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First Report of Association of Phytoplasma Strain belonging to 16SrI-D Group with Phyllody Disease of Niger (*Guizotia abyssinica* Cass.)

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Niger [*Guizotia abyssinica* Cass.] is one of the important minor oilseed crops grown in India. Its production is challenged by numerous diseases affecting production and quality, of which phyllody is emerging as constraint. During June 2021, niger plants exhibiting typical symptoms of phytoplasma infection were observed at the experimental field of Zonal Agricultural Research Station, Bengaluru (13.0801N,77.570E), Karnataka, India. The infected plants showed excessive proliferation of shoots, reduced leaf size and stunted growth leading, phyllody symptoms with a disease incidence of 10 per cent. The association of phytoplasma with the niger phyllody samples was confirmed by PCR using 16S rRNA, *Sec*Y and ribosomal protein (rp) gene-specific primers. The amplified PCR products were cloned and sequenced. The 16S rRNA, SecY and rp genes sequences analysis showed that niger phyllody (NiPP) phytoplasma shared a maximum nucleotide identity of 96.10 to 99.80 per cent (16S rDNA), 93.40 to 98.60 per cent (SecY gene) and 93.30 to 95.50 per cent (rp gene) with '*Candidatus* Phytoplasma asteris' group (16SrI-D) isolates reported from different countries across the world. This was well supported by close clustering of NiPP phytoplasma with '*Ca.* P. asteris' phytoplasmas belonging to the 16SrI-D group in the phylogenetic analysis. Further, the virtual RFLP pattern generated for the NiPP phytoplasma showed a similarity coefficient of 1.0 with '*Ca.* P. asteris', 16SrI enclosed in 16Sr group I and subgroup D. As per our knowledge this is first report of '*Ca.* P. asteris' belonging to 16Sr I-D group association with phyllody disease of niger in India.

Keywords : Niger, Multilocus gene, Nested PCR, *Ca.* P. asteris, 16SrI-D group

Niger (*Guizotia abyssinica* Cass.) is one of the important minor oil seed crops in India. It belongs to the family, Asteraceae. It is an annual herb native to Africa and later introduced to many tropical and subtropical regions of the world (Vaali *et al.,* 2011). Oil extracted from the niger seeds is used in human food preparation, paints, soaps, as an illuminant, drying oil and rheumatism treatment. Pre-flowering stages of whole plant is used as fodder and green manure and seeds as food for cage birds (Vaali *et al.,* 2011). India ranks first in the area, production and export of niger in the world. In India, it is mainly cultivated in tribal areas of Madhya Pradesh, Odisha, Bihar, Karnataka, Maharashtra and Andhra Pradesh (Anita Itnal *et al.,* 2022). Area, production and productivity of niger in India are about 156.46 thousand ha, 45.42 thousand tonnes and 290 kg/ha, respectively (Anonymous, 2019). In Karnataka, niger is cultivated over an area about 0.99 thousand ha with production and productivity of 0.20 thousand tonnes and 200 kg/ha, respectively (Anonymous, 2019).

Some pathogens including fungi and bacteria are known to infect the niger crop (Kolte, 1985). Natural

occurrence and successful transmission of niger phyllody through the leafhopper vector *Orosius albicinctus,* as well as the association of phytoplasma measuring 100-800 nm in ultra thin sections of diseased niger phloem sieve tubes were confirmed by electron microscope (Rangaswamy and Muniyappa, 1993). Phytoplasma diseases are emerging as a threat to the cultivation of many crops resulting in huge losses (Rao *et al.,* 2018 and Venkataravanappa *et al.,* 2019). Phytoplasmas induce an array of disease symptoms in infected plants.

