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RESEARCH ARTICLE

New Efficient Natural Leafhopper Vectors of Sugarcane Grassy Shoot Phytoplasma in India

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Abstract Sugarcane grassy shoot (SCGS) disease is associated with the presence of 16SrXI group phytoplasmas that are transmitted by leafhoppers; limited studies have been performed in India toward its natural transmission. To determine the insect vectors that transmit the disease in nature, leafhopper species from SCGS-infected fields at Shahjahanpur, Central Uttar Pradesh, India, were collected and analyzed for phytoplasma presence using nested polymerase chain reaction with phytoplasma-specific primers. An ~1.2-kb amplified DNA fragment was detected in nested PCR from the three major leafhopper species, viz. *Maiestas portica* (Melichar), *Exitianus indicus* (Ross) and *Cofana unimaculata* (Signoret), and the symptomatic sugarcane leaves of variety CoS 07250. BLASTn analysis of ~1.2-kb 16S rDNA partial sequences obtained from symptomatic sugarcane plants and these leafhoppers revealed 99–100 % sequence identities among themselves and 99 % identity with other reported strains of '*Candidatus* Phytoplasma oryzae' (16SrXI group). Phylogenetic analysis of 16S rRNA sequences of SCGS, *M. portica*, *C. unimaculata* and *E. indicus* phytoplasma strains also indicated the closest phylogenetic relationship with those of '*Ca*. P. oryzae' group. Transmission tests and population sampling study further confirmed that *M. portica* and *C. unimaculata* were vectors of the SCGS phytoplasma from diseased to healthy sugarcane plants. The identification of new vectors of SCGS phytoplasma suggested that these leafhopper species may be responsible for secondary spread of SCGS phytoplasma.

Keywords *Maiestas portica* · *Exitianus indicus* · *Cofana unimaculata* · Sugarcane grassy shoot disease · Phytoplasma · Transmission · '*Ca*. P. oryzae'

Introduction

Sugarcane is one of the most important industrial crops in India occupying about 5 million hectares in area. The Indian sugar industry plays a key role in global sugar market producing around 300–350 MT cane and 23–25 MT white sugar. Besides, about 3 billion liters of alcohol and 2330 MW power and many by-products are also produced. At present, the Indian share in global sugar production and consumption is 15 and 13 %, respectively (Solomon 2014).

The sugarcane grassy shoot (SCGS) is a major phytoplasma disease of sugarcane in Asian countries, viz. Bangladesh, India, Malaysia, Nepal, Pakistan, Sri Lanka and Vietnam (Rao et al. 2008, 2012). The disease is characterized by the production of a large number of thin, slender, adventitious tillers from the base of the affected plants and typical leaf chlorosis (Rao et al. 2012). In India, SCGS disease on sugarcane crop has been reported on regular intervals from different parts of the country and is responsible for significant yield losses (Nasare et al. 2007; Rao

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et al. 2008, 2014; Viswanathan et al. 2011; Tiwari et al. 2012). Phytoplasmas are phloem-limited microorganisms and are transmitted through leafhoppers (Cicadellidae) and planthoppers (Delphacidae) (Weintraub and Beanland 2006). Planting of infected setts and spread of phytoplasma by vectors may be responsible for an increase in the incidence of this disease particularly in Uttar Pradesh, India (Srivastava et al. 2006; Rao et al. 2008, 2014).

Limited attempts have been made on transmission of SCGS phytoplasma in India by leafhoppers/planthoppers, and the disease so far is only reported to be transmitted by *Deltocephalus vulgaris* (Cicadellidae: Deltocephalinae) (Srivastava et al. 2006). However, in Taiwan and Thailand, sugarcane white leaf phytoplasma was transmitted by *Matsumuratettix hiroglyphicus* (Cicadellidae: Deltocephalinae) and *Yamatotettix flavovittatus* (Cicadellidae: Deltocephalinae) (Matsumoto et al. 1968; Hanboonsong et al. 2002, 2006). Another delphacid planthopper *Saccharosydne saccharivora* (Delphacidae: Delphacinae) was reported to transmit the sugarcane leaf yellows phytoplasmas in Cuba (Arocha et al. 2005).

