234	Cuba
Ca. P. americanum	16SrXVIII	DQ174122	USA
Ca. P. castaneae	16SrXIX	AB054986	South Korea
Ca. P. rhamni	16SrXX	X76431	Europe
Ca. P. pini	16SrXXI	AJ632155	Spain

Phytoplasma sp. strain	16SrXXII	Y14175	Nigeria
Grapevine yellows	16SrXXIII	AY083605	Australia
Sorghum bunchy shoot phytoplasma	16SrXIV	AF509322	Australia
Tea witches broom	16SrXXV	AF521672	Australia
Sugarcane phytoplasmaD3T1	16SrXXVI	AJ539179	Mauritius
Sugarcane phytoplasmaD3T2	16SrXXVII	AJ539180	Mauritius
Derbid phytoplasma	16SrXXVIII	AY744945	Cuba
Ca. P. malaysianum	16SrXXXII-A	EU371934	Malaysia

Table 1: 16SrRNA gene sequences of phytoplasma employed in analysis.

Phytoplasma species	**Sub-group**	**Accession No.**	**Country**
Tomato big bud	16SrII	KT970083	India
Tomato big bud	16SrII	KT970081	India
Cauliflower phyllody	16SrII	KC953012	China
Tomato big bud	16SrII	KC953016	China
Tomato big bud	16SrII	KT970084	India
Tomato big bud	16SrII	KT970082	India
Tomato big bud	16SrII	KT970080	India
Tomato big bud	16SrII	KT970078	India
Tomato big bud	16SrII	KC953016	China
Sword bean witches-broom	16SrII	KC953015	China
Parthenium virescence	16SrII	KC953014	China
Cauliflower witches-broom	16SrII	KC953012	China
Sesame phyllody	16SrII	GU004322	Thailand
Cowpea virescence	16SrII	KC953013	China
Sesame phyllody	16SrII	GU004362	Thailand
Brinjal little leaf	16SrVI-D	GU004356	India
Potato witches'-broom	16SrVI-A	GU004316	Canada
Clover phyllody	16SrVI-A	GU004315	Canada
Vinca virescence	16SrVI-A	GU004317	France
Ash yellows	16SrVI-A	GU004329	New York, USA
Milkweed yellows	16SrIII-F	GU004340	New York, USA
Potato purple top-MT	16SrIII-M	GU004333	Montana, USA
Clover yellow edge	16SrIII-B	GU004332	Lithuania
Spirea stunt	16SrIII-E	GU004326	New York, USA
Poinsettia branch-inducing	16SrIII-H	GU004328	USA
Peach X-disease	16SrIII-A	GU004327	Canada
Walnut witches-broom	16SrIII-G	GU004325	Georgia, USA
Apple proliferation	16SrX-A	GU004335	Italy
Mexican periwinkle virescence	16SrXIII-A	GU004336	Mexico
Tomato big bud	16SrI-A	AY803178	Arkansas, USA
Chrysanthemum yellows	16SrI-A	AY803170	Germany
Hydrangea phyllody	16SrI-A	AY803181	Belgium
Chrysanthemum yellows	16SrI-B	DQ787851	Italy
Primrose virescence	16SrI-B	AY803176	Germany
Clover phyllody	16SrI-C	AY803183	Germany
Paulownia witches-broom	16SrI-D	AY803184	Taiwan
Blueberry stunt	16SrI-E	AY803169	Michigan, USA
Apricot chlorotic leaf roll	16SrI-F	AY803166	Spain
Strawberry multiplier	16SrI-K	AY803180	Florida, USA
Aster yellows	16SrI-M	AY803168	Germany
Ipomoea witches-broom	16SrI-N	AY803182	Taiwan
Peanut witches-broom	16SrII-A	GU004331	Taiwan
Soybean phyllody	16SrII-C	GU004324	Thailand
Picris echioides	16SrII-E	GU004348	Italy
Australian tomato big bud	16SrII-D	GU004347	Australia
Elm yellows	16SrV-A	AY197690	New York, USA
Cherry lethal yellows	16SrV-B	AY197693	China
Alder yellows	16SrV-C	AY197692	Germany
Flavescence doree	16SrV-D	AY197685	Italy
Rubus stunt	16SrV-E	AY197696	Italy

American potato purple top wilt	16SrXVIII-B	GU004338	Nebraska, USA
Stolbur-It	16SrXII-A	GU004355	Italy
Pear decline	16SrX-C	GU004363	Italy
Candidatus Phytoplasma fraxini	16SrVII-A	GU004329	USA
Loofah witches'-broom	16SrVIII-A	GU004319	Taiwan

Table 2: *SecY* gene sequences of different phytoplasma employed in analysis.

