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Molecular diagnostics and characterisation of leafhopper species associated with phytoplasma strains in legume ecosystems

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Abstract

The field survey conducted between 2021 and 2025 across various districts includes Thanjavur (33 %), Pudukkottai (69–79 %), Tiruchirappalli (36 %), Madurai (27 %), Dindigul (17 %), Coimbatore (79 %) and Tiruppur (57 %) of Tamil Nadu aimed to examine the prevalence of phytoplasma infections in leguminous crops. The survey identified four key leafhopper species associated with or putative vectors unless transmission was experimentally demonstrated. The identified species were *Orosius albicinctus*, *Orosius orientalis*, *Hishimonus phycitis* and *Amrasca biguttula biguttula*. These species were identified using both taxonomical and morphological characterisation methods. Taxonomically, the species were distinguished based on male genitalia characteristics, while morphological features such as wing patterns, leg spines, spur and distinct spots and markings on the wings were used for identification. Furthermore, a mitochondrial cytochrome oxidase I (*mtCOI*) gene-based genetic study confirmed the identities of the leafhoppers. Polymerase chain reaction (PCR) assays were conducted using universal phytoplasma primer pairs P1/P7 and R16F2n/R16R2 to detect phytoplasma infection in the crops. Restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified 16S rDNA sequences indicated that isolates from red gram, green gram, black gram and horse gram were identified as '*Candidatus Phytoplasma aurantifolia*'. In contrast, sequences from Bengal gram, cowpea, soybean and faba bean isolates matched '*Candidatus Phytoplasma asteris*'. The affected leguminous crops exhibited various phytoplasma symptoms, including bushy growth, small leaves, phyllody, shortened internodes and yellowing of leaves, further confirming the presence of phytoplasma infections. This study provides significant insights into the spread of phytoplasma diseases and highlights the role of specific leafhopper species as vectors in the transmission of these pathogens.

Keywords: phytoplasma; symptoms; survey; taxonomy; vectors

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Introduction

Pulses are a rich source of protein, thereby rendering especially important in vegetarian and vegan diets. They are also packed with essential vitamins and minerals, including iron, potassium, zinc and folate and provide a plant-based alternative to animal protein, which is vital for growth, tissue repair and immune functions. Pulses are a staple crop in many developing countries, particularly in Africa, Asia and Latin America, where they not only serve as an important dietary component (1). In 2024, the global production of pulses was approximately 87.7 million metric tonnes, with an average productivity of around 800–850 kg/ha. India, being the largest producer and consumer of pulses, contributed a significant portion, with an estimated pulse production of 27.4 million metric tonnes in the same year. In Tamil Nadu, the state's pulse production was around 1.3 million metric tonnes, with a productivity ranging from 650–700 kg/ha. Tamil Nadu plays a crucial role in India's pulse production, especially in growing pulses such as red gram, black gram and green gram, contributing significantly to both national and global pulse markets (2).

With the global population continuing to rise and food systems under increasing pressure, pulses have emerged as a critical and sustainable food source to meet the growing demand for protein.

Pulses provide an efficient way to address protein deficiencies while being environmentally sustainable. However, recent challenges have arisen due to phytoplasma disease infections, which have significantly reduced pulse crop yields. Phytoplasma is a cell-wall-less bacterium that inhabits the plant's phloem and causes severe damage to pulse crops, leading to lower yields and affecting the economic viability of these crops in many countries. The disease is primarily spread through insect vectors, particularly leafhoppers (Cicadellidae: Hemiptera), which are known to transmit the phytoplasma bacteria from infected to healthy plants. This insect-mediated transmission is a major factor in the horizontal spread of the disease, making it difficult to control and leading to widespread crop losses. Infected plants exhibit stunted growth, yellowing of leaves and a general decline in plant health, which ultimately results in lower productivity and availability of pulses (3). These are not only impacts the local economies of farming communities but also disrupts the supply of pulses, which are essential for food security in many parts of the world.

Globally, several leafhopper species are reported as vectors of phytoplasma, including *Hishimonus phycitis* in India (3), *Neoaliturus haematoceps* in Iran and Turkey (4), *Orosius albicinctus* in Iran and India (4) and *Orosius cellulosus* and *Orosius orientalis* in Iran, Pakistan, Turkey and India (5). Among these, *H. phycitis* and *O. albicinctus* are considered the primary vectors of phytoplasma (3), with *H. phycitis* being particularly widespread across several regions in India (6). Recent changes in environmental factors have led to increased complexity in pest and disease dynamics, particularly in the tropical regions of India, such as Tamil Nadu (3).