Based on the analysis of highly conserved 16S rRNA gene sequences, 41 '*Candidatus*' species, 34 ribosomal groups and 160 subgroups were reported (Bertaccini and Lee, 2018). Out of these 34 ribosomal groups, 11 have been reported from India including 16SrI, II, VI and XI groups, which are considered major strains (Rao *et al.,* 2017). Finer classification and description of the biology and ecology of phytoplasmas that are closely related but belong to distinct strains cannot be easily resolved by the highly conserved 16S rRNA gene sequence alone (Duduk and Bertaccini, 2011). Hence, there is a need of the application of other housekeeping genes including SecA, imp, tuf, ribosomal protein (rp), *Sec*Y and SAP11 genes for finer classification and categorizing phytoplasmas into diverse ribosomal groups and subgroups (Martini *et al.,* 2007; Hodgetts *et al.,* 2008; Ashwathappa *et al.,* 2019 and Rihne *et al.,* 2021). With this backdrop, in the present study, attempt was made to identify and characterize the strain of the phytoplasma associated with phyllody disease of niger based on PCR assays and 16S rRNA, *Sec*Y and rp gene sequence comparison analysis.

Material and Methods

Collection of Diseased Plant Samples

During June 2021, leaf samples from naturally infected niger plants showing typical symptoms of phytoplasma (little leaves, virescence and phyllody) were collected from the experimental field of Zonal Agricultural Research Station, Bengaluru (13.0801N,77.570E), Karnataka, India. The Incidence of niger phyllody (NiPP) was recorded by scoring infected plants over healthy plants. Five symptomatic and one healthy leaf samples were collected from the same location and nucleic acid was extracted.

DNA Extraction and PCR Amplification of 16Sr RNA, SecY Gene and Ribosomal Protein (rp) Gene

To confirm the phytoplasma infection in niger, total DNA was isolated from five infected niger leaf samples using Cetyl-trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). DNA isolated from sesame and brinjal and previously confirmed for presence of phytoplasma was used as positive control and samples without symptoms were used as a negative control. Both direct and nested PCR assays were performed for the detection of the phytoplasma using the DNA isolated from infected niger samples using phytoplasma universal primers (P1/P7) (Deng and Hiruki, 1990 and Schneider, 1995), and nested PCR primers (R16F2n/R16R2) (Gundersen and Lee, 1996), respectively. After general detection, the rp operon encompassed with rps19, rpl22 and rps3 genes was amplified using rp gene specific *rp*(I)F1A/ *rp*(I)R1A) primer pair (Martini *et al.,* 2007). Similarly, the *Sec*Y gene was amplified by PCR using primers *Sec*YF1/*Sec*YR1 (Lee *et al.,* 2010). PCR reactions were carried out in a thermal cycler (Master cycler, Profelex, Hamburg, Germany) and the cycling protocols used for PCR assay was followed as earlier described (Venkataravanappa *et al.,* 2019; Bandakanavara *et al*., 2021). PCR amplified products of 16S rRNA (1.8 kb), rp gene (1.2 kb) and *Sec*Y gene (1.5 kb) were purified and cloned into the pTZ57R/T vector (MBI Fermentas, USA) as per the manufacturer instructions. The transformation was performed using *Escherichia coli* (DH5α) cells. Recombinant plasmids were purified and confirmed clones were sequenced in both directions at Eurofins Genomics Pvt. Ltd., Bengaluru, India.

Sequence Analysis

Full-length 16S rRNA gene, ribosomal protein (rp) gene and SecY gene sequences of NiPP phytoplasma isolates obtained were subjected to BLAST search to find similar sequences in the NCBI database. Sequences of different phytoplasma infecting crops

showing more homology with 16Sr RNA (Table 1), *Sec*Y gene (Table 2) and ribosomal protein (rp) gene (Table 3) were retrieved from the NCBI database and aligned using BioEdit (Hall, 1999) and ClustalW (Thompson *et al.,* 1994) programs. A phylogenetic tree was constructed using MEGA X software using the Neighbour-Joining method with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously (Kumar *et al.,* 2016). *Acholeplasma laidlawii* (Accession number U14905) for 16S rRNA and *Leptospira interrogans* (Accession number MH376290) for *Sec*Y genes and *A. laidlawii* (Accession number M81465) for rep gene sequences were used as outgroups in the phylogenetic tree analysis.

