Association of 16SrII-C and 16SrII-D subgroup phytoplasma strains with witches' broom disease of *Parthenium hysterophorus* and insect vector *Orosius albicinctus* in India

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Abstract *Parthenium* weed (*Parthenium hysterophorus*, *Asteraceae*) is a common, invasive weed found throughout the Indian subcontinent, Africa, America and Australia. The observed disease, *Parthenium* Witches' Broom (PWB), characterised by phylloidy, stunting and prolific branching was observed during a field survey conducted between September 2014 to June 2015. PWB phytoplasma was detected in all symptomatic plant samples and in the previously reported insect vector, *Orosius albicinctus* belonging to family *Cicadellidae* (subfamily *Deltocephalinae*) and other Hemipteran insect samples by amplifying its 16S rRNA gene. To our knowledge, this is the first report of a 'Ca. Phytoplasma aurantifolia'-related strains associated with witches' broom disease of *P. hysterophorus* and insect vector *Orosius albicinctus*.

Keywords 'Ca. Phytoplasma aurantifolia' · 16Sr II group · *Parthenium hysterophorus* · *Cicadellidae* · *Deltocephalinae* · Insect Vectors · *Orosius albicinctus*

Parthenium hysterophorus is a widely spread and exotic weed in India. It is known as one of the most dangerous weeds and several attempts have been made to control its spread by various methods (Sileshi and Tessema 2010). Foliar leachates from *Parthenium* have been demonstrated to have allelopathic effects, including low germination rates and reduced yields in several pulse crops (Evans 1997). *Parthenium hysterophorus* is a known secondary host for many bacterial, viral diseases and insect pests of crop plants (Lakshmi and Srinivas 2007). It is known to harbour the faba bean phyllody phytoplasma (Taye et al. 2004), Aster Yellow phytoplasma (Raj et al. 2008), *Pseudomonas solanacearum* (Kishun and Chand 1998), *Xanthomonas campestris* pv. Phaseoli (Evans 1997), Groundnut bud necrosis virus (Prasada et al. 2005) and Tomato yellow leaf curl virus (Govindappa et al. 2005).

Parthenium weed has been shown to be an alternative host of black bean aphid (*Aphis fabae*) in southern India and scarab beetle (*Pseudoheteronyx* sp.), which is a pest of sunflower in central Queensland, Australia (Evans 1997). *Parthenium hysterophorus* also serves as a reservoir for Lepidopteran and Hemipteran insect pests of crop plants between cropping seasons (Sileshi et al. 2008). Another insect pest, *Liriomyza trifolii* which oviposit on *P. hysterophorus* in Texas, USA is a known pest of bell pepper (*Capsicum annuum*, *Solanaceae*) (Chandler and Chandler 1988).

Parthenium hysterophorus plants typically showing symptoms of phylloidy were observed during a field survey conducted between September 2014 to June 2015. Plant samples were collected from barren lands, dumping grounds and farmlands around Pune, Satara and Naldurga (Osmanabad) cities of Maharashtra, India. Simultaneously, insect samples, mainly plant hoppers and leafhoppers (targeting *Cicadellidae* family), were collected using a white light trap as previously described (Steiner and Hauser 2010), as they are known plant sap-feeders and vectors of phytoplasmas (Wilson and Weintraub

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2007). A total of 48 insect samples were collected in eppendorf tubes and preserved in 95 % ethanol until DNA extraction.

Two grams of compound leaves from symptomatic and asymptomatic plants (Fig. 1) were used for total DNA extraction using the CTAB method (Doyle and Doyle 1990). Total DNA extraction from collected insect samples were carried out using DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's protocols, using the whole individual as starting material.

To determine the presence of phytoplasma in *P. hysterophorus* and insect samples, the PCR reaction was performed with 1X PCR buffer, 1.5 mM MgCl₂, 50 ng DNA from either plant or insect samples, 200 µM of dNTPs, 1 U of Taq DNA Polymerase (Invitrogen, Germany), 0.5 % DMSO, 2 µg per µl BSA, 1 µM of P1 and P7 primers (Smart et al. 1996) followed by nested PCR primer R16F2n and R16R2 (Gundersen and Lee 1996). For nested PCR reaction, 10 fold diluted template generated by P1/P7 primers were used. PCR amplification of partial mitochondrial *COX I* (Cytochrome Oxidase I, COI) gene was performed using universal invertebrate primers, HCO and LCO (Folmer et al. 1994). All purified phytoplasma 16S rRNA gene PCR products were sequenced directly by using primers viz. 343R, 704F, 907R, 1028F (Baker et al. 2003), R16F2n, R16R2 while COI gene was sequenced directly using LCO and HCO primers.

