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Molecular Characterization of Selected Weed Host and Crop Plants Infected by Phytoplasma

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ABSTRACT

Amplified 1.8 kb and 1.2 kb fragment corresponding to the 16S rDNA and intergenic transcribed spacer region of the phytoplasma infecting pigeonpea, brinjal, sesamum, chickpea, sunhemp, buckwheat, datura and parthenium tissue using phytoplasma specific universal primers P1/P7 and R16F2n/R16FR2. Phylogenetic analysis of 16S rDNA gene of phytoplasma revealed that, *Daturastramonium* phytoplasma Shivamogga, pigeonpea phyllody GKVK (Accession No. MW559977), chickpea phyllody phytoplasma Doddaballapur (Accession No. MW583034) sunhemp phyllody GKVK (Accession No. MW543302) and buckwheat phyllody GKVK (Accession No. MW559978) were clustered with periwinkle little leaf phytoplasma (Accession No. AF228053.1) and shared nucleotide identity of 62.1 to 85.5 per cent. Similarly, sesamum phyllody GKVK (Accession No. MW559980) and parthenium phyllody GKVK (Accession No. MW559981) with *'Setaria verticillata'* little leaf phytoplasma SVLL-GOK1 (Accession No.MW077129.1) with sequence similarity of 88.4 to 96.1 per cent, whereas, brinjal little leaf phytoplasma GKVK (Accession No.MW547094) clustered with Iranian safflower phyllody phytoplasma (Accession No. EF534205.1) and shared 92.4 per cent nucleotide identity.

Keywords : Phytoplasma, Molecular characterization, PCR and Phylogenetic analysis

THE rapid increasing population and change in dietary habits associated with urbanization lead to increased demands for food and fuel. A wide range of crops are grown in different agro-climatic zones of India (Naingroo, 2014). Selected few crops *viz*., sesamum, pigeonpea, buckwheat, brinjal (Egg plant), chickpea, sunhemp and the associated with weeds *viz*., parthenium and datura. There are several constraints in production of above crops such as abiotic and biotic stresses. Among them, the phytoplasma-associated diseases represent one of their major constraints in several parts of the world causing significant yield losses and quality of produce deteriorating. In India, phytoplasma associated with plants including field crops, fruits, trees, ornamental, sugarcane, grasses and weeds is increasing at an alarming rate. Phytoplasma cause diseases in several plant species and resulted in serious crop loss. They pose threat as alternate natural hosts for the spread of phytoplasma pathogen to other economically important plants and thereby chances of causing severe losses (Rao *et al*., 2011). Phytoplasma are obligate prokaryotic wall-less bacteria which multiply in isotonic niches of plant phloem tissues and insect haemolymph. They are pleomorphic, with size variations from 200 to 800 nm and possess a very small genome of about 680-1600 kbp. Phytoplasmas are associated with over 600 diverse plant diseases worldwide, mainly transmitted by phloem-feeding insects, especially leaf hoppers and plant hoppers (Bertaccini *et al*., 2014). Phytoplasmas are associated with a wide variety of symptoms in a diverse range of plant hosts. Characteristic symptoms associated with phytoplasma infection includes stunting, shoot proliferation, witches' broom of developing tissues (clustering of branches), phyllody (retrograde metamorphosis of the floral organs to leaf like structures), virescence (green coloration of non-green flower parts), formation of bunchy fibrous secondary roots, reddening of leaves and stems, generalized yellowing, decline, phloem necrosis and fasciation that may be due to the imbalance of plant growth regulators (Bertaccini, 2007; Hoshi *et al*., 2009; Omar *et al*., 2014 and Hogenhout *et al*., 2008). Some plant species are tolerant or resistant to phytoplasma infections; when infected, these plants may be asymptomatic or exhibit mild symptoms (Lee *et al*., 2000).

Material and Methods

Collection of Phytoplasma Disease Sample

Samples were collected from naturally phytoplasma infected plants showing typical symptoms of phytoplasmaduring 2018-19 and 2019-20.