Detailed knowledge of the factors affecting population and the dispersal of vectors is a prerequisite not only for understanding SCGS disease epidemiology, but also for developing an integrated pest management program against the disease. Since the SCGS disease is spreading at alarming rate in different parts of the country (Viswanathan et al. 2011), the present investigation was carried out to identify any additional insect vectors of SCGS disease in the major sugarcane grown area of Uttar Pradesh (2.3 million ha area under sugarcane cultivation with annual cane production of 135.64 million tons), where SCGS disease is a regular recurrence in most of the important commercially grown sugarcane varieties (Tiwari et al. 2012). In this study, leafhoppers were collected in SCGS-affected sugarcane fields of variety CoS 07250 at Shahjahanpur, central Uttar Pradesh, to identify the leafhopper species present and to confirm their role in the natural transmission of SCGS disease.

Materials and Methods

Experimental Location

This study was carried out at Shahjahanpur (27.35 N latitude and 79.37 E longitude) a major sugarcane growing area in a central district of Uttar Pradesh, India. The site is characterized by rainfall distribution of 89.8 to 489 mm with peaks of the leafhopper populations from July to November. Many commercial sugarcane varieties in Shahjahanpur district are severely affected by SCGS disease (Tiwari et al. 2012).

Survey and Sample Collection

Survey was made in the research field of Sugarcane Research Institute, Shahjahanpur, UP, India, in 2013 in ratoon crop of variety CoS 07250 showing SCGS symptoms (6–8 months old). Disease incidence was recorded, and leaves of SCGS-infected and healthy sugarcane plants were collected.

Insect Sampling, Identification and Insect Population

The leafhopper species feeding on sugarcane crops in SCGS-infected and healthy fields were collected at 10-days interval using sweep net method (Rao et al. 2014). Insects captured in the net were then separated out, counted and sent for identification during the period, June–November 2013. The collected insects were identified at Network Project on Insect Biosystematics, Department of Entomology, GKVK, Bangalore, India. For population sampling, yellow sticky cards were used in the field at height of 50 cm and population sampling of leafhopper species was calculated at 10-day intervals from July to November 2013.

DNA Extraction and PCR Assay

DNA was extracted from leaf midrib of healthy and symptomatic sugarcane leaves and ten individuals' [9 in case of *Nephotettix cirescens* (Distant)] leafhopper by CTAB method (Ahrens and Seemuller 1992) and was used as template in PCR assays by universal primer pair P1/P6 (Deng and Hiruki 1991; Schneider et al. 1995) followed by R16F2n/R16R2 in nested PCR assays (Gundersen and Lee 1996).

PCR was performed in a Mastercycler (Eppendorf, Germany), and the cycling protocol was followed as described by Rao et al. (2014). The product of direct PCR was diluted 1:10 with sterile water, and 2 µl was used as template in nested PCR. Reaction mixture and condition of nested PCR were as follows: 94 °C:5 min (1 cycle) 30 cycles of 94 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 2 min, with extension in the final cycle for 10 min. Volume of each reaction was 25 µl containing 5 pM of each forward and reverse primers, 200 ng of DNA template, 1.5 mM $MgCl_2$, 1× *Taq* buffer, 0.2 mM dNTP and 1U *Taq* polymerase enzyme (G Bioscience, USA).

The DNA extracted from periwinkle infected with sugarcane grassy shoot phytoplasma (Rao et al. 2014) was used as a positive control. The DNA extracted from non-symptomatic sugarcane leaves was used as negative control. Five microliters of each PCR product was subjected to electrophoresis in a 1.0 % (w/v) agarose gel, stained with ethidium bromide and observed under UV transilluminator.