The obtained 16S rRNA sequences were then analysed using online databases viz. EzTaxon database (Kim et al. 2012) and GenBank, using NCBI-BLAST (Altschul et al. 1990) to find the closest match. COI gene sequences were compared in Barcode of Life (BOLD) database. All obtained sequences were deposited in the GenBank database. The phylogenetic tree based on R16F2n and R16R2 sequence was computed by neighbour-joining method using MEGA 6® software package (Tamura et al. 2013) with 1000 bootstrap replications. The genetic distances and similarities were calculated by the Tamura-Nei method.

All symptomatic *P. hysterophorus* samples showed positive amplification of phytoplasma 16S rRNA gene using P1, P7 and nested R16F2n, R16R2 primer pairs. No amplification was observed in DNA isolated from asymptomatic plants.

Phytoplasma 16S rRNA gene sequences obtained from symptomatic *P. hysterophorus* samples viz. PR01 to PR06 (GenBank accession numbers LN879437, LN878982, LN879438 to LN879441 respectively) showed 99.3 % similarity with a '*Ca. Phytoplasma aurantifolia*' strain WBDL (U15442) when compared using the EzTaxon 16S rRNA database. Alternatively, NCBI-BLAST results showed 100 % sequence similarity with Sesame Phylloidy Phytoplasma (KF322278), Coconut Phytoplasma (JQ868437), Soybean witches'-broom phytoplasma (HQ840717), Cactus Witches'-Broom phytoplasma (EU099565) and Parthenium Virescence

Phytoplasma (KP899066); all related to '*Ca. Phytoplasma aurantifolia*' species. However, samples PR07 and PR08 (GenBank accession numbers LN879442 and LN879443) showed 98.78 % sequence similarity with '*Ca. Phytoplasma aurantifolia*' strain WBDL (U15442) in EzTaxon database while NCBI-BLAST results showed 100 % sequence similarity with Scaevola witches'-broom phytoplasma (AB257291) and Eggplant phytoplasma (JX441321) indicating that genetically different strains are associated with *P. hysterophorus*. These two strains showed 100 % sequence similarity with '*Ca. Phytoplasma australasia*' strain Carica papaya (Y10097) and over 99.9 % similarity with *Parthenium* phytoplasma sequences deposited in GenBank database (KJ676961 and KC855731) from various places in India (unpublished).

The virtual RFLP (Zhao et al. 2009) pattern derived from the PWB Phytoplasma sequences PR01, PR03 and PR04 (LN879437 to LN879439) was found similar to a reference sequence of Cactus witches'-broom phytoplasma (AJ293216) with a similarity coefficient of 0.99, confirming PWB phytoplasma belong to 16SrII group, subgroup C, while PWB Phytoplasma sequences of strains PR07 (LN879442) and PR08 (LN879443) belonged to 16SrII group, subgroup D. In the present study, we found two phytoplasma subgroup strains (16SrII- C and D) showed similar phenotypic symptoms but showed sequence similarity of 98.6 %. It is important to note that these samples were collected from an area where phytoplasma strains related to '*Ca. Phytoplasma aurantifolia*' were dominant among *Parthenium* plants. These strains also showed phylogenetic similarity with Bamboo Witches' Broom (Yadav et al. 2015) and Tephrosia Witches broom Phytoplasma (Yadav et al. 2014), all belonging to 16Sr II group, notably collected from the same geographic regions (Fig. 2). Overall, '*Ca. Phytoplasma aurantifolia*' is known to infect more than 55 plant species reported from 24 countries as per over 150 16S ribosomal RNA records found in GenBank database. The phylogenetic tree constructed based on R16F2n and R16R2 sequences also confirmed the genetic variability among the phytoplasma strains studied (Fig. 2).

Leafhoppers and plant hoppers are pests of many temperate and tropical crops which cause significant yield losses and increased mortality in infected crop plants due to phytoplasmas vectored by them. Knowledge of the mechanisms of phytoplasma transmission is critical for understanding of the epidemiology of diseases caused by them in crop plants and secondary hosts. Among the 48 insects collected, we could successfully amplify and sequence COI gene of 45 samples (GenBank Accession numbers, LN878983 to LN879027). The identification results of these insects are given in Table 1. We could identify 7 insect species from 6 families based on COI gene sequences and delineated them to their taxonomic designation. However, 10 of them could not be distinguished beyond family level and 5 of them could not be distinguished beyond phylum level (Table 1). The

Fig. 1 The excised branch of *Parthenium hysterophorus* showing typical witches' broom symptoms after infection of '*Candidatus* Phytoplasma aurantifolia' at Naldurg, India (a, b and c), asymptomatic branch of *Parthenium hysterophorus* plant (d)