Total Genomic DNA Extraction

Total nucleic acid was isolated from infected leaf tissue by modified cetyl trimethyl ammonium bromide (CTAB) (Sunard *et al*., 1991) method and used for PCR amplification by using degenerated oligonucleotide universal primers (Deng and Hiruki, 1991). The DNA concentrations were measured with nanodrop spectrophotometer.

Polymerase Chain Reaction

The DNA obtained was subjected to PCR amplification using primer designed to amplify 16S rDNA from the infected weed hosts and crop plants. PCR amplifications were conducted using phytoplasma specific universal P1 (5'-AAGAGTTTGATCCTGGCTCAGGA TT-3') (Deng and Hiruki, 1991) and P7 (5'-CGTCCTTCATCGGCTCTT-3') (Smart *et al*., 1996) amplifying ~1,800 bp fragment of phytoplasma in first round PCR. Further, first round PCR products were used in nested PCR amplification using universal primer pair R16F2n (5'-GAAACGACTGCTAAGACTGG-3') and R16R2 (5' -TGACGGGCGGTGTG TACAAA CCCC-3) (Lee *et al*., 1993).

The DNA was amplified by an initial denaturation of 94 °C for 2 min followed by 35 cycles of 94 °C for 2 min denaturation, 55 °C for 2 min primer annealing (56 °C for 1 min for N-PCR), 72 °C for 3 min primer extension and final extension at 72 °C for 10 min.

Analysis of PCR products by one per cent agarose gel electrophoresis. Sequencing of amplified PCR product and sequence analysis. The products were sent to Chromous Biotech Pvt. Ltd., Bengaluru for the sequencing by Sanger's primer walking method. Sequencing was done in both directions using forward and reverse primers. The sequences retrieved were subjected to BLAST analysis.

Construction of Phylogenetic Tree

The sequence homology obtained in BLAST (www.ncbi.nih.gov /BLAST) and Neighbor joining phylogenetic tree was generated using MEGA 6.06 software tool. Sequences downloaded from NCBI database used for phylogenetic analysis (Table 1).

Results and Discussion

During field survey recorded the symptoms of phytoplasma infection on five crops *viz*., pigeonpea (*Cajanus cajan*), brinjal (*Solanum melongena*), sesamum (*Sesamum indicum*), chickpea (*Cicer arietinum*), sunhemp (*Crotalaria juncea*) and weed hosts *viz*., parthenium (*Parthenium hysterophorus*) and *Datura stramonium* in different parts of Southern Karnataka. The plants infected with phytoplasma were pale green and bushy due to excessive stunting, reduction in leaf size, reduced internodal length, excessive axillary proliferation and floral malformation like abnormal green structures developed by replacing normal flowers (Plate 1). Similar types of symptoms were reported on pigeonpea (Suryanarayana *et al.,* 1997 and Raj *et al*., 2006), brinjal (Rathnamma & Patil, 2017 and Vijay Kumar *et al.*, 2018), sesamum (Manjunatha *et al*., 2012; Palanna *et al*., 2012; Venkataravanappa *et al*., 2017; Devanna *et al.*, 2020), chickpea (Pallavi *et al*., 2012; Naik *et al*., 2018), sunhemp (Yang ,1979) and parthenium (Padmanabhan,1984 & Sanjeeth Kumar, 1989).

Characterization of Phytoplasma Associated with Selected Weed Hosts and Crop Plants using 16S rDNA

The presence or absence of phytoplasma was confirmed by PCR from symptomatic plants using the 16S rDNA gene specific phytoplasma universal primers. The PCR amplification from eight samples