Sequencing

Nested PCR product (~1.2-kb amplicon) was purified using the PCR Clean-up System (Promega, USA). The purified amplified products were sequenced directly in both directions and were assembled using DNA baser v4 program and further aligned using Clustal W method of BioEdit software (Hall 1999). The 16S rDNA sequences generated from present study were submitted in NCBI GenBank and used as query sequence in BLASTn search analysis.

The sequence generated from the present study and reference phytoplasma strains sequence retrieved from GenBank were used to construct phylogeny by neighbor-joining method with 1000 replications for each bootstrap value using MEGA 7.0 software version (Tamura et al. 2011). *Acholeplasma laidlawii* was used as out group to root the phylogenetic tree.

Insect Rearing

Eggs, nymphs and adults of three leafhopper species under study (*Maiestas portica*, *Exitianus indicus* and *Cofana unimaculata*) were collected from the sugarcane fields, and their colonies were established in insect-proof greenhouse-grown healthy sorghum plants in pots until the emergence of next generation. Ten individual leafhoppers from each established colony were tested by nested PCR assays as reported above to ensure that leafhopper colonies were phytoplasma free.

A total of 20 adult leafhoppers after acquisition access feeding of 72 h in SCGS-infected plants in pots were transferred to five pots each containing 4 healthy sugarcane plants (tested free of phytoplasmas by PCR assays) variety CoS 07250 (3 weeks old) followed by an inoculation access period of 7 days in an insect-proof greenhouse. One cage with 4 healthy sugarcane plants was used for control where no leafhopper was released. The leafhopper species in the inoculated pots were killed after 72 h using imidacloprid (1 ml/3l water), and plants in each cage were continuously monitored for symptom expression up to 60 days post-inoculation. All the killed insects in inoculated pots were collected, stored at −20 °C and further analyzed for the presence of phytoplasmas through nested PCR assays as reported above. The insect-inoculated sugarcane plants were also analyzed by PCR assays after 60 days in experimental pots under cages for phytoplasma presence.

Insect Transmission Assays

Healthy cane setts of variety CoS 07250 were sown in pots in greenhouse and covered with nylon mesh. A total of 24 sugarcane plants were grown in six pots with 4 healthy sugarcane plants each. After 2 months, sugarcane growing leaves were tested with phytoplasma-specific primers (P1/P6 followed by R16F2n/R16R2) to confirm their phytoplasma-free status. Three pots with four sugarcane plants were inoculated independently with three different species of leafhoppers (*M. portica*, *E. indicus* and *C. unimaculata*) from the established colonies on sorghum plants. The rest three pots with four sugarcane plants were kept free from leafhoppers as a control.

Results

Survey and Symptomatology

During survey of commercial sugarcane fields of variety CoS 07250 in 2013, 7–12 % disease incidence (on the basis of visual observation of symptoms in fields) of SCGS disease in plant crops and over 45 % incidence in ratoon crop were recorded in the months from June to November 2013 (data not shown). The major symptoms observed were tiller proliferation, stunted growth and soft-textured chlorotic leaves in affected clumps (Fig. 1b, c).

Leafhopper Identification

A total of 941 leafhoppers were collected with yellow trap paper over a period of 5 months (July–November 2013) in SCGS-infected fields. Out of the collected insects, five leafhopper species were identified as Deltocephalinae, as *Exitianus indicus* (Distant), *Nephotettix cirescens* (Distant), *Hishimonus phycitis* (Distant), *Cicadulina bipunctata* (Melichar) and *Maiestas portica* (Melichar), one as Typhlocybinae as *Empoascanara prima* and three as Cicadellinae, viz. *Cofana spectra* (Distant), *C. unimaculata and Hecalus porrectus* (Walker). *M. portica*, *C. unimaculata*, *E. prima* and *E. indicus* were identified as major species found in sugarcane fields on the basis of number of insects trapped which enclosed the 87.7 % of total leafhopper specimens identified (Table 1).

Seasonal Variation in Population Densities of Leafhoppers

The peak of population density of *M. portica* occurred in July and August followed by *C. unimaculata* and *E. indicus* (Fig. 2). The maximum incidence of SCGS disease symptoms in the field was recorded in September–October, which could correlate with high leafhopper population peaks of *M. portica* and *C. unimaculata* in earlier months in the same fields.