Virtual RFLP Analysis

The *in-silico* RFLP patterns of the 16S rRNA gene from NiPP phytoplasma isolates were generated using iPhyClassifier online tool (https://plant pathology.ba.ars.usda.gov/cgi-bin/resource/ iphyclassifier.cgi) (Zhao *et al.,* 2009) to identify the phytoplasma to group and subgroup level. The virtual RFLP patterns were compared and the similarity coefficient was calculated (Wei *et al.,* 2008). Restriction pattern obtained for the phytoplasmas associated with niger phyllody was compared with already published RFLP patterns of phytoplasma representatives of available ribosomal groups and 16SrI subgroups (Lee *et al.,* 1998).

Results and Discussion

During June 2021, the niger plants exhibiting symptoms such as reduced leaf size, stunted growth, phyllody and excessive proliferation of shoots (Fig.1) indicative of witches' broom diseases caused by phytoplasmas was noticed at an experimental field, the college of agriculture, Bengaluru, Karnataka, India. The incidence of niger phyllody (NiPP) was 10 per cent. Phytoplasma presence in all the five symptomatic samples was confirmed through PCR amplification in direct and nested PCR assays which resulted in expected amplicon size of 1.8 kb and 1.2 kb with universal primer pair P1/P7 and R16nF2/ R16nR2, respectively. No amplification was observed in asymptomatic plant sample collected from same location. (data not shown). Amplified PCR fragments of each gene were cloned separately and sequenced in both directions. The consensus sequences of 16S rRNA (accession number OP057904), *Sec*Y gene (accession number OP082232) and rp gene (accession number OP082231) were deposited in NCBI GenBank.

Nucleotide sequence of the 16S rRNA gene of NiPP was compared with 32 phytoplasma 16S rRNA gene sequences retrieved from the NCBI database. Results revealed that 16S rRNA gene sequence of NiPP shared maximum nt identity of 99.80 per cent with aster yellows (AY180957) and less than 98.20 per cent nt identity with several phytoplasmas belonging to '*Ca*. P. asteris' group (16Sr I) (Table 1). These results

Fig. 1 : Niger plant with phyllody and little leaf symptoms (a) and Healthy plant (b) under field condition

TABLE 1

16S rRNA gene sequences of phytoplasmas employed in analysis

Phytoplasma disease / country	16S rDNA group/subgroup	Gen Bank accession number	Identity with NiPP phytoplasma
Aster yellows / Germany	16SrI-L	AY180957	99.80
Cucumber viresence / Taiwan	16SrI-K	GU361755	96.50
Aster yellows / USA	16SrI-B	M30790	98.00
Potato purple top / Russia	16SrI-P	EU333397	97.90
Apricot aster yellows / Spain	16SrI-F	AY265211	97.20
Aconitum proliferation / Lithuania	16SrI-A	AF510323	98.50
Aster yellows-AVUT / Germany	16SrI-M	AY265209	98.30
Blueberry stunt-BBS3 / USA	16SrI-E	AY265213	98.40
Cherry little leaf / Lithuania	16SrI-Q	AY034089	97.70
Japanese maple witches' broom	16SrI-B	JQ015183	96.10
Clover phyllody / Germany	16SrI-C	AY265217	98.20
Valeriana yellows / Lithuania	16SrI-M	AY102274	96.00
Western X-disease / USA	16SrIII-A	L04682	90.00
Coconut lethal yellowing / USA	16SrIV-A	U18747	90.10
Ash yellows / USA	16SrVII-A	AF092209	91.80
Apple proliferation / Germany	16SrX-A	AJ542541	91.90
Spartium witches' broom / Italy	16SrX-B	X92869	92.00
Pear decline / Germany	16SrX-C	AJ542543	92.00
Stone fruit yellows / Germany	16SrX-F	AJ542544	88.00
Rice yellow dwarf / Germany	16SrXI-A	AB052873	57.00
Cucumis sativus phytoplasma / Iran	16SrVI-A	MH004458	97.00
Strawberry yellows / Lithuania	16SrXII-E	DQ086423	96.20
Mexican periwinkle virescence / Mexico	16SrXIII-A	AF248960	89.90
Bermudagrass white leaf / Italy	16SrXIV-A	AJ550984	88.80
Chestnut witches' broom / South Korea	16SrXIX-A	AB054986	89.60
Hibiscus witches' broom / Brazil	16SrXV-A	AF147708	93.40
Sugarcane yellow leaf / Cuba	16SrXVI-A	AY725228	95.90
American potato purple top wilt / USA	16SrXVIII-A	DQ174122	89.90
Pine shoot proliferation / Europe	16SrXXI-A	AJ632155	89.30
Awka wilt / Nigeria	16SrXXII-A	Y14175	96.70
Grapevine yellows / Australia	16SrXXIII-A	AY083605	85.90
Sorghum bunchy shoot / USA	16SrXXIV-A	AF509322	85.50

