Development and utilization of *gyrA* and *gyrB* gene-based diagnostics for the phytoplasma classified under 16Sr I group in plants and insects

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Abstract

In the present study, a new set of primers of *gyrA* and *gyrB* genes of the phytoplasma genome were designed and validated for the successful detection and taxonomic classification of the previously identified phytoplasma strains of ‘*Candidatus P. asteris*’ (16SrI-B subgroup) associated with Catharanthus leaf yellows, sesame phyllody and the leafhopper (*Hishimonus phycitis*). Our results suggested the ability and sensitivity of *gyrA* and *gyrB* genes as an alternative molecular marker to identify the *Ca. P. asteris* strain up to subgroup level associated both with plants and insects.

Keywords Multilocus genes · Validation · Phytoplasma detection · Leafhopper

Phytoplasma classification established using 16S ribosomal groups and ‘*Candidatus Phytoplasma*’ taxon is mainly based on 16S rDNA properties and do not always provide molecular distinction of the closely related strains. More variable single copy genes, such as ribosomal proteins (*rpl22* and *rps3*), *secY*, *secA*, *tuf*, *dnaB* and *groEL* were employed for finer classification of phytoplasma and differentiation (Schneider et al. 1997; Langer and Maixner 2004; Lee et al. 2007; Martini et al. 2007; Hodgetts et al. 2008; Hodgetts and Dickinson 2010; Bertaccini et al. 2014). To further improve detection and taxonomic classification of phytoplasma, we have designed primers for PCR assays based on *gyrA* and *gyrB* genes and tested them on aster yellows phytoplasma strains (16SrI) from field-infected samples of periwinkle (*Catharanthus roseus*) and sesame (*Sesamum indicum*). The detection and validation of *gyrA* and *gyrB* gene specific primers were also attempted to detect the phytoplasma in samples of leafhopper (*Hishimonus phycitis*) collected from infected sesame fields.

The DNA was extracted from samples of two isolates of *Catharanthus* with symptoms of little leaf (one each from Gorakhpur, Cr-GKP isolate and Shahajahanpur, Cr-SJP

isolate, Uttar Pradesh, India), sesame showing phyllody from Kushinagar (S-Kus isolate, Uttar Pradesh, India) and ten individual leafhoppers, *H. phycitis* collected from infected sesame fields at Kushinagar (HP-Kus). The protocol of Ahrens and Seemüller (1992) and Marzachi et al. (1998) was followed for total DNA extraction from the plants and leafhopper samples, respectively. The extracted DNAs from the earlier identified phytoplasma strains in our lab as 16SrI-B subgroup associated with the periwinkle (Cr-GKP and Cr-SJP), sesame (S-Kus) and *H. phycitis* (HP-Kus) (Madhupriya et al. 2015; Madhupriya 2016) were tested for the presence of phytoplasmas for direct PCR amplification with newly designed set of primers for *gyrA* and *gyrB* genes, *gyrAF/R* (5′-TGCTTATCACACCGGACAAG-3′/5′-CCAAAGCCGTTTTTCAGTCAT-3′) and *gyrBF/R* (5′-GGA GCCTCTGTGGTAAATGC-3′/5′-GCATCTTTGAGGCTT GCT TT-3′) that amplified an expected size amplicon of about 1.4 kb (*gyrA*) and 1.5 kb (*gyrB*) from all the four phytoplasma isolates of the plants and leafhopper samples. PCR assay was carried out in a thermal cycler (Eppendorf, Germany) and the cycling protocol used for the PCR assays was 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 45 s, annealing at 55 °C (*gyrAF/R*) and 56 °C (*gyrBF/R*) for 1 min, and extension at 72 °C for 2 min, with extension in the final cycle for 10 min.

Expected amplified products of 1.4 kb (*gyrA*) and 1.5 kb (*gyrB*) were obtained with DNA extracted from both the test plants (periwinkle and sesame) and the leafhopper (*H. phycitis*) confirming the specificity of *gyrA* and *gyrB*

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Fig. 1 First-round PCR assay with *gyrA* gene primer results lane 1: positive control; lane 2: *C. roseus* (Cr-SJP isolate); lane 3: negative control; lane 4: leafhopper DNA (HP-Kus); lane 5: S-Kus isolate; lane 6: *C. roseus* (Cr-GKP isolate) and lane 7: (M) 1 kb DNA ladder

Fig. 2 First round PCR assay with *gyrB* gene primer results lane 1: positive control; lane 2: *C. roseus* (Cr-SJP isolate); lane 3: negative control; lane 4: *C. roseus* (Cr-GKP isolate); lane 5: S-Kus isolate; lane 6: leafhopper DNA (HP-Kus isolate) and lane 7: (M) 1 kb plus DNA ladder

gene primer-based PCR system to identify the 16SrI group phytoplasma (Figs. 1, 2). PCR-purified products were bidirectionally sequenced to obtain maximum length of sequence and the contig sequences were deposited in the GenBank database with accession number KC495561 (*H.*

phycitis HP-Kus), KM272592 (sesame phyllody S-Kus), JQ747504 (Cr-SJP), KT306673 (Cr-GKP) for *gyrA* gene and KC495563 (HP-Kus), KM272593 (S-Kus), JQ747509 (Cr-SJP), KT306674 (Cr-GKP)) for *gyrB* gene. Pairwise sequence comparison of *gyrA* and *gyrB* gene sequences of *C. roseus* little leaf and yellows, sesame phyllody and *H. phycitis* revealed 99–100% sequence identity with that of 16SrI group of phytoplasmas. Following amplification with these primers, a phylogenetic analysis was constructed for the *gyrA* and *gyrB* gene sequences obtained from plant and leafhopper samples in the present study (Fig. 3a, b) using the neighbor-joining method with MEGA 7.0 (Kumar et al. 2016) using 1000 bootstrap replications. The phylogeny analysis revealed that the phytoplasma strains from periwinkles, sesame and *H. phycitis* clustered together with identified 16Sr I subgroup B strains. The ability to differentiate 16Sr groups and subgroups using *gyrA* and *gyrB* gene-based primers suggests that these genes may provide an informative alternative sensitive molecular marker for authentic identification and diagnosis of phytoplasma diseases in plants and insects. Recently, *gyrB* housekeeping gene fragments combined with 16S rDNA sequences were employed to analyze the genetic variation and phylogenetic interrelatedness of phytoplasma strains classified into 16SrI-B and-D subgroups in China (Yu et al. 2017). However, in the present study, a new set of primers were designed and PCR assay was validated for utilization of *gyrA* and *gyrB* gene sequences to identify and characterize the phytoplasma strains associated with plants and insect. These genes can be further tested and utilized for identification of other phytoplasma groups infecting important crops for its utility as promising molecular detection tools for phytoplasma strains in plants and insects.

Fig. 3 a, b Phylogenetic tree constructed by recombination detection and analysis methods by neighbor-joining method showing the relationships between *Catharanthus* (Cr-SJP, Cr-GKP), sesame (S-Kus) and *H. phycitis* (HP-Kus) phytoplasma strains (highlighted) and reference phytoplasma strains based on the nucleotide sequences of *gyrA* and *gyrB* gene sequence fragment (Bar 0.05)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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