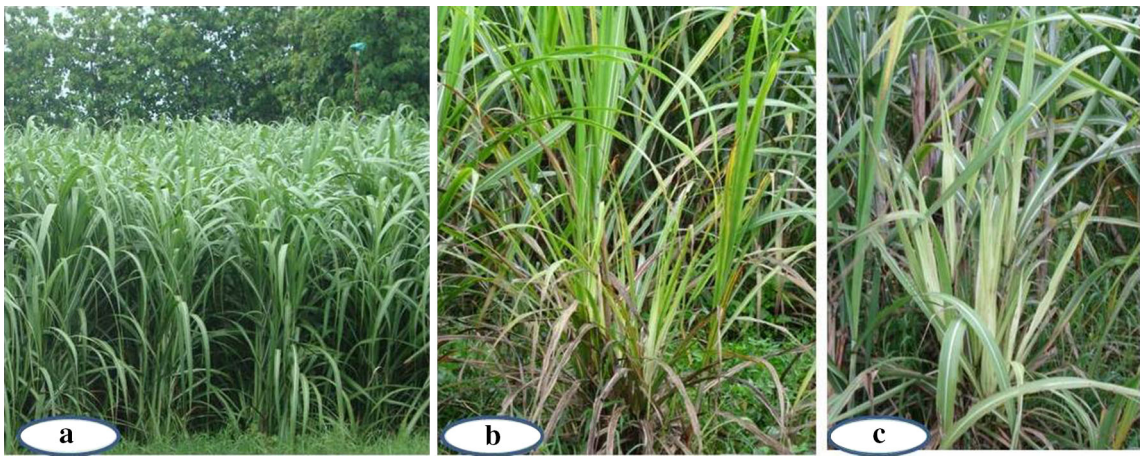

Fig. 1 Sugarcane grassy shoot symptoms on variety CoS 07250. **a** Healthy sugarcane plants, **b** grassy shoot and chlorotic leaves in affected clump; **c** production of soft-textured chlorotic leaves in affected clump

Table 1 Leafhopper species identified and phytoplasma PCR detection results

Subfamily	Species name	Total no. of insect collected	PCR assay result*
Cicadellinae	1. *Cofana unimaculata* (Signoret)	229	Positive**
	2. *Cofana spectra* (Distant)	28	–
	3. *Hecalus porrectus* (Walker)	21	–
Deltocephalinae	4. *Exitianus indicus* (Distant)	177	Positive*
	5. *Nephotettix cirescens* (Distant)	09	–
	6. *Hishimonus phycitis* (Distant)	35	–
	7. *Maiestas portica* (Melichar)	307	Positive*
	8. *Cicadulina bipunctata* (Melichar)	22	–
Typhlocybinae	9. *Empoascanara prima* (Distant)	113	–

+ = Positive for phytoplasma in nested PCR assays; − = negative for phytoplasma in nested PCR assays; * ten individual specimens of leafhoppers per species were analyzed in PCR assays (except *Nephotettix cirescens*, where only nine individuals were tested); ** amplification was achieved with phytoplasma-specific primer pair P1/P6 followed by R16F2n/R16R2

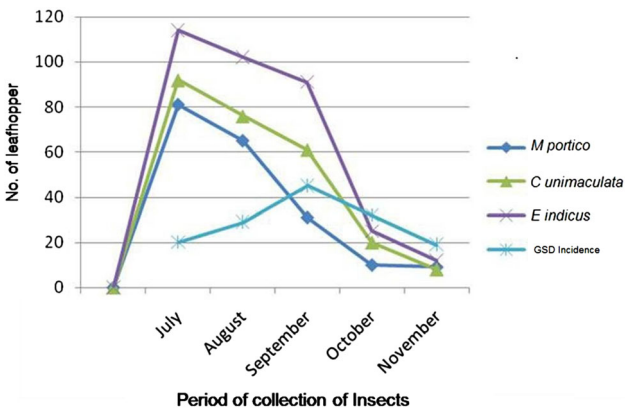

Fig. 2 Leafhopper species found in sugarcane fields and their relationship with sugarcane grassy shoot disease incidence in the fields at Shahjahanpur