Fig. 2 Phylogenetic tree constructed based on 16S rRNA sequences showing the relationships between the Parthenium Witches' Broom Phytoplasma with phytoplasmas strains reported earlier as being associated with *Parthenium hysterophorus* and with other reference strains. Symbol ⊕ denotes strains of phytoplasma species found associated with *Parthenium hysterophorus* from current and earlier studies. The evolutionary history was inferred using the Neighbor-Joining method, with the bootstrap values (1000 replicates) shown next to the branches. The evolutionary distances were computed using the Tamura-3 Parameter method using MEGA6 (Tamura et al. 2013). The 16S rRNA gene sequence of *Acholeplasma laidlawii* (M23932) was used as out group

Table 1 List of Insect Samples collected from *Parthenium hysterophorus* dominated sites of vegetation during June- July 2015

Closest match (BOLD Database)	Percent similarity	No of samples	PCR positives	Accession No. (partial COI gene)
Hemiptera				
Unknown Hemiptera				
Unidentified species 1	85.26	1	1	LN879020
Unidentified species 2	86.79	1	0	LN879024
Unidentified species 3	86.97	1	1	LN878994
Unidentified species 4	87.54	3	3	LN878995, LN879011, LN879022
Unidentified species 5	85.93	1	1	LN879016
<i>Cicadellidae</i>				
<i>Exitianus indicus</i>	100	2	2	LN878998, LN879017
<i>Orosius albicinctus</i>	100	1	1	LN879006
Unidentified species 1	99.85	4	4	LN879018, LN879019, LN879012, LN879021
Unidentified species 2	99.1	2	1	LN879009, LN878985
Unidentified species 3	98.31	2	2	LN879014, LN879015
Unidentified species 4	99.68	1	0	LN879005
Unidentified species 5	99.85	2	2	LN879013, LN878991
Unidentified species 6	99.85	1	0	LN879010
Unidentified species 7	100	1	1	LN878993
<i>Cydnidae</i>				
<i>Geotomus</i> sp.	98.1	1	0	LN878984
<i>Fulgoridae</i>				
Unidentified species 1	96.55	1	0	LN878987
Unidentified species 2	97.42	1	1	LN878997
Unidentified species 3	97.71	2	0	LN878999, LN879001
<i>Lygaeidae</i>				
<i>Nysius plebeius</i>	100	3	3	LN878996, LN879003, LN879008
<i>Membracidae</i>				
<i>Leptocentrus</i> sp.	100	3	0	LN879025, LN879026, LN879027
<i>Miridae</i>				
<i>Taylorilygus apicalis</i>	100	10	10	LN878983, LN878986, LN878988, LN878989, LN878990, LN878992, LN879002, LN879004, LN879007, LN879023
<i>Trigonotylus tenuis</i>	100	1	0	LN879000

difficulty in identifying Hemipteran pests demand the requirement for the development of systemic DNA barcoding efforts which will help in identifying them at their various stages of development and facilitate the protection measures to be taken at appropriate stages of plant growth.

From the survey and sampling, it was clear that *Taylorilygus apicalis* (Miridae) was dominating the insect fauna followed by members of *Cicadellidae* family. Insects *Taylorilygus apicalis* (Miridae), *Exitianus indicus*, *Orosius albicinctus* (*Cicadellidae*), *Nysius plebeius* (*Lygaeidae*) and other unidentified Hemipteran insects tested positive for phytoplasma presence (Table 1) in phytoplasma specific PCR assays. *Orosius albicinctus* has been reported previously as a vector of alfalfa witches' broom (Salehi et al. 1995), cucumber phyllody (Azadvar et al. 2005), garden beet witches' broom

(Mirzaie et al. 2007) and sesame phyllody (Esmailzadeh-Hosseini et al. 2007) which suggest its role in transmitting PWB phytoplasma. *Exitianus indicus* were reported as putative phytoplasma vectors in sugarcane (Kumar et al. 2015). It is important to note that the presence of phytoplasmas (16Sr II group) found in this study were also found in *Tephrosia perpurea* and *Dendrocalamus strictus* (Yadav et al. 2014 and 2015) which indicates transmission of these strains through putative common insect vector(s). The presence of phytoplasma DNA in most of the tested Hemipteran insects indicated that these insects are reservoirs for phytoplasma and are its putative vectors. However, vectoring capability can be established further by transmission assays. The insects from *Cicadellidae* family had been reported as phytoplasma vectors worldwide and the probability is that the number will increase

with the additional transmission studies. Barcoding and systematics of insect pests from Indian farms in various crops and weeds needs immediate and serious attention. Results obtained after PCR screening of Hemipteran, especially *Cicadellidae* insects demands in depth studies in the finding vectoring ability of these insects.

Other DNA sequences related to this study are deposited in GenBank database with accession numbers LN879028 to LN879031, LN811709 and LN879033.

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