Phytoplasma species		**Accession No.**	**Crop**	**Country**
Picris echioides phyllody		EF193381	Picris echioides	Italy
Flavescence doree	16SrV-D1	AY197664	Grapevine	Italy
Alder yellows	16SrV-C	AY197666	Periwinkle	Italy
Spartium witches-broom	16SrV-C	AY197672	Spartium junceum	Italy
Hemp dogbane yellows	16SrV-C	AY197674	Apocynum cannabinum	USA
Ca. P. ulmi	16SrV-A	AY197675	Ulmus americana	USA
Illinois elm yellows	-	EF183490	Elm	USA
Vinca virescence	-	EF183491	Vinca virescence	USA
Catharanthus phyllody	16SrVI	EF183494	Catharanthus rosea	Sudan
Ca. P. fraxini'	16SrVII-A	EF183492	Ash yellows	New York, USA
Pigeon pea witches-broom	16SrIX-A	EF183495	Pigeon pea	Florida, USA
Gliricidia little leaf	16SrIX	EF186800	Gliricidia	Honduras
Knautia arvensis phyllody	16SrIX	EF186801	Knautia arvensis	Italy
Picris echioides phyllody	16SrIX	EF186802	Picris echioides	Italy
Ca. P. phoenicium	16SrIX-B	EF186803	Almond	Lebanon
Palm lethal yellowing	16SrIV	DQ297677	Palm	Florida
Peach X-disease	16SrIII-A	EF186813	Peach	California, USA
Poinsettia branch-inducing	16SrIII-H	EF186811	Poinsettia	USA
Goldenrod yellows	16SrIII-D	EF186810	Goldenrod	New York, USA
Vaccinium witches-broom	16SrIII-F	EF186809	Vaccinium	Germany
Faba bean phyllody	16SrII-C	EF186817	Faba bean	Sudan
Crotalaria phyllody	16SrII-C	EF186818	Crotalaria	Thailand
Ca. P. aurantifolia	16SrII-B	EF186815	Lime	Oman
Italian alfalfa witches-broom	16SrII-E	EF193380	Alfalfa	Italy
Sesame phyllody	16SrII-A	EF193378	Sesame	Thailand
Ca. P. australasia	16SrII-D	EF193373	grapevine	Australia
Ca. P. mali	16SrX-A	EF193366	Apple	Italy
Ca. P. pyri	16SrX-C	EF193370	Pear	Germany
Apricot aster yellows	-	AY264866	Apricot	Spain
Aster yellows	16SrI-L	AY183686	Tomato	Germany
Blueberry stunt	16SrI-E	AY264863	Blueberry	Michigan, USA

Ca. P. australiense	16SrXII-B	AY376666	Grapevine	Australia
American potato purple top wilt	16SrXVIII-B	EF193362	Potato	Nebraska, USA
Tomato stolbur	16SrXII-A	EF193364	Tomato	Italy
Mexican periwinkle virescence	16SrXIII-A	EF193365	Periwinkle	Mexico
Ca. P. australiense	16SrXII-B	EF193372	Grapevine	Australia
Alfalfa witches-broom	16SrII-D	EF193371	Alfalfa	Oman
Sesame phyllody	16SrII-A	EF193377	Sesame	Thailand
Tomato big bud	16SrI-A	EF193373	Tomato	Arkansas, USA
Tephrosia witches-broom	16SrXII	JF441272	Tephrosia	China
Cleome phyllody	16SrII-A	EF193379	Cleome viscosa	Thailand
Peanut witches'-broom	16SrII-A	EF193375	Peanut	Taiwan
Crotalaria witches-broom	-	EU650182	Crotalaria	China
Cleome witches-broom	-	EU409599	Cleome viscosa	China
Sunn hemp witches-broom	16SrII-A	EF193374	Sunn hemp	Thailand
Sweet potato witches-broom	16SrII-A	EF193376	Sweet potato	Taiwan
Spermacoce exilis phyllody	16SrII-A	KX650616	Spermacoce exilis	China
Celosia argentea	16SrII-A	KX426375	Celosia argentea	China
Tomato big bud	-	KC953010	Tomato	China
Cowpea virescence	-	KC953007	Cowpea	China
Corchorus aestuans	16SrII-A	KX645866	Corchorus aestuans	China
Ipomoea aquatica witches-broom	-	KJ735784	Ater spinach	China
Lactuca sativa witches-broom	-	KJ735772	Lettuce	China
Cauliflower phyllody	16SrII-A	KC953006	Cauliflower	China
Pigeon pea witches-broom	-	FJ560598	Pigeon pea	China
Green gram phyllody	16SrII	AB703244	Green gram	Myanmar
Black gram witches-broom	16SrII	AB703243	Black gram	Myanmar
Parthenium weed witches-broom	16SrII	KU958723	Parthenium	Yunnan
Sword bean witches-broom	16SrII	KC953009	Sword bean	Yunnan
Peanut witches-broom	16SrII	JX871469	Peanut	Yunnan
Cucumber phyllody	16SrII	KY365523	Cucumber	Iran
Sesame phyllody	16SrII	AB703247	Sesame	Myanmar
Lime witches'-broom	16SrII	EF186815	Lime	Oman
Faba bean phyllody	16SrII	EF186817	Faba bean	Sudan
Alfalfa witches'-broom	16SrII	KY365520	Alfalfa	Iran
Crotalaria juncea	16SrII	KJ806618	Crotalaria juncea	Brazil

Mycoplasma-like organism		M74770	Crop	Country

Table 3: Ribosomal protein gene sequences of different phytoplasmas employed in analysis.