TABLE 1

List of phytoplasma strains used for phylogenetic analysis of 16S rRNA

Phytoplasma strains	Accession Number
'Pelargonium capitatum' phytoplasma clone 61	HM583346.1
'Exitianus indicus' phytoplasma EiAPN6	MT500683.1
Empoasca sp. phytoplasma EmND2	MT500682.1
Sweet potato witches' - broom phytoplasma	DQ777762.2
'Desmodium ovalifolium' witches'-broom Hainan-Chengmai	MK956144.1
'Catharanthus roseus' phytoplasma CR-ND	MT420679.1
'Parthenium hysterophorus' phytoplasma PR24	MG748740.1
'Phaseolus vulgaris' phytoplasma RJ	MN700647.1
'Croton sparsiflorus' phytoplasma CSAP-P9	MN551491.1
'Albizia lebbeck' witches' - broom phytoplasma AWB 1	MN121115.1
'Allium cepa' phytoplasma On-Gharb 2	LT715991.1
'Tylophora indica' little leaf yellowing phytoplasma Gurgaon-1	KF773149.1
Candidatus Phytoplasma aurantifolia 204	MN611950.1
'Cannabis sativa' leaf yellowing phytoplasma CSLY-FAZ1	MW077131.1
'Setaria verticillata' little leaf phytoplasma SVLL-GOK1	MW077129.1
Brinjal little leaf phytoplasma strain BLL	EF186820.1
Periwinkle little leaf phytoplasma	AF228053.1
Iranian safflower phyllody phytoplasma	EF534205.1
Bushehr sesame phyllody phytoplasma Bu10	KC429652.1
Cleome witches'-broom phytoplasma ClWB	EU513212.1
'Emiliasonchifolia' witches'-broom phytoplasma T1	MT420682.1
Phytoplasma sp. PR08	LN879443.1
'Helianthusannuus' phyllody	MK421430.1
Bougainvillea proliferation phytoplasma New Delhi	MF509776.1

of symptomatic pigeonpea, brinjal, sesamum, chickpea, sunhemp, buckwheat, datura and parthenium, yielded the expected products of 1.8 kb in direct PCR by using P1/P7 primers pairs (Plate 2). Further, direct PCR product was subjected to the nested PCR using universal phytoplasma primer pair R16F2n/R16R2, which yielded the expected amplicon size of 1.2kb (Plate 3). All the samples collected from symptomatic plants gave a PCR amplification confirming that phytoplasma as disease causative agent. Previous efforts successfully amplified DNA fragments of ~1.8 and ~1.2 kb, respectively, from direct and nested PCR assays, respectively (Salehi *et al.,*2015; Benequen *et al.*, 2016; Mulpuri and Muddanuru, 2016; Hosseini *et al.,* 2016; Akhtar *et al.*, 2016; Ahmad *et al.,* 2017; Omar, 2017; Hemmati *et al.,* 2017; Madhupriya *et al.*, 2017 and Devanna *et al.*, 2020).

16S rDNA Gene Sequence Analysis

The phylogenetic analysis of 16S rDNA gene of phytoplasma associated with pigeonpea, brinjal, sesamum, chickpea, sunhemp, buckwheat, datura and parthenium revealed that, *Datura stramonium* phytoplasma Shivamogga, pigeonpea phyllody GKVK (Accession No. MW559977), chickpea phyllody phytoplasma Doddaballapur (Accession No. MW583034) sunhemp phyllody GKVK (Accession No. MW543302) and buckwheat phyllody GKVK (Accession No. MW559978) were clustered with periwinkle little leaf phytoplasma (Accession No.

Plate 1: Symptoms of phytoplasma infection recorded on selected weed hosts and crop plants (A) Sesamum (B) Chickpea (C) Pigeonpea (D) Brinjal (E) Buckwheat (F) Sunhemp (G) Parthenium (H) Datura