Detection of SCGS Phytoplasma in Symptomatic Sugarcane Plants and Leafhopper Species

Universal phytoplasma-specific primer pair P1/P6 yielded an amplicon of ~1.5 kb in three sugarcane leaf samples showing chlorotic symptoms and positive control (data not shown), while no amplification was observed in any of the nine tested leafhoppers. However, nested PCR with primer pair R16F2n/R16R2 yielded ~1.2-kb amplicon in all the three symptomatic sugarcane samples as well as in three leafhopper species *M. portica*, *C. unimaculata* and *E. indicus* along with the positive control (Fig. 3a, b; Table 1). Insect from these three positive leafhopper species was therefore further used for transmission studies. No amplification was observed in the DNA extracted from asymptomatic sugarcane samples, and the other six

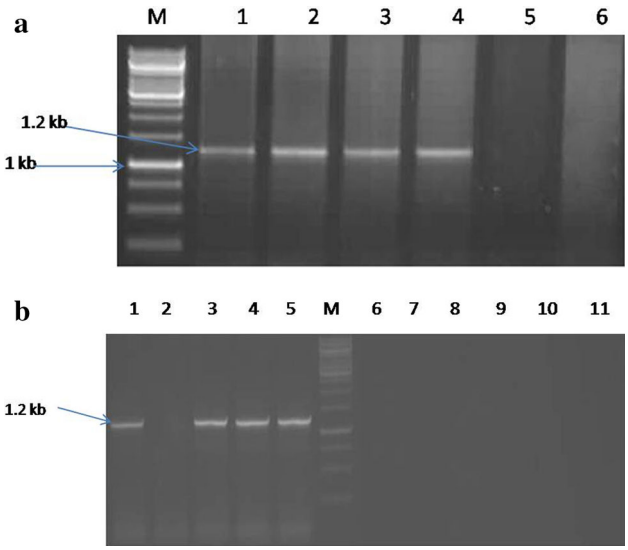

Fig. 3 **a** Nested PCR assay results of SCGS-infected sugarcane plants M. 1-Kb DNA marker (Fermentas, Germany); *1–3* SCGS-infected samples (var CoS 07250); *lane 4* positive control; *lane 5* negative control (without template); *lane 6* healthy sugarcane. **b** Nested PCR results of leafhoppers tested with R16F2n/R16R2 primers. *Lane 1* positive control (SCGS); *lane 2* negative control; *lane 3 Exitianus indicus*; *lane 4 Cofana unimaculata; lane 5 Maiestas portica*; *lane 6 Cofana spectra* (Distant); *lane 7 Hecalus porrectus* (Walker); *lane 8 Nephotettix cirescens*; *lane 9 Hishimonus phycitis*; *lane 10 Cicadulina bipunctata; lane 11 Empoascanara prima;* M. 1-Kb DNA marker (Fermentas, Germany)

identified leafhopper species collected from SCGS-affected fields (Fig. 3b; Table 1).

BLASTn analysis of the ~1.2-kb 16S rRNA partial gene sequence of phytoplasma strains from symptomatic sugarcane (GenBank Acc. No.: KP406155) and PCR-positive leafhopper species (Acc. No.: KP406156, KP406157, KP406158) revealed 99–100 % identity among themselves and with identified strains of 16SrXI group ('*Candidatus* Phytoplasma oryzae'). Phylogenetic analysis using MEGA 7.0 software also supported BLASTn analysis results where all the phytoplasma strains from leafhoppers and symptomatic sugarcane clustered together with members of '*Ca.* P. oryzae' group (Fig. 4).