were well supported by the phylogenetic analysis showing the 16S rRNA gene sequence of NiPP closely clustering with members of '*Ca*. P. asteris' group (16SrI) infecting different crops (Fig. 2a). Further, *in silico* RFLP analysis of F2nR2 fragment of 16S rRNA sequence of NiPP phytoplasma using aid of online tool iPhyClassifier indicated that the virtual RFLP pattern derived from the query of F2nR2 fragment of 16S rRNA sequence of NiPP phytoplasma is identical (similarity coefficient of 1.0) to the reference strain phytoplasma enclosed in 16SrI-D. (GenBank accession: Y10097). Therefore, the

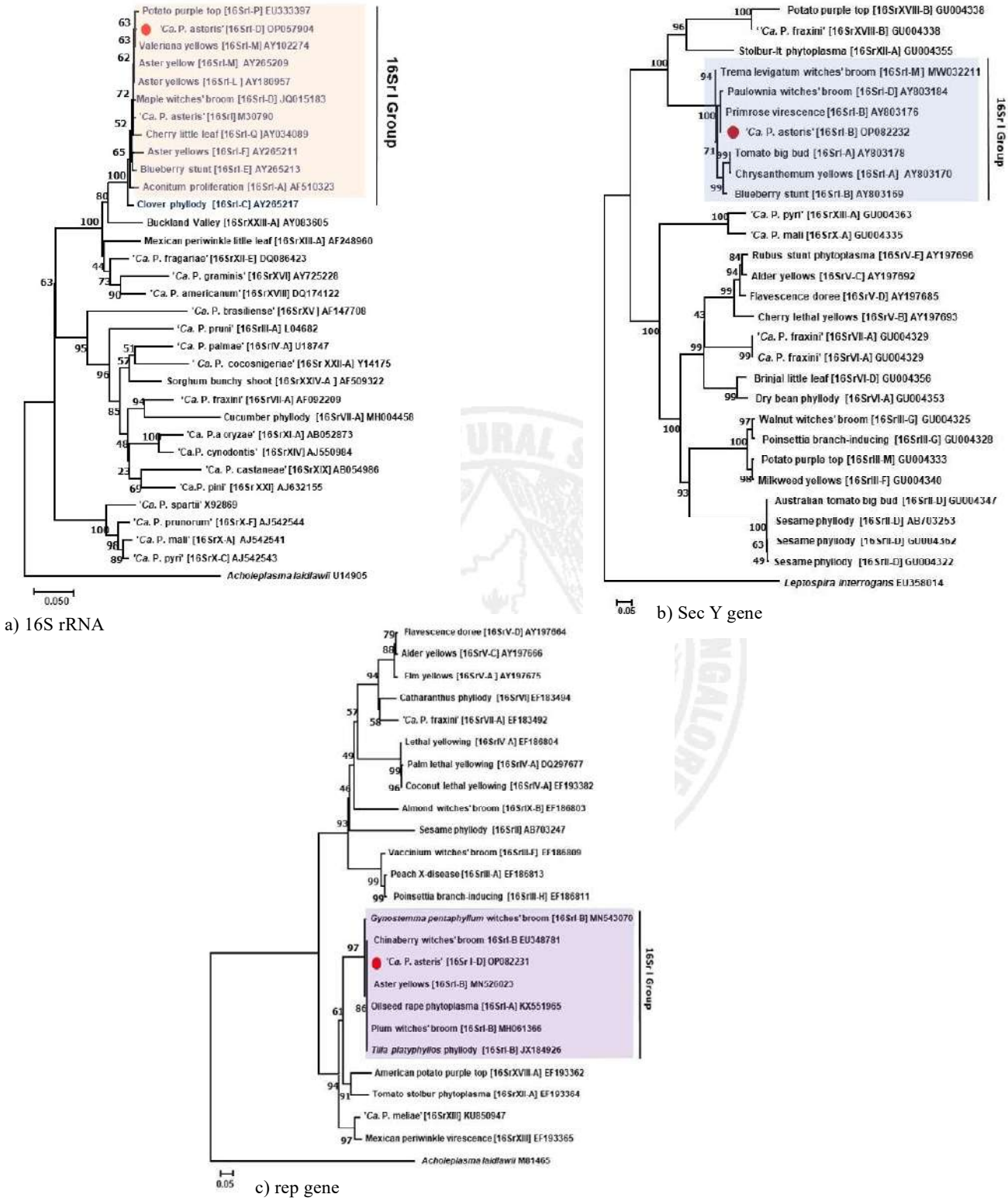

Fig. 2 : Phylogenetic tree based on sequences of 16S rRNA (a) *Sec*Y genes (b) and rp gene (c) from niger phytoplasma (NiPP) with the sequences of phytoplasma strains listed in table 1, 2 and 3 by Neighbor-Joining method using MEGA X Software. The tree was rooted with sequences of *Acholeplasma laidlawii* (U14905), *Leptospira interrogans* (MH376290) and *Acholeplasma laidlawii* (M81465) for 16S rRNA, *Sec*Y gene and rp gene analysis, respectively. A bootstrap analysis with 1,000 replicates was performed and the bootstrap per cent values above 50 are showed along the branches.

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TABLE 2

SecY gene sequences of phytoplasmas employed in the analysis

Phytoplasma Species /Country	subgroup	Gen Bank accession number	Identity with NiPP phytoplasma
Tomato big bud phytoplasma / USA	16SrI-A	AY803178	93.50