Recent data from India has revealed a concerning increase in the diversity of phytoplasmas affecting a wide range of crops, including pulses, oilseeds, fibre crops, fruits, vegetables, flowers and weeds (7). Phytoplasma diseases in leguminous crops like pigeonpeas and chickpeas have been reported across various regions, associated with different phytoplasma groups. The pigeonpea witches' broom phytoplasma (16SrIX group) has been found in countries such as China, Mexico, Myanmar, India and Puerto Rico (8). In India, the 16SrI group and 16SrIX-C subgroup are known to cause symptoms like stunted growth, small leaves and shortened internodes (9). The chickpea phyllody phytoplasma (16SrII group) has also been recorded in countries such as Pakistan (10), India (11), Sudan (12), Oman (13) and Australia. The diversity of phytoplasma diseases affecting soybeans across different regions shows varied symptoms and associated phytoplasma groups. Soybean phytoplasma diseases, such as severe bud proliferation and delayed maturity, are transmitted by the leafhopper *Scaphytopius acutus* (14). Phytoplasma infections have been reported in Thailand, Malawi, Mozambique, Tanzania and India, with the 16SrII-C subgroup found in Thailand, Malawi and Mozambique. In India, phytoplasmas are associated with the 16SrI-B and 16SrII-D subgroups (15).

These pathogens infect a variety of important leguminous plants, including black gram, green gram, cowpea, chickpea and pigeon pea, posing a substantial threat to agricultural productivity (16). Two major phytoplasma species, *'Candidatus Phytoplasma aurantifolia'* and *'Candidatus Phytoplasma asteris'*, are responsible for significant phytoplasma diseases affecting pulse crops in India (11).

Molecular techniques have suggested a potential link between the presence of phytoplasmas and the occurrence of unusual wilt symptoms in host crops. Traditional methods are not effective at distinguishing between phytoplasma strains, which makes DNA-based molecular techniques, especially those utilising the 16S rRNA gene, crucial in current research. These methods are now indispensable for detecting, classifying and analysing the phylogeny of phytoplasmas (17). DNA-based approaches offer enhanced specificity and sensitivity in identifying different phytoplasma strains compared to traditional methods. The 16S rRNA gene is commonly targeted for phytoplasma identification because it is highly conserved across various phytoplasma groups (18).

In this study, we examined the widespread phytoplasma infestation in various leguminous crops in the Tamil Nadu region using 16S rRNA gene-specific primers. The identification of leafhoppers at the species level is particularly challenging due to their subtle morphological differences. To address this, the study aimed to investigate both the morphological and molecular characterisation of leafhopper species, including *O. albicinctus, H. phycitis* and *A. biguttula biguttula*, which were predominantly found in pulses. The study also assessed the phylogenetic diversity of these leafhoppers and explored their interactions with phytoplasmas on leguminous crops.

Materials and Methods

Survey to collect phytoplasma-infected crops and their vectors

The field survey conducted between 2021 and 2025 in various districts includes Thanjavur, Pudukkottai, Tiruchirappalli, Madurai, Dindigul, Coimbatore and Tiruppur of Tamil Nadu aimed to examine phytoplasma infections in leguminous crops like red gram (*Cajanus cajan* (L.) Huth), green gram (*Vigna radiata* (L.) R.Wilczek), black gram (*Vigna mungo* (L.) Hepper), bengal gram (*Cicer arietinum* L.), horse gram (*Macrotyloma uniflorum* (Lam.) Verdc.), cowpea (*Vigna unguiculata* (L.) Walp.), soybean (*Glycine max* (L.) Merr.) and faba Bean (*Vicia faba* L.). The survey was conducted at vegetative stage across several districts once a year, totalling five times between 2021 and 2025

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and the phytoplasma disease-infected samples were collected by walking diagonally across a one-hectare area within continuous fields, with 15 plants randomly selected from each area for inspection. The focus was on assessing hopper populations and identifying phytoplasma-infected plants. Phytoplasma disease-infected plant samples and phytoplasma disease causing hoppers were collected for further analysis, which included both morphological examination and molecular confirmation in the laboratory.