Transmission Assays

The three leafhopper species positive for SCGS phytoplasma (*M. portica, E. indicus* and *C. unimaculata*) were used for the transmission assays. After 10 weeks of incubation, none of the sugarcane plants fed by the three leafhopper species showed SCGS symptoms. Further, no amplification was obtained in any of the sugarcane plants inoculated by any of the three leafhopper species in direct PCR assays (data not shown). However, in nested PCR expected length amplified product was obtained in 9 out of 20 and 5 out of 20 sugarcane plants that were used to feed *M. portica* and *C. unimaculata*, respectively (Table 2). Sequence analysis of PCR amplicons from experimental caged insects and sugarcane plants indicated the presence of 16SrXI phytoplasma in transmission materials. No phytoplasma was detected in plants inoculated with *E. indicus* and in control plants that were not exposed to leafhoppers (Table 2).

Discussion

SCGS disease is a major constraint in sugarcane cultivation, causing severe losses to sugarcane industry all over India (Vishwanathan and Rao 2011). Effective management of the SCGS disease requires a good understanding of disease epidemiology with knowledge of the insect vectors and their plant host reservoirs. So far, only *D. vulgaris* was reported as natural vector and *E. indicus* as putative vector of SCGS phytoplasma from India (Srivastava et al. 2006; Rao et al. 2014). In the present study, two additional leafhopper vectors, viz. *M. portica* and *C. unimaculata,* are reported.

The results on insect population in sugarcane fields revealed that *M. portica* was the major available species in SCGS-affected fields at Shahjahanpur from July to November 2013 and was also found positive for phytoplasma presence in nested PCR assays. Obura et al. (2009) reported the vector capability of *Maiestas banda* for transmission of Napier grass stunt phytoplasma disease. In the present study, the phytoplasma association with *M. portica* and its transmitting capability suggested that it may be responsible for SCGS epidemics. Leafhoppers in the genus *Maiestas* are mostly grass feeders (Webb and Viraktamath 2009) and reported to transmit phytoplasmas infecting graminaceous crops. For example, the zigzag leafhopper *Maiestas dorsalis* (Motschulsky) is a vector of rice orange leaf phytoplasma in Asia (Rivera et al. 1963). However, *M. distinctus* (Motschulsky) and *M. dorsalis* have been shown to be associated with sugarcane white leaf phytoplasma in Thailand (Hanboonsong et al. 2006). All the reports on *Maiestas* species as vector for phytoplasmas belong to members of the rice yellow dwarf group (16SrXI) suggesting complex interactions between this group and leafhoppers of the genus *Maiestas*. It is worth noting that the results of this study showed *M. portica*, as a vector of SCGS phytoplasma, which is a new report in the world.

Several species identified in this study belong to the genera *Exitianus* and *Cofana*, the members of which were also reported as possible phytoplasma vectors. *Exitianus capicola* has been reported as a vector of phytoplasma in *Limonium* hybrids in Israel (Weintraub et al. 2004). Rao