Chrysanthemum yellows phytoplasma / Germany	16SrI-A	AY803170	93.40
Trema' witches'-broom / China	16SI-B	MW032211	88.70
Primrose virescence phytoplasma / Germany	16SrI-B	AY803176	98.60
Paulownia witches-broom phytoplasma / Taiwan	16SrI-D	AY803184	97.80
Blueberry stunt phytoplasma / Michigan, USA	16SrI-E	AY803169	94.60
Sesame phyllody phytoplasma / Thailand	16SrII	GU004322	31.90
Sesame phyllody phytoplasma / Thailand	16SrII	GU004362	42.20
Milkweed yellows phytoplasma / New York, USA	16SrIII-F	GU004340	42.40
Potato purple top phytoplasma / Montana, USA	16SrIII-M	GU004333	42.50
Poinsettia branch-inducing phytoplasma / USA	16SrIII-H	GU004328	34.00
Walnut witches-broom / Georgia, USA	16SrIII-G	GU004325	33.90
Cherry lethal yellows phytoplasma / China	16SrV-B	AY197693	33.60
Alder yellows phytoplasma / Germany	16SrV-C	AY197692	33.60
Flavescence doree phytoplasma / USA	16SrV-D	AY197685	44.10
Dry bean phyllody phytoplasma / Washington, USA	16SrVI-A	GU004353	45.00
Brinjal little leaf phytoplasma / India	16SrVI-D	GU004356	44.80
Candidatus Phytoplasma fraxini / USA	16SrVII-A	GU004329	44.80
Apple proliferation phytoplasma / Italy	16SrX-A	GU004335	43.30
Pear decline phytoplasma / Italy	16SrX-C	GU004363	32.60
Mexican periwinkle virescence / Mexico	16SrXIII-A	GU004336	32.90
Potato purple top wilt phytoplasma / USA	16SrXVIII-B	GU004338	32.60
Stolbur-It phytoplasma / Italy	16SrXII-A	GU004355	31.70
'Candidatus Phytoplasma americanum' / USA	16SrXVIII-B	MN227134	39.50