Taxonomical and morphological identification of leafhoppers

Live leafhoppers were captured in the field using aspirators and sweep nets, then transported to the laboratory for taxonomical analysis. Following the protocol described by previous researchers, the male genitalia of each species were dissected for identification (19). The process involved removing the abdomen of the leafhopper by inserting a sharp pin between the thorax and abdomen. The abdomen was then treated with 10 % KOH for 2–4 hr to dissolve unsclerotised tissues. After this, the internal tissues were carefully removed under a stereo zoom microscope (Leica M205 A, Leica Microsystems, Wetzlar, Germany) using a hooked pin to examine the genital area. Once identified, the dissected genitalia were stored in glycerol vials and deposited in the Insect Museum, Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, India, for further study. Additionally, dissected male genitalia and various developmental stages of leafhoppers (eggs, nymphs and adults) were photographed using stereo-zoom (Leica M205 A, Leica Microsystems, Wetzlar, Germany) and phase contrast (Euromex iScope, The Netherlands) microscopes.

Molecular analyses

Leafhoppers

The collected leafhoppers, both immature and adult, were surface sterilised using 3 % NaOCl and rinsed 2–3 times with distilled water before genomic DNA extraction. The DNA extraction followed the Hot Sodium Hydroxide and Tris (HotSHOT) method as described by previous researchers (20, 21). The purity and concentration of the extracted DNA were assessed using a NanodropOne™ spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and stored at -20 °C for future use. The mitochondrial cytochrome c oxidase subunit I (mtCOI) gene (658 bp) was amplified via polymerase chain reaction (PCR) (Eppendorf MastercyclerTM, Hamburg, Germany) in a 20 µL reaction volume using the universal primers LCO 1490 and HCO 2198 (22, 23), as per the (24) protocol. The amplified DNA was separated on a 1 % agarose gel, stained with ethidium bromide (10 mg/mL) and quantified using the NanodropOneTM spectrophotometer at A260/280 nm to determine concentration and purity. The PCR products were then purified with the PureLinkTM PCR Purification Kit (ThermoFisher Scientific) and sequenced using double-pass Sanger dideoxy sequencing at Biokart India Pvt. Ltd. (Bengaluru, India). The raw sequences were manually edited using DNA MAN software and annotated (25). Reference sequences from the National Centre for Biotechnology Information (NCBI) were compared for sequence similarity. Species identification was confirmed through a BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the *mtCOI* gene sequences generated in this study were submitted to NCBI GenBank.

Phytoplasma pathogen analysis

The total DNA from leaf tissues of field-collected leguminous crops exhibiting phytoplasma symptoms, as well as asymptomatic plants, was extracted using the method described previously (26). Two sets of species-specific primers, P1/P7 and R16F2n/R16R2, were used for direct and nested PCR amplification targeting the 16S rRNA region, which includes the 16S-23S intergenic spacer (ITS) region, tRNA-Ile and partial 23S rRNA region of the phytoplasma (27). Direct PCR with P1/P7 primers was performed under the following conditions: an initial denaturation step at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 3 min; with a final extension step at 72 °C for 10 min. For nested PCR with R16F2n/R16R2 primers, the direct PCR product was diluted (1 : 30) with sterile distilled water and used as a template for a reaction consisting of an initial denaturation at 94 °C for 3 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 2 min and extension at 72 °C for 2 min; with a final extension at 72 °C for 10 min. Finally, the PCR products (5–10 µL) were electrophoresed in a 1.0 % (w/v) agarose gel, stained with ethidium bromide, visualised under UV light and the resulting nested PCR product (1.25 kb amplicon) was purified using the Wizard® SV Gel and PCR Clean-up System (Promega, USA).

Phylogenetic analyses

Phylogenetic analyses are conducted to determine the evolutionary relationships and ancestral history among different organisms, genes or pathogens. By comparing molecular sequences (such as DNA, RNA or proteins), this analysis constructs a "family tree" that allows to accurately identify unknown or emerging species. Sequences of the 16S rRNA gene from phytoplasma strains affecting leguminous crops, along with leafhopper mtCOI sequences, were retrieved from the NCBI GenBank. These sequences were edited in BioEdit software (version 7.0.5.3) and aligned using the ClustalW algorithm (28, 29). The phylogenetic tree was constructed using the neighbour-joining method in MEGA-12, with a bootstrapping value of 1000 iterations. The final tree was visualised and colored using the iTOL web-based software (30).