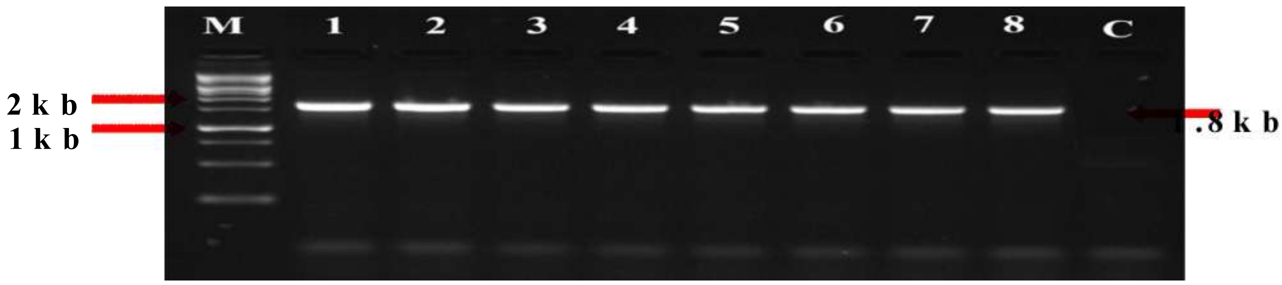

Plate 2 : PCR amplification of 16S rDNA gene of phytoplasma using P1/P7 primers

Lanes:

Lane M: 1 kb Ladder
Lane 2: Brinjal little leaf
Lane 3 : Sesamum phyllody
Lane 4: Chickpea phyllody
Lane 5: Sunhemp phyllody
Lane 6: Parthenium phyllody
Lane 7 : Buckwheat phyllody
Lane 8: Datura phyllody

TABLE 2

Analysis of the sequence similarities among the 16S rDNA gene sequences from the phytoplasma

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	ID																															
2	0.890	ID																														
3	0.914	0.914	ID																													
4	0.814	0.814	0.852	ID																												
5	0.871	0.870	0.855	0.928	ID																											
6	0.763	0.698	0.736	0.653	0.623	ID																										
7	0.835	0.835	0.882	0.759	0.780	0.672	ID																									
8	0.959	0.959	0.876	0.789	0.834	0.875	0.797	ID																								
9	0.961	0.960	0.877	0.790	0.835	0.687	0.798	0.998	ID																							
10	0.637	0.637	0.714	0.603	0.608	0.786	0.776	0.601	0.601	ID																						
11	0.778	0.817	0.918	0.780	0.930	0.737	0.734	0.733	0.783	0.735	ID																					
12	0.834	0.834	0.881	0.759	0.781	0.842	0.996	0.796	0.797	0.776	0.668	ID																				
13	0.888	0.888	0.968	0.827	0.831	0.670	0.908	0.850	0.851	0.736	0.787	0.907	ID																			
14	0.804	0.804	0.761	0.707	0.746	0.895	0.694	0.791	0.79	0.656	0.803	0.692	0.739	ID																		
15	0.639	0.639	0.714	0.604	0.609	0.786	0.778	0.602	0.602	0.997	0.916	0.778	0.736	0.807	ID																	
16	0.835	0.835	0.882	0.760	0.782	0.654	0.997	0.797	0.798	0.777	0.769	0.998	0.908	0.693	0.779	ID																
17	0.784	0.784	0.864	0.728	0.731	0.857	0.939	0.747	0.748	0.826	0.853	0.938	0.890	0.646	0.826	0.939	ID															
18	0.784	0.784	0.864	0.728	0.731	0.698	0.939	0.747	0.748	0.826	0.653	0.938	0.890	0.646	0.826	0.939	0.794	ID														
19	0.914	0.914	0.765	0.852	0.855	0.730	0.882	0.876	0.877	0.714	0.791	0.881	0.968	0.761	0.714	0.882	0.864	0.864	ID													
20	0.867	0.867	0.851	0.924	0.996	0.635	0.777	0.831	0.831	0.605	0.893	0.778	0.828	0.742	0.606	0.779	0.728	0.728	0.851	ID												
21	0.879	0.879	0.960	0.818	0.823	0.685	0.911	0.841	0.842	0.738	0.884	0.910	0.989	0.732	0.738	0.911	0.892	0.892	0.96	0.819	ID											
22	0.978	0.978	0.894	0.802	0.85	0.913	0.815	0.96	0.961	0.617	0.918	0.814	0.868	0.803	0.619	0.815	0.765	0.765	0.894	0.846	0.859	ID										
23	0.784	0.784	0.863	0.727	0.73	0.785	0.938	0.746	0.747	0.826	0.852	0.937	0.889	0.646	0.826	0.938	0.998	0.998	0.863	0.727	0.892	0.764	ID									
24	0.869	0.869	0.854	0.926	0.998	0.621	0.779	0.834	0.835	0.607	0.793	0.780	0.830	0.746	0.608	0.781	0.730	0.730	0.854	0.994	0.822	0.851	0.729	ID								
25	0.888	0.888	0.969	0.828	0.832	0.688	0.909	0.851	0.852	0.736	0.887	0.908	0.999	0.740	0.736	0.909	0.891	0.891	0.969	0.829	0.989	0.869	0.89	0.831	ID							
26	0.914	0.914	0.756	0.852	0.855	0.730	0.882	0.876	0.877	0.714	0.791	0.881	0.968	0.761	0.714	0.882	0.864	0.864	0.723	0.851	0.960	0.894	0.863	0.854	0.969	ID						
27	0.853	0.850	0.609	0.876	0.621	0.853	0.843	0.716	0.671	0.625	0.755	0.832	0.753	0.655	0.863	0.733	0.916	0.816	0.656	0.661	0.735	0.781	0.715	0.661	0.854	0.756	ID					
28	0.914	0.914	0.969	0.852	0.849	0.720	0.864	0.883	0.884	0.689	0.893	0.863	0.941	0.761	0.689	0.864	0.837	0.837	0.969	0.846	0.932	0.899	0.837	0.848	0.942	0.969	0.757	ID				
29	0.960	0.960	0.877	0.790	0.835	0.654	0.798	0.998	0.732	0.601	0.733	0.797	0.851	0.790	0.602	0.798	0.748	0.748	0.877	0.831	0.842	0.961	0.747	0.835	0.852	0.877	0.671	0.884	ID			
30	0.783	0.785	0.664	0.698	0.672	0.889	0.763	0.783	0.839	0.651	0.823	0.823	0.841	0.945	0.851	0.924	0.730	0.708	0.746	0.753	0.838	0.879	0.707	0.853	0.842	0.946	0.890	0.749	0.739	ID		
31	0.637	0.637	0.714	0.603	0.608	0.770	0.776	0.601	0.601	0.997	0.895	0.776	0.736	0.706	0.997	0.777	0.826	0.826	0.714	0.605	0.738	0.617	0.826	0.607	0.736	0.714	0.761	0.689	0.601	0.725	ID	
32	0.834	0.902	0.914	0.814	0.870	0.689	0.835	0.959	0.96	0.637	0.717	0.834	0.888	0.804	0.639	0.835	0.784	0.784	0.914	0.867	0.879	0.978	0.784	0.869	0.888	0.914	0.785	0.914	0.960	0.878	0.637	ID