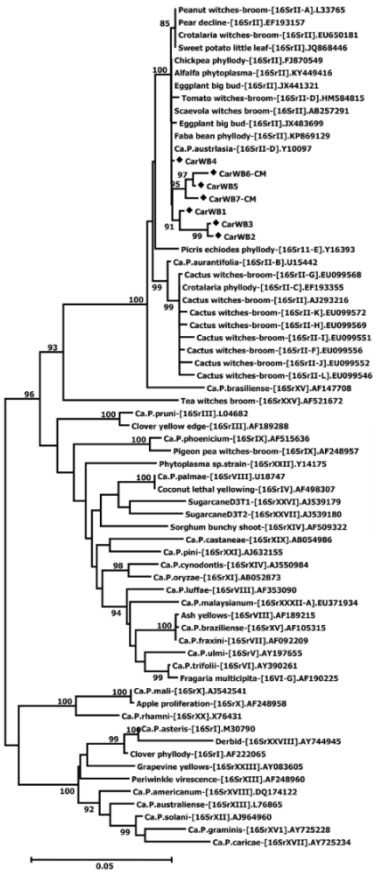
Results

Detection of phytoplasma

The seven CarWB phytoplasma samples collected from different farmers' fields gave positive amplification in PCR for the primer pairs designed to amplify 16srRNA (P1/P7), *SecY* gene (*SecYF2*/*SecYR1*), and *rep* gene (*rp (II) F1A/rp (II) R1A*) and confirmed the association of phytoplasma with the carrot witch's broom samples. No amplification was obtained from the non-symptomatic samples. The amplified PCR products of *16SrRNA*, *SecY* gene, and *rep* gene were cloned and sequenced. The sequences of 16SrRNA, rp gene, and *SecY* gene of seven CarWB phytoplasma isolates (CarWB1, CarWB2, CarWB3, CarWB4, CarWB5, CarWB6-CM and CarWB7-CM) were deposited in the GenBank (Accession No. MG013978, MH816941, MH816942, MH816943, MH816944, MH816945, MH816946 (16srRNA), MH816947, MH816948, MH816949, MH816950, MH816951, MH816952, MH816953 (Ribosomal protein gene), MG013981, MH844537, MH844538, MH844539, MH844540, MH844541, MH844542 (*SecY* gene)). The sequence analysis showed that the seven CarWB phytoplasma isolates shared nucleotide similarity of 88.2% to 97.6%, 95.8% to 100% and 93.6% to 100% with 16srRNA, *SecY* gene, and *rp* gene, respectively among themselves.

Carrot witches broom phytoplasma 16SrRNA gene sequence analysis

The *16SrRNA* gene sequences obtained from the seven isolates of CarWB phytoplasma (CarWB1, CarWB2, CarWB3, CarWB4, CarWB5, CarWB6-CM, and CarWB7-CM) from Karnataka in the current study were compared with twenty-three *16SrRNA* gene sequences of *Ca.* P. australasiae (16SrII) and thirty-eight 16SrRNA gene sequences of different phytoplasma sequences available in the database (Table 1). The *16SrRNA* gene sequence of CarWB phytoplasma isolates (CarWB1, CarWB2, CarWB3, CarWB4, CarWB5, CarWB6-CM and CarWB7-CM) shared highest nt identity of 92.9% to 97.9% with Eggplant big bud (JX441321, JX483699), Scaevola witches broom (AB257291), Crotalaria witches-broom (EU650181), Faba bean phyllody (KP869129), Alfalfa phytoplasma (KY449416), *Ca.* P. australasiae (Y10097), Pear decline (EF193157), Sweet potato little leaf (JQ868446), *Ca.* P. aurantifolia (U15442) phytoplasmas. Further, CarWB phytoplasma isolates showed very low nt identity of 78.9% to 94.0% with Peanut witches-broom (L33765), Crotalaria phyllody (EF193355) and Cactus witches'-broom (EU099568, EU099556, EU099569, EU099551, EU099552, EU099572 and EU099546) belongs to *Ca.* P. australasiae (16SrII) group. The phytoplasmas belonged to the different groups showed less than 85% nucleotide identity with CarWB phytoplasma isolates. The result is well supported by phylogenic analysis showing newly characterized CarWB phytoplasma grouped with previously identified phytoplasmas belonging to the *Ca.* P. australasiae (16SrII) infecting different crops in Indian subcontinents, Australia and Saudi Arabia (Figure 2a). The analysis also showed that Indian carrot infecting phytoplasmas form a monophyletic cluster with Asian-Australasian- Saudi Arabia originated phytoplasmas and established the close relationship with subgroup 16SrII-D.



Figures 2a: Phylogenetic tree based on sequences of the 16SrRNA.