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Virtual restriction fragment length polymorphism analysis

Virtual restriction fragment length polymorphism (RFLP) analysis was conducted using the iPhyClassifier online tool to classify phytoplasma subgroups based on the DNA fragment patterns obtained through in silico digestion with specific restriction enzymes. In this method, phytoplasma DNA samples were virtually digested with 17 different restriction enzymes: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI *(Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I *(Sau*3AI), *Mse*I, *Rsa*I, *Ssp*I *and Taq*I. These enzymes cut the DNA at their respective recognition sites, generating fragment patterns of various lengths. The enzymes chosen are widely used for conducting 16S rRNA RFLP analysis of phytoplasmas (31).

Result

Survey of phytoplasma disease prevalence and vector populations in leguminous crops

Table 1 summarises the data on the prevalence of phytoplasma diseases and the population densities of leafhopper species, including the *O. albicinctus*, *O. orientalis H. phycitis* and *A. biguttula biguttula,* that were associated with leguminous crops in various districts of Tamil Nadu. The table highlights the percentage of plants affected by phytoplasma disease in various districts includes Thanjavur (33 %), Pudukkottai (69–79 %), Tiruchirappalli (36 %), Madurai (27 %), Dindigul (17 %), Coimbatore (79 %) and Tiruppur (57 %), which is characterised by symptoms such as stunted growth and abnormal leaf development. It also includes the population counts of these leafhopper species, which were known vectors of the phytoplasma pathogens. The data provides valuable insights into the correlation between leafhopper populations and the spread of phytoplasma infections, helping to identify regions with higher disease incidence and potential risks to leguminous crop production.

Taxonomic description of the leafhopper species

In *O. albicinctus*, the pygofer was broadly rounded with irregular setae on its posterior half. The sub-genital plates were triangular, ending in a finger-like process and bear five to six macrosetae along with fine, hair-like setae. The style has an enlarged apex with a bend and features a finger-like preapical lobe. The connective stem forms a characteristic 'Y' shape where it articulates with the aedeagus. The ventral view of the aedeagus shows shafts originating from the base and branching extensively, displaying substantial divergence (Fig. 1a).

The male genitalia of *H. phycitis* were characterised by a trapezoidal pygofer, whose length was twice its width, with macrosetae arranged along the mid-lateral region. The sub-genital plates were triangular, bearing two rows of macrosetae near the median margin and a finger-like extension at the tip. The connective is inverted 'Y'-shaped, with the arms equal in length to the stem. The aedeagus features a prominent dorsal apodeme (Fig. 1b). In *A. biguttula biguttula*, the connective was fused with a short aedeagus, which was attached to paired anal tubes tipped with slender, curved hooks. The sub-genital plates were elongated, narrowing towards the apex, with stout macrosetae located in the basal half and long, hair-like microsetae covering the distal half (Fig 1c). The male genitalia of the leafhopper species *O. albicinctus*, *H. phycitis* and *A. biguttula biguttula* are distinctive and useful for species identification.

Table 1. Field incidence of phytoplasma disease and associated vectors in leguminous crops across different districts of Tamil Nadu, India

District surveyed in Tamil Nadu	Geo-coordinates	Crop	Mean (± SE) Number of vectors (Adults) / 3 leaves*	Phytoplasma percent disease incidence (PDI)
Thanjavur	10.6277° N, 79.2525° E	Soybean	28.01 ± 0.03	33 %
Pudukkottai	10.2132° N, 78.5426° E	Red gram	11.09 ± 1.40	73 %
Pudukkottai	10.2132° N, 78.5426° E	Black gram	19.42 ± 1.02	79 %
Pudukkottai	10.2132° N, 78.5426° E	Green gram	09.71 ± 0.08	69 %
Tiruchirappalli	10.8712° N, 78.8197° E	Bengal gram	27.86 ± 1.48	36 %
Madurai	9.96491° N, 77.7885° E	Cowpea	13.3 ± 1.06	27 %
Dindigul	10.2261° N, 78.2295° E	Faba bean	2.02 ± 0.04	17 %
Coimbatore	11.0122° N, 76.9354° E	Bengal gram	36.04 ± 1.77	79 %
Trippur	11.0961° N, 77.2664° E	Horse gram	19.05 ± 1.63	57 %

*Mean of 15 observations in 3 leaves selected at the top, middle and bottom canopy of the plants.