1 Albizialebbeck witches-broom_phytoplasma AWB 1
2 Alliumcepa phytoplasma On-Gharb 2
3 Bougainvillea proliferation phytoplasma New Delhi
4 Brinjal little leaf phytoplasma GKVK
5 Brinjal little leaf phytoplasma strain BLL
6 Buckwheat phyllody GKVK
7 Bushehr sesame phyllody phytoplasma Bu10
8 Candidatus Phytoplasma aurantifolia 204
9 Cannabissativa leaf yellowing phytoplasma CSLY-FAZ1
10 Catharanthus roseus phytoplasma CR-ND
11 Chickpea phyllody phytoplasma Doddaballapur
12 Cleome witches-broom phytoplasma ClWB
13 Crotonsparsiflorus phytoplasma CSAP-P9
14 Daturastramonium Shivamogga
15 Desmodiumovalifolium witches-broom Hainan-Chengmai
16 Emiliasonchifolia witches-broom phytoplasma T1
17 Empoasca sp. Phytoplasma EmND2
18 Exitianusindicus phytoplasma_EiAPN6
19 Helianthusannuus phyllody
20 Iranian safflower phyllody phytoplasma
21 Partheniumhysterophorus phytoplasma PR24
22 Parthenium phyllody GKVK
23 Pelargoniumcapitatum phytoplasma clone 61
24 Periwinkle little leaf phytoplasma
25 Phaseolusvulgaris phytoplasma RJ
26 Phytoplasma sp. PR08
27 Pigeonpea GKVK
28 Sesamum phyllody GKVK
29 Setariaverticillata little leaf phytoplasma SVLL-GOK1
30 Sunhemp phyllody GKVK
31 Sweet potato witches-broom phytoplasma
32 Tylophoraindica little leaf yellowing phytoplasma Gurgaon-1