Carrot witches broom phytoplasma *SecY* gene sequence analysis

The *SecY* gene (1.6 kbp) of seven CarWB1 phytoplasma isolates (CarWB1, CarWB2, CarWB3, CarWB4, CarWB5, CarWB6-CM and CarWB7-CM) sequences obtained in the current study were compared with nineteen *SecY* gene sequences of *Ca.* P. australasiae 16SrII group isolates and thirty-six *SecY* gene sequences of different phytoplasmas available in the database (Table 2). The sequence analysis result revealed that, the carrot isolates (CarWB1, CarWB2, CarWB3, CarWB4, CarWB5, CarWB6-CM and CarWB7-CM) shared maximum not identity of 96.3% to 99.5% with Tomato big bud (KT970083, KC953016, KT970078, KT970080, KT970082, KT970084, KC953016, KT970081), Sesame phyllody (GU004362), Cauliflower phyllody (KC953012), Sword bean witches-broom. (KC953015), Parthenium virescence (KC953014), Cauliflower witches-broom (KC953012), Cowpea virescence (KC953013); This indicates the *SecY* gene

amplified from carrot infected witches broom disease belongs to the *Ca.* P. australasiae (16SrII), more specifically to the subgroup 16Sr II-D. The phylogenetic analysis showed that the *SecY* gene of CarWB phytoplasma is more closely clustered to the previously identified phytoplasmas belongs to the group of 16SrII (Figure 2b).

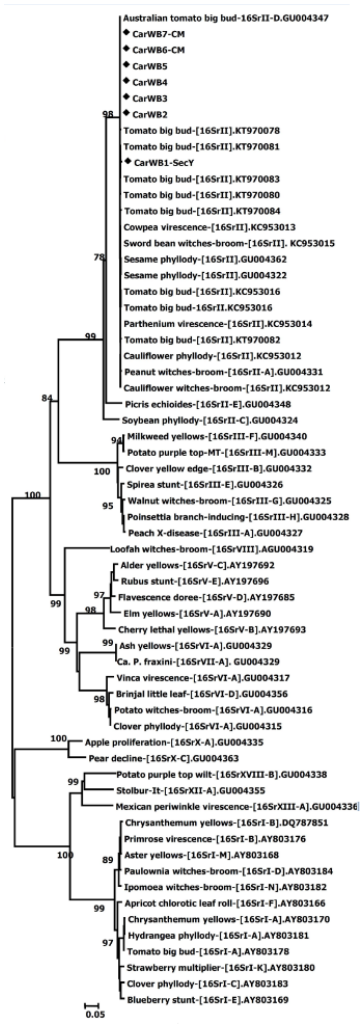


Figure 2b: Phylogenetic tree based on sequences of the SecY gene.

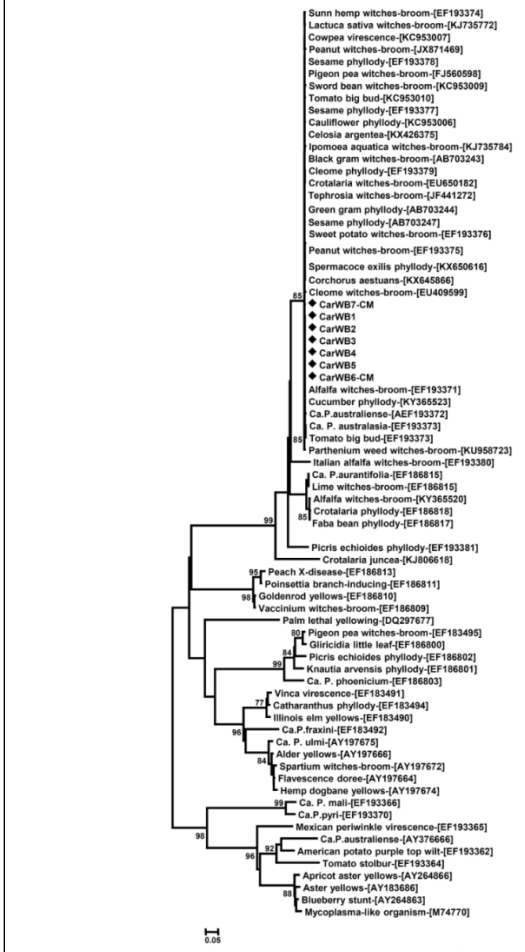


Figure 2c: Phylogenetic tree based on sequences of the ribosomal gene of the CarWB phytoplasma isolates with other phytoplasma strains using the Neighbor-joining algorithm. Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees are un-rooted. A bootstrap analysis with 1000 replicates was performed and the bootstrap percent values more than 50 are numbered along branches.

Carrot witches broom phytoplasma ribosomal protein (rp) gene sequence analysis

The *rep* gene has more variability than the *16SrRNA* gene and a better molecular tool for differentiation of genetically closely related but ecologically distinct phytoplasma strains that are not readily separated on the basis of the highly conserved *16SrRNA* gene sequence. The *rep* gene (1.2 kb) of seven CarWB phytoplasma isolates (CarWB1, CarWB2, CarWB3, CarWB4, CarWB5, CarWB6-CM and CarWB7-CM) sequences obtained were compared with the corresponding region of eighteen *rep* gene sequences of *Ca.* P. australasiae (16SrII) and fourty-nine *rep* gene sequences of different phytoplasmas available in the database (Table 3).