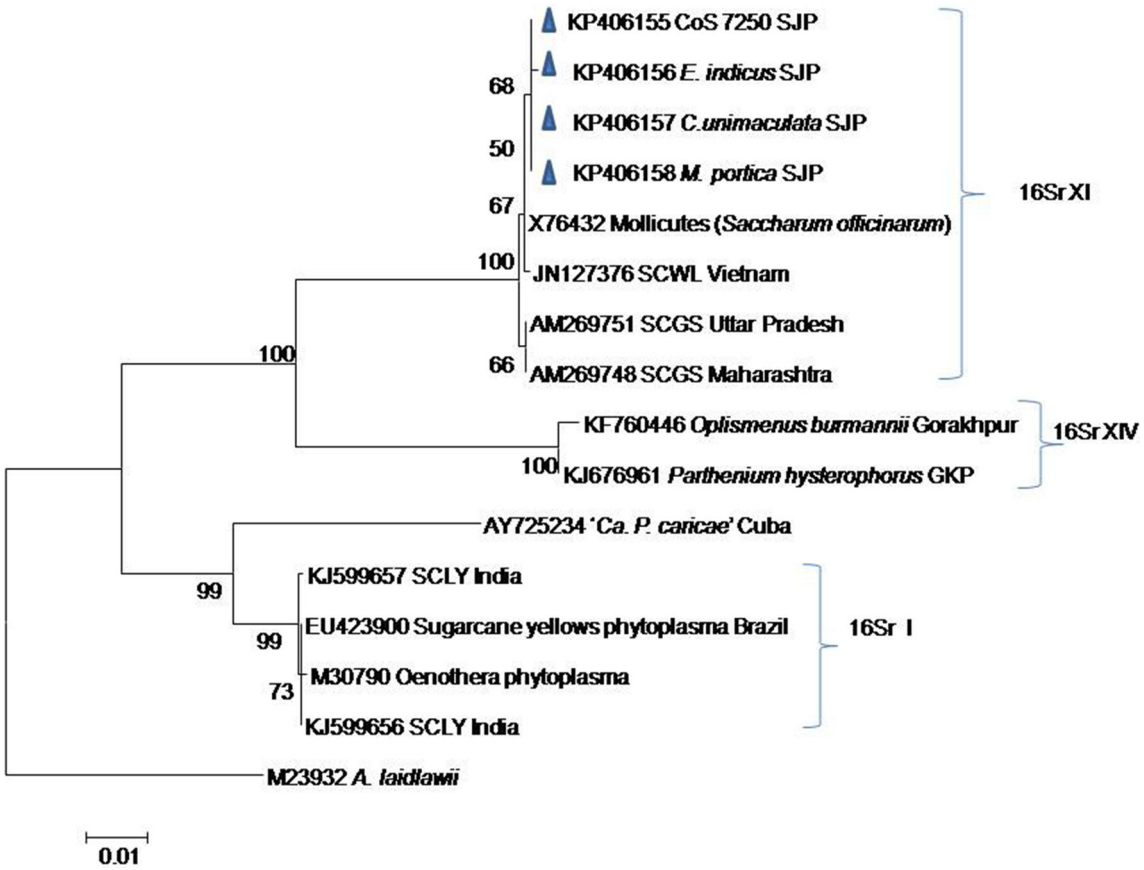

Fig. 4 Phylogenetic tree showing the phylogenetic relationships among the SCGS phytoplasma, phytoplasma detected in *M. portica, C. unimaculata, E. indicus* and reference phytoplasma strains. Accession numbers are specified in the tree. '*Ca*. P.' stands for '*Candidatus* Phytoplasma sp.' *Achleoplasma laidlawii* was used as a out group

Table 2 Results of leafhopper transmission experiments

Insect vector	Plants showing symptoms after 60 days from transmission/total no of plants	Plants positive with nested PCR assays after 60 days from insect transmission/total no of plants	PCR-positive insects after inoculation period/total no of insect used
Leafhoppers with 72-h acquisition feeding on SCGS-infected plants			
1. *M. portica*	0/20	9/20	12/20
2. *E. indicus*	0/20	0/20	0/20
3. *C. unimaculata*	0/20	5/20	6/20
Leafhoppers with 72-h acquisition feeding on healthy sugarcane plants			
1. *M. portica*	0/5	0/5	0/20
2. *E. indicus*	0/5	0/5	0/20
3. *C. unimaculata*	0/5	0/5	0/20

et al. (2014) demonstrated that SCGS-infected field-collected leafhopper species, *E. indicus,* was carrying 16SrXI-B group phytoplasmas, and suggested this leafhopper as a putative vector for SCGS phytoplasma. In the present study, *E. indicus* was again found positive with SCGS phytoplasma but resulted unable to transmit the phytoplasma to the healthy sugarcane plants; however, it may have capability to act as a vector under suitable environmental conditions which needs further study. The alternative/reservoir plants harboring the SCGS phytoplasma are unknown, and hence understanding of the host range of SCGS phytoplasma and knowledge of other potential insect vector is also desirable for planning sustainable management strategies for SCGS disease.

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