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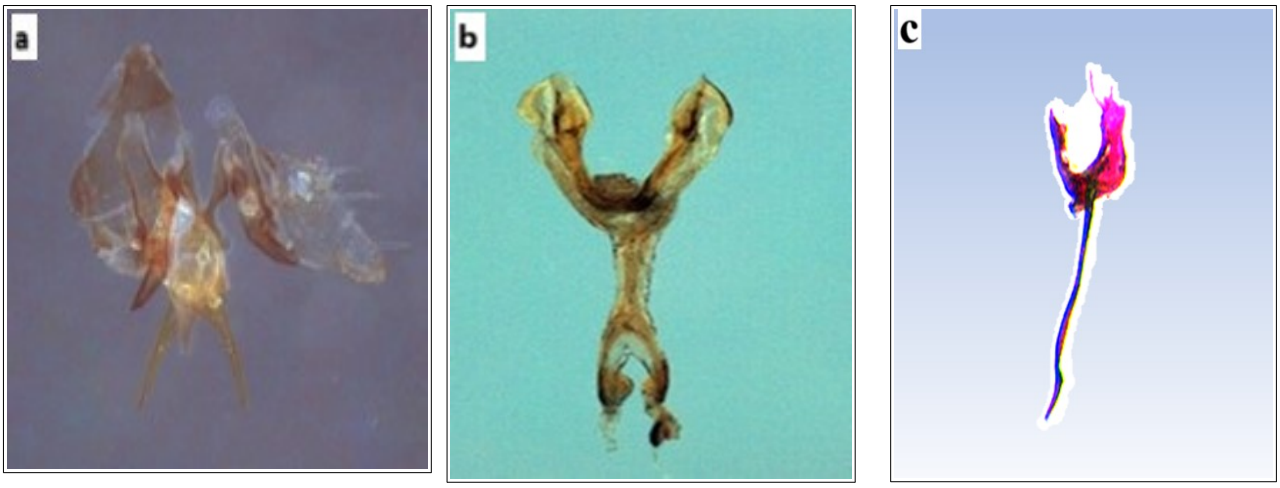


Fig. 1. Taxonomical description of male aedeagus in leafhopper species. a: *Orosius albicinctus;* b: *Hishimonus phycitis*; c: *Amrasca biguttula biguttula*.

Morphological and molecular identification of the leafhopper species

Four major leafhopper species, *O. albicinctus, O. orientalis, H. phycitis* and *A. biguttula biguttula,* were collected from pulses-growing regions of Tamil Nadu and identified based on their morphological and taxonomical features. In *O. albicinctus*, the eggs were whitish and transparent, laid on the midrib or leaf sheath. The body was light ochraceous with dark brown and black mottling and irregular striations. Nymphs have yellowish-red ocelli and the vertex features two spots near the inner margin of each eye, along with two additional spots at the anterior and posterior margins and a curved, inverted 'V' shape line (Fig. 2a). In *H. phycitis*, the eggs are yellowish, 'C'-shaped and deposited on the leaf blade or sheath. The nymphs possess transparent white ocelli with a yellowish white crown and two yellow dots. The pronotum was greenish yellow with brown specks, while the scutellum was yellowish with a brown scutoscutellar suture. Adults have a light yellowish-green head, white forewings with brown mottling and a dark brown median spot (Fig. 2b).

For *A. biguttula biguttula*, the eggs are greenish and laid in a single row inside the leaf tissues along the leaf midribs. Nymphs are pale green, like adults. The adults were yellowish green with intricate markings on the pronotum and vertex of the head and the hind tibiae are equipped with strong setae and spurs, aiding in jumping (Fig. 2c). *Orosius orientalis* can be identified by its yellowish-brown body marked with dark brown spots or linear patterns (Fig. 2d). Its compound eyes are brown and slightly concave, complementing a pale-yellow face dotted with a few brown spots. The prothorax, which was longer than the head crown, features a slightly concave posterior margin with darker markings near the anterior edge. The scutellum was broader, yellowish in colour and marked with distinct brown spots. The male genitalia of *O. orientalis* were characterised by a distinctively split, Y-shaped reproductive system essential for identifying this leafhopper species. The main organ, the aedeagus, features a single base that divides into two separate, long, narrow shafts that spread widely apart and curve upward. Additionally, the elongated sub genital plates possess a single row of stiff hairs along their outer edge and a small, fleshy, finger-like projection on the inner edge, while the internal clasping styles are relatively small with rounded, slightly swollen tips.