The *rep* gene sequences analysis showed that CarWB phytoplasma isolates showed maximum nt identity of 94.2 to 98.8 with Spermacoce exilis phyllody (KX650616), Green gram phyllody (AB703244), Black gram witches-broom (AB703243) and Cucumber phyllody (KY365523). While CarWB phytoplasma showed very low nt identity of 92.6% to 95.1% with Celosia argentea (KX426375), Tomato big bud (KC953010), Cowpea virescence (KC953007), Corchorus aestuans phyllody-(KX645866), Ipomoea aquatica witches-broom (KJ735784), Lactuca sativa witches-broom-(KJ735772), Cauliflower phyllody (KC953006), Pigeon pea witches-broom (FJ560598), Parthenium weed witches-broom (KU958723), Sword bean witches-broom (KC953009), Peanut witches-broom (JX871469) and Sesame phyllody (AB703247) phytoplasma sequences. However, the phytoplasmas belonged to the different groups showed less than 85% of nucleotide identity with CarWB phytoplasma. This indicates the *rep* gene amplified from infected carrot witches broom samples belongs to the *Ca.* P. australasiae (16SrII). The phylogenetic analysis showed that the *rep*

gene of CarWB phytoplasma isolates are more closely clustered with the previously identified phytoplasmas belongs to the group of 16SrII (Figure 2c).

Virtual RFLP analysis

The virtual RFLP patterns of the 16SrRNA gene of seven carrot witches phytoplasma isolates revealed that the CarWB1 (coefficient of similarity 0.93), CarWB2 (0.89), CarWB3 (0.88), CarWB5 (0.89) and CarWB6-CM (0.83) represent a previously un-described subgroup in the group 16SrII. They exhibit different restriction patterns from (Figures 3A-3F) that of the 16SrRNA gene from subgroup 16SrII-D (accession no. Y10097, *Ca.* P. australasiae). However, the isolate CarWB7-CM (0.87) exhibited restriction pattern different from that of the 16SrRNA gene from subgroup 16SrII-B (accession no. U15442, *Ca.* P. aurantifolia), while isolate CarWB4 exhibited similar restriction pattern to that of *Ca.* P. australasiae (accession no. Y10097, similarity coefficient of 1.00). Based on the threshold similarity coefficient for the new subgroup, delineation is set at 0.97 [36]. Therefore, carrot witches broom phytoplasma isolates (CarWB1, CarWB2, CarWB3, CarWB5, CarWB6-CM, CarWB7-CM) showing similarity coefficient less than 0.97 may be considered as a new subgroup under *Ca.* P. australasiae 16SrII group. The enzymes that distinguish other *Ca.* P. australasiae 16SrII group isolates reported so far are BstUI and RsaI for CarWB1 (Figure 3A), RsaI, Sau3AI (MboI), HaeIII, TaqI, SspI, HpaI, HhaI distinguishes CarWB2 (Figure 3B and 3B1), Sau3AI (MboI) and AluI distinguishes CarWB3 (Figure 3C), Sau3AI (MboI) and AluI distinguishes CarWB5 (Figure 3d), Sau3AI (MboI), SspI and AluI distinguishes CarWB6-CM (Figure 3E), Alu I and HpaI distinguishes CarWB7-CM (Figure 3F). This was further supported by restriction map of carrot witches phytoplasma isolates obtained from pDRAW32 (AcaClone Software; http://www.acaclone.com) analysis showing significant differences in AluI, BstUI, RsaI, Sau3AI (MboI), HaeIII, TaqI, SspI, HpaI and HhaI with Eggplant big bud (JX483699) and other 13 representatives of subgroups (16SrII- A, B, C, D, F, G, H, I, J, K, L, S, T) belong to 16SrII (Figure 4). This is the first report of *Ca.* P. australasiae belonged 16SrII affecting carrot in India.

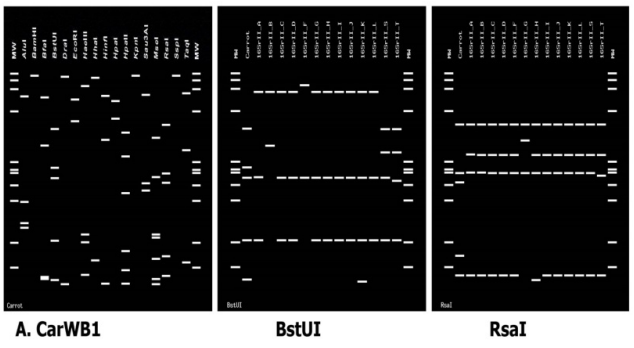


Figure 3A: Virtual RFLP patterns derived from *in silico* digestions, using iPhyClassifier, of F2n/R2 fragments of *16S rRNA* gene from strains of A. CarWB1 (accession no. MG013978).