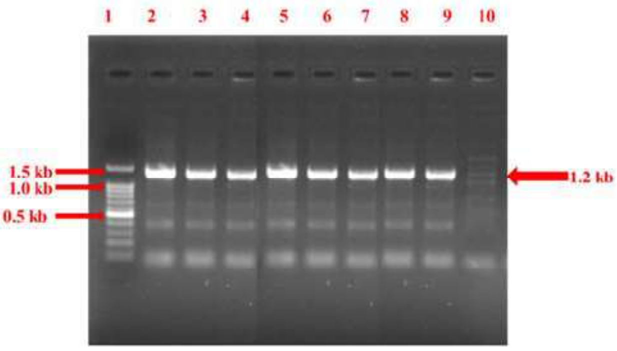

Plate 3: Nested- PCR amplification of 16S rDNA of phytoplasma

Lanes:

Lane 1 : 1.5 kb Ladder	Lane 6 : Sunhemp phyllody
Lane 2 : Pigeonpea phyllody	Lane 7 : Parthenium phyllody
Lane 3 : Brinjal little leaf	Lane 8 : Buckwheat phyllody
Lane 4 : Sesamum phyllody	Lane 9 : Datura phyllody
Lane 5 : Chickpea phyllody	Lane 10 : Control (Healthy)

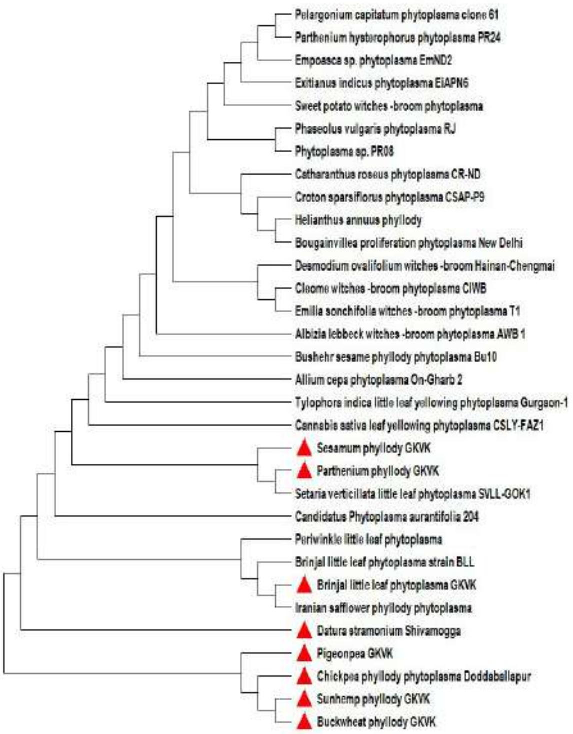

Fig. 1: Phylogenetic relationship of phytoplasma diseases on the basis of the 16Sr DNA gene sequences

AF228053.1) and shared nucleotide identity of 62.1 to 85.5 per cent. Similarly, sesamum phyllody GKVK (Accession No. MW559980) and parthenium phyllody GKVK (Accession No. MW559981) with *'Setaria verticillata'* little leaf phytoplasma SVLL-GOK1 (Accession No. MW077129.1) with sequence similarity of 88.4 to 96.1 per cent.whereas, brinjal little leaf phytoplasma GKVK (Accession No. MW547094) clustered with Iranian safflower phyllody phytoplasma (Accession No. EF534205.1) and shared 92.4 per cent nucleotide identity (Table 2 and Fig. 1). These results are in agreement with previous work of Salehi *et al.* (2016); Gopala and Rao (2018); Naik *et al.* (2018) and Gurupada *et al.* (2019).

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