To confirm the presence of phytoplasmas in the salivary glands of various insect species, we performed the following steps: Salivary glands were dissected from the insects and DNA was extracted from the dissected salivary glands. Then the PCR was used to amplify the 16S rRNA gene region of phytoplasmas, a common marker for identifying these bacteria. The PCR products (amplicons) were sequenced to confirm the presence of phytoplasmas. *A. biguttula biguttula* did not have detectable phytoplasmas in its salivary glands. Both *O. albicinctus* (Fig. 3 a and b) and *H. phycitis* (Fig. 3 c and d) were found to have phytoplasmas in their salivary glands. The sequences confirming the presence of phytoplasmas in *O. albicinctus* (accessions PP151601 and PP151602) and *H. phycitis* (accessions PP151599 and PP151600) were submitted to the National Center for Biotechnology Information (NCBI) database for public access and further reference. The study demonstrates that *O. albicinctus* and *H. phycitis* can harbour phytoplasmas in their salivary glands, which is crucial for the transmission of these bacteria to new plants. In contrast, *A. biguttula biguttula* does not carry phytoplasmas in its salivary glands, suggesting differences in the vector competency among these insect species.

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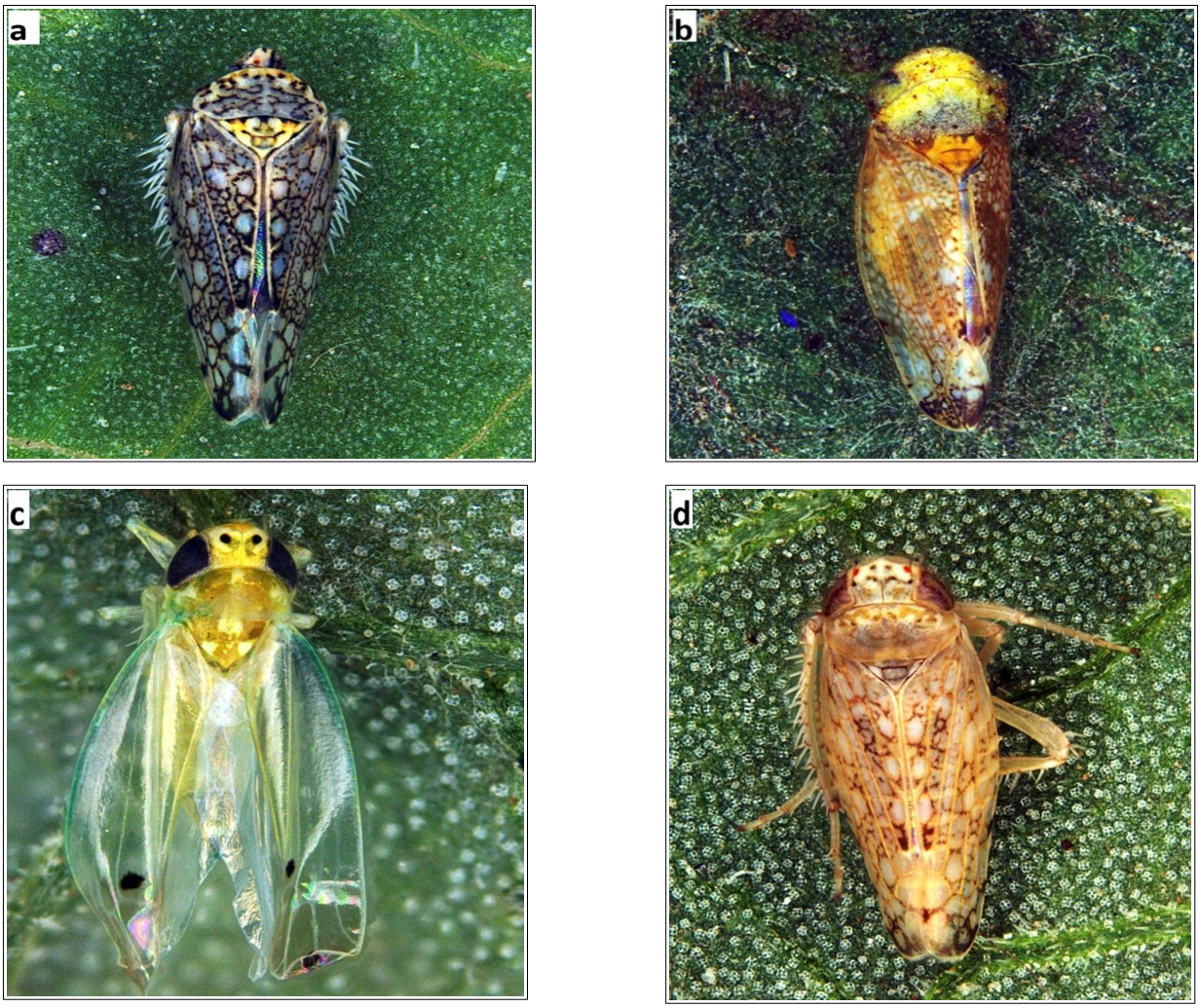


Fig. 2. Morphological identification of leafhopper species. a: *Orosius albicinctus;* b: *Hishimonus phycitis;* c: *Amrasca biguttula biguttula;* d: *Orosius orientalis*.