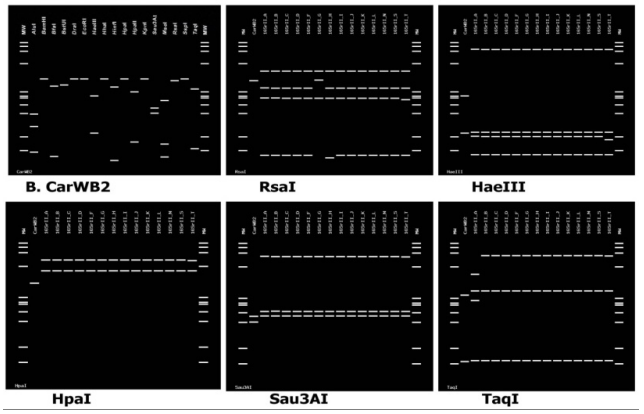


Figure 3B: Virtual RFLP patterns derived from *in silico* digestions, using iPhyClassifier, of F2n/R2 fragments of *16S rRNA* gene from strains of CarWB2 (accession no. MH816941).

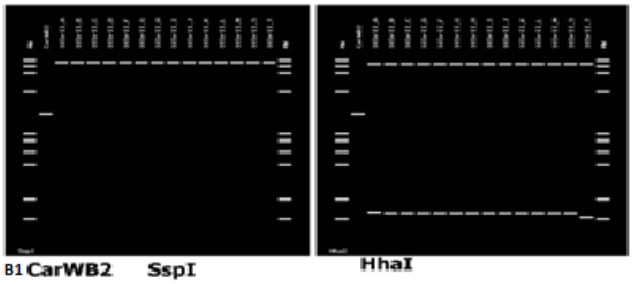
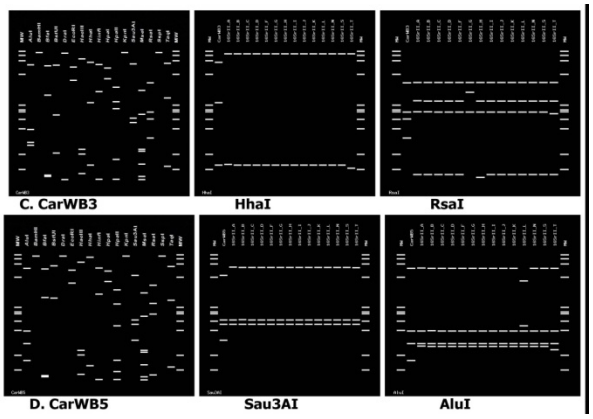


Figure 3B1: Virtual RFLP patterns derived from *in silico* digestions, using iPhyClassifier of F2n/R2 fragments of *16S rRNA* gene from strains of CarWB2.



Figures 3C and 3D: Virtual RFLP patterns derived from in silico digestions, using iPhyClassifier, of F2n/R2 fragments of 16S rRNA gene from strains of (C) CarWB3 (accession no. MH816942); (D) CarWB5 (accession no. MH816944).

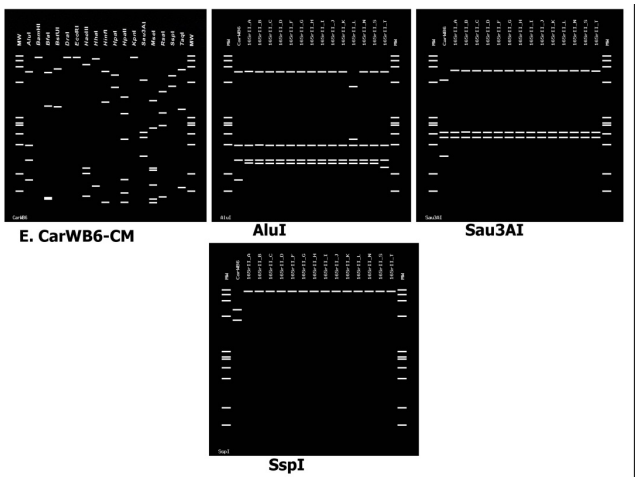


Figure 3E: Virtual RFLP patterns derived from in silico digestions, using iPhyClassifier, of F2n/R2 fragments of 16S rRNA gene from strains of CarWB6 (accession no. MH816945).