phytoplasma identified in the present study belongs to '*Ca*. P. asteris' (16SrI) group and subgroup D. and is a '*Ca*. P. asteris'-related strain belongs to subgroup D (Lee *et al.,* 1998 and Wei *et al.,* 2008).

Similarly, the SecY gene of NiPP was compared with the corresponding region of twenty-four phytoplasmas sequences (Table 2) belonging to different groups (Lee *et al.,* 2010). This comparison showed that NiPP has maximum nt identity of 93.40 to 98.60 per cent with several phytoplasmas infecting tomato (AY803178), chrysanthemum (AY803170), trema plant (MW032211), Primrose (AY803176), Paulownia (AY803184) and blueberry (AY803169) belonging to the '*Ca*. P. asteris' group (16Sr I). This was well supported by phylogenetic analysis (Fig. 2b) in which the *Sec*Y gene sequence of NiPP phytoplasma closely clustered with several phytoplasmas enclosed in the 16SrI group.

The rp gene sequence of NiPP comparison with the corresponding region of twenty-eight phytoplasmas from different groups showed highest nucleotide identity of 94.30 to 99.50 per cent with aster yellows phytoplasma (MN526023) and other phytoplasmas belonging to the '*Ca*. P. asteris' group (16Sr I) (Table 3). This result was also well supported by a phylogenetic analysis showing rp gene sequence of

TABLE 3

rp gene sequences of phytoplasmas employed in analysis

Phytoplasma Species / Country	subgroup	Gen Bank accession number	Identity with NiPP phytoplasma
Gynostemma pentaphyllum' witches'-broom / China	16SrI-B	MN543070	96.20
Aster yellows phytoplasma / Hungary	16SrI-B	MN526023	99.50
Oilseed rape phytoplasma / China	16Sr1-A	KX551965	94.30
Tilia platyphyllos' phytoplasma / Lithuania	16SrI-B	JX184926	97.50
Sesame phyllody / Myanmar	16SrI-M	AB703247	96.50
Peach X-disease / USA	16SrI-A	EF186813	99.00
Goldenrod yellows / USA	16SrIII-D	EF186810	55.40
Vaccinium witches' broom / Germany	16SrIII-F	EF186809	58.70
Poinsettia branch inducing / USA	16SrIII-H	EF186811	58.40
Pigeon pea witches' broom / USA	16SrIX-A	EF183495	59.00
Almond witches' broom / Lebanon	16SrIX-B	EF186803	63.20
Picris echioides phyllody / Italy	16SrIX-C	EF186802	58.40
Elm yellows / USA	16SrV-A	AY197675	58.00
Alder yellows / Italy	16SrV-C	AY197666	58.20
"Flavescence dorée" / Italy	16SrV-D	AY197664	60.00
Catharanthus phyllody / Sudan	16SrVI	EF183494	60.30
Ash yellows / USA	16SrVII-A	EF183492	78.60
Apple proliferation / Italy	16SrX-A	EF193366	80.50
Pear decline / Germany	16SrX-C	EF193370	48.50
Mexican periwinkle virescence / Mexico	16SrXIII-A	EF193365	60.90
American potato purple top wilt / USA	16SrXVIII-A	EF193362	60.60
Tomato "stolbur" / Italy	16SrXII-A	EF193364	79.00
Palm lethal yellowing / Florida	16SrIV-A	DQ297677	79.00
Lethal yellowing phytoplasma / USA	16SrIV-A	EF186804	79.00
Coconut lethal yellowing / USA	16SrIV-A	EF193382	79.00
Mexican periwinkle virescence / Mexico	16SrXIII	EF193365	68.40
'*Candidatus* Phytoplasma meliae' / Argentina	16SrXIII	KU850947	69.00
'*Candidatus* Phytoplasma meliae' / South Korea	16SrXIII	KU850945	69.00