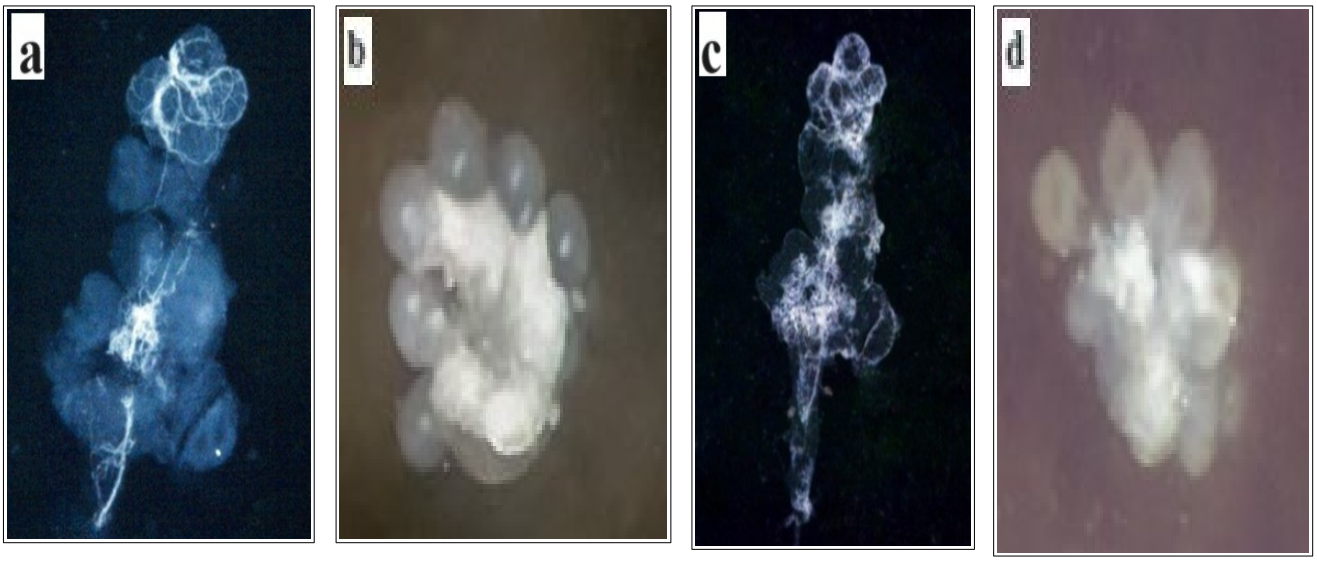


Fig. 3. Dissection of the salivary gland for confirmation of phytoplasma transmission. a, b: the salivary gland of *Orosius albicinctus;* c, d: the salivary gland of *Hishimonus phycitis*.

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Molecular insights and sequence-based characterisation

In the phylogenetic tree (Fig. 4), the genetic sequence OR880916, obtained from the green gram isolate named TN-CBE and the sequence OR884077 from the black gram isolate named TN-CBE-BG, were found to be 100 % matched with the green gram populations. This result indicates that both sequences are identical to those found in the green gram populations, demonstrating that the genetic material from the TN-CBE and TN-CBE-BG isolates was closely related to and consistent with, the green gram reference populations. This close match highlights the genetic uniformity and potential similarity in the strains infecting these crops.