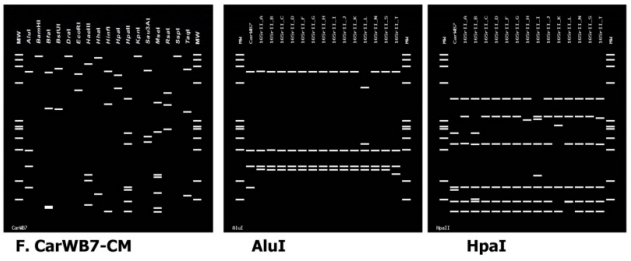


Figure 3F: Virtual RFLP patterns derived from *in silico* digestions, using iPhyClassifier, of F2n/R2 fragments of *16S rRNA* gene from strains of CarWB7 (accession no. MH816946) using 17 restriction endonuclease enzymes (left): AluI, BamHI, BfaI, BstUI, DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, Sau3AI, MseI, RsaI, SspI, and TaqI. Virtual RFLP patterns of CarWB1 (BstUI and RsaI), CarWB2 (RsaI, Sau3AI (MboI), HaeIII, TaqI, SspI, HpaI and HhaI), CarWB3 (Sau3AI (MboI) and AluI), CarWB5 (Sau3AI (MboI) and AluI), CarWB6 (Sau3AI (MboI), SspI and AluI) and CarWB7 (AluI and HpaI) to distinguishing strain Indian phytoplasmas from other strains in group 16SrII. The restriction fragments were resolved by *in-silico* electrophoresis through 3% agarose gel. MW, WX174 DNA-HaeIII digest.

Recombination analysis

The Recombination analysis was carried by using RDP4, based on the alignment of different (*16SrRNA*, ribosomal protein gene and *SecY* gene) gene sequences of seven CarWB phytoplasma isolates with other respective genes of phytoplasma retrieved from the database was done. The analysis indicated the presence of inter-specific recombination only in 16SrRNA (Figure 5) and there is no recombination was detected in ribosomal protein gene and *SecY* gene of seven CarWB phytoplasma isolates.

The recombination breakpoint analysis for 16SrRNA gene of seven CarWB phytoplasma isolates revealed that most parts of their 16SrRNA F2nR2 fragments being descendent from as major *Ca. P. solani*-(16SrXII) (AJ964960) and minor parent *Ca. P. caricae*-(16SrXVII) (AY725234). In the case of CarWB2, the recombination breakpoints were predicted at nucleotides position of 36 and 1630 with an average probability value of 8.319 x10^{-3}.

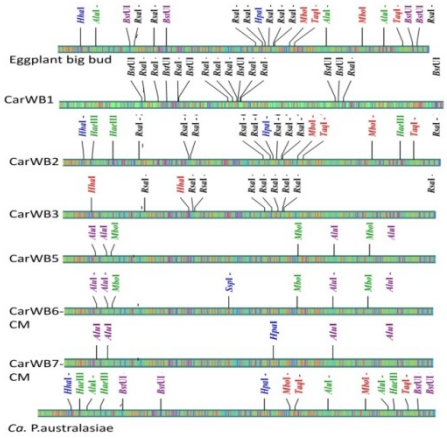


Figure 4: Comparative analysis of virtual restriction sites in *16SrRNA* gene. Sequences of the *16SrRNA* gene of phytoplasma from Carrot are CarWB1 (accession no. MG013978), CarWB2 (accession no. MH816941), CarWB3 (accession no. MH816942), CarWB5 (accession no. MH816944), CarWB6 (accession no. MH816945), CarWB7 (accession no. MH816946) 'Eggplant big bud (JX483699) phytoplasma (AY265209) and *Ca.* P. australasiae phytoplasma (accession no. Y10097,) the important difference in the restriction site of different phytoplasmas.

Further, the recombination breakpoints were detected in CarWB3 at predicted nucleotides position of 36 and 1659 with an average probability value of 7.636 x 10^{-32}. Similarly, in CarWB4, the recombination breakpoints were detected at nucleotides position of 51 and 1040 with an average probability value of 1.465 x 10^{-1}. While in case of CarWB5 the breakpoints for recombination were detected at predicted nucleotides position of 36 and 1659 with an average probability value of 8.25 x 10^{-30}. Further, in case of CarwB6 and CarwB7, the breakpoints were detected at nucleotides position of 36-1659 and 46-1256 with average probability values of 1.573 x 10^{-30} and 6.620 x 10^{-34}, respectively.

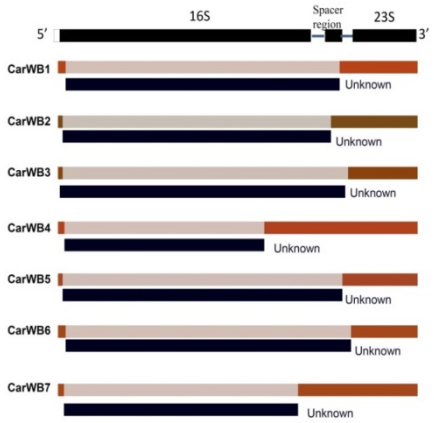


Figure 5: Analysis of recombination of *16SrRNA* gene of phytoplasma isolates (CarWB1, CarWB2, CarWB3, CarWB4, CarWB5, CarWB6-CM, and CarWB7-CM) from Carrot: The phytoplasma acronyms given are: Carrot witches broom Phytoplasma (CarWB), *Ca.*P.solani-(16SrXII) (AJ964960) and *Ca.* P. caricae-(16SrXVII) (AY725234). The sequence of indeterminate origin is indicated as "unknown". The bars below the isolate name indicate their genome and the boxes below this with phytoplasma acronyms indicate the approximate position at which recombination has occurred in the genome of phytoplasma isolates.