NiPP closely clustering with phytoplasmas belonging to the '*Ca*. P. asteris' group (16Sr I) (Fig. 2c).

The phytoplasma associated with phyllody disease of niger was identified based on conserved 16S rRNA gene and less conserved genes *Sec*Y, ribosomal protein gene sequences and *in-silico* restriction analysis (Zhao *et al*., 2009). These analyses revealed that the phytoplasma causing phyllody in niger in Karnataka (India) is a member of *'Ca*. P. asteris' and belongs to the 16Sr I group.

Finer classification and description of the biology and ecology of phytoplasmas that are closely related but belong to distinct strains cannot be easily resolved by the highly conserved 16S rRNA gene sequence alone (Duduk and Bertaccini, 2011). Therefore, less conserved markers including SecA, imp, tuf, ribosomal protein (rp), *Sec*Y, and SAP11 genes, have been previously utilized for finer classification of closely related phytoplasma within or between the existing 16S group or subgroups (Martini *et al.,* 2007 and Hodgetts *et al.,* 2008).

Sequence analysis of *Sec*Y and ribosomal protein (rp) in our study confirmed that the NiPP phytoplasma has a close evolutionary relationship with the '*Ca*. P. asteris' (Hodgetts *et al.,* 2008). A literature survey showed that the '*Ca.* P. asteris' is associated with a wide host range compared to other phytoplasma groups and known to infect different crops in India including tomato, strawflower, gaillardia, gerbera, carrot and coriander (Venkataravanappa *et al.,* 2019; Ashwathappa *et al.,* 2019 and Reddy *et al.,* 2020). Recent disease reports showed that 16SrI phytoplasma possesses a wide host range of crops (Thorat *et al.,* 2016). These phytoplasma diseases are becoming major problem in many crops worldwide, including India (Bertaccini and Lee, 2018). More than 700 economically important plant species are affected by these pathogens inducing symptoms such as stunting, shoot proliferation, phyllody, viresence, witches' broom and fasciation in different crops (Rao *et al.,* 2018). According to previous studies phyllody disease in niger was caused by distinct aster yellows phytoplasma groups including 16SrI-B or rpI-B, (Babaie *et al.,* 2007 and Vali *et al.,* 2014), 16SrII-D (Hosseini *et al.,* 2014) from Iran, 16SrI from India (Mahalingappa *et al.,* 2019).

In the present investigation, we have identified and classified phytoplasma infecting niger based on16S rRNA gene sequence and *in-silico* restriction analysis using iPhyClassifier online tools (Zhao *et al.,* 2009). The evidence suggests that, Phytoplasma NiPP isolate causing niger phyllody India is a member of 16Sr I-D subgroup belongs to the group *'Ca*. P. asteris'. Applications of housekeeping genes like ribosomal protein (rp) gene, SecY gene provides a reliable means for the differentiation of broad array of phytoplasma and has become the most comprehensive and widely accepted phytoplasma classification system.

The phytoplasma diseases of niger are becoming a major constraint for its production in India and other parts of the world. More studies are required to determine the source of inoculum, vectors involved in transmission and the economic impact of *'Ca*. P. asteris' will help find strategies to control this pathogen. To our knowledge, this is first report of *'Ca.* P. asteris' belonging to 16SrI-D group association with phyllody disease of niger in India and world based on the multilocus genes sequences analysis.

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