Discussion

Plant-pathogenic phytoplasmas are unique prokaryotic microbes that lack a cell wall and belong to the class Mollicutes and have been recently classified within the provisional genus "*Candidatus* phytoplasma" based on the *16SrRNA* gene sequence analysis [30]. These phytopathogenic phytoplasmas are known to infect more than 700 economically important plant species belongs to vegetables, cereals, fruits, ornamentals, and forest plants causing huge economic losses worldwide. The infected plants exhibit various kind of symptoms, may be due to the imbalance in different intrinsic factors of the host during host-pathogen interaction [31]. Accurate detection of phytoplasma is a prerequisite for the management of the disease. The nucleic-acid based technique such as polymerase chain reaction (PCR) is routinely used for detecting phytoplasmas in different plants. At present, the molecular analysis of conserved genes, in particular, the *16SrRNA* gene is used for the detection, identification, and classification of phytoplasmas [32-34].

In the present study, seven CarWB samples collected from the different farmer's field showed positive amplification with phytoplasma specific primers designed for different genomic segments (*16SrRNA*, *SecY* gene and *rep* gene). Based on sequence information of *16SrRNA*, *SecY* gene and *rep* gene, the CarWB phytoplasmas infecting carrot from Karnataka, India were identified as members of the 16SrII-D and 16SrII-B subgroup belongs to the *Ca.* P. australasiae (16SrII) group [29]. Further, *In-silico* restriction analysis of F2n/R2 fragment of *16SrRNA* gene of CarWB using iPhyClassifier online tools showed that the CarWB phytoplasma isolates are different with respect to having restriction sites for the nine restriction enzymes (AluI, BstUI, RsaI, Sau3AI (MboI), HaeIII, TaqI, SspI, HhaI, and HpaI) [35], which are routinely used for classification of the phytoplasma into groups and subgroups. According to the proposed 16SrRNA-based phytoplasma classification scheme by applying computer simulated RFLP analysis and the similarity coefficient calculation [36,37], the value of the similarity coefficient between CarWB phytoplasma isolates and *Ca.* P. australasiae (16SrII-B and16Sr II-D) is ranged from 0.83 to 0.93 and the enzymes that differentiate the CarWB phytoplasma isolates from that of 16SrII phytoplasma group isolates are AluI, BstUI, RsaI, Sau3AI (MboI), HaeIII, TaqI, SspI, HhaI, and HpaI. Generally, it has been accepted that even one restriction site difference (within the 16SrRNA gene F2nR2 region) between phytoplasma strains from previously established subgroups may be considered as a new subgroup [35]. The strains under investigation showed similarity coefficient ranged from 0.83 to 0.93, which is less than to 0.97. Thus the strains (CarWB1, CarWB2, CarWB3, CarWB5, CarWB6-CM, and CarWB7-CM) are new and significantly distinct from those of representative strains of *Ca.* P. australasiae belongs to the 16SrII group reported so far.

For the finer classification within or between the existing 16S group or subgroup of closely related phytoplasma species infecting different crops, several studies have used less-conserved and variable genes (*secA, imp, tuf, ribosomal protein (rp), secY*, and *SAP11* genes) as molecular markers in conjunction with the *16SrRNA* gene [24,38-43]. The member of phytoplasma, *Candidatus Phytoplasma australasiae* were known to infect chickpea, tomato and sesame in the Indian subcontinent [44-47], The findings from the sequence analysis of *secY* and *ribosomal protein (rp)* genes confirmed that CarWB phytoplasma in the current study is having a close relationship to the *Candidatus* Phytoplasma australasiae, revealing the rapid expansion of host range by phytoplasma belongs to *Ca.* P. australasiae 16SrII subgroup.

The recombination analysis suggested that seven CarWB phytoplasma isolates have obtained at least some of its sequence by recombination between *Ca.* P. solani (AJ964960) and *Ca.* P. caricae (AY725234) for the 16SrRNA like ancestors reported from Spain and Cuba, respectively [48,49] but not from India. This suggested that recombination between the parents of the CarWB phytoplasma isolates either occurred before introduction to India or the CarWB phytoplasma isolates present in the country, but is yet to be identified. Recombination is a major mechanism in creating genetic diversity in phytoplasmas and has played a key role in the evolution of wild-type line (OY-W) and mild-symptom line (OY-M) of onion yellows phytoplasma [46,50].

In conclusion, the phytoplasma infection in carrot is increasing in Karnataka State, India. This is one of the most important and alarming signal for its seed production. In the present study, we have characterized phytoplasma belongs to *Ca.* P. australasiae 16SrII group related strain association with carrot witches broom disease from Karnataka, India. This is the first evidence of association of phytoplasma '*Ca.* P. australasiae belonging to an a16SrII group with a carrot from Karnataka State, India. These findings would be more useful for future studies on the disease and its vector management.

Competing Interests

The authors declare that they have no competing interests.

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