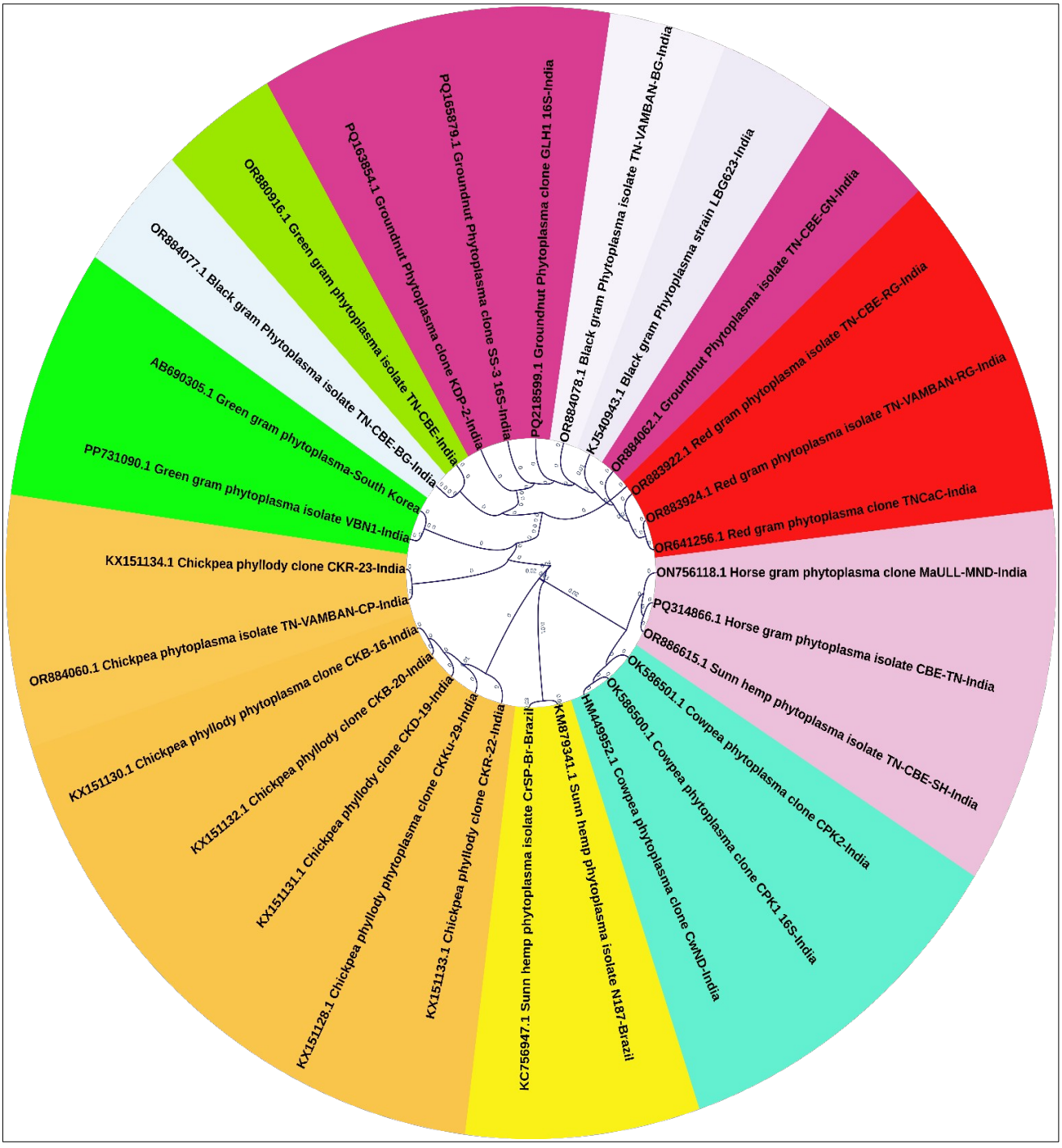


Fig. 4. A phylogenetic tree, constructed using the neighbour-joining method in Mega 12, shows the relationships among *Candidatus Phytoplasma* species infecting leguminous crops. This tree was based on the 16S rRNA gene and includes GenBank accession numbers for each representative species, indicating the specific genetic sequences used.

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The genetic sequence OR884060, obtained from the chickpea (Bengal gram) isolate named TN-VAMBAN-CP in Tamil Nadu, was found to be in close relation to the sequence KX151134 from the clone named CKR-23, which was part of Indian populations.

This close genetic relationship suggests that the TN-VAMBAN-CP isolate and the CKR-23 clone share significant genetic similarities. This indicates that the chickpea isolate from Tamil Nadu may have a genetic background closely related to the Indian clone CKR-23, reflecting potential similarities in their genetic makeup or evolutionary history.

The genetic sequence PQ314866, derived from the horse gram isolate named CBE-TN and the sequence OR886615, obtained from the Sunn hemp isolate TN-CBE-SH, were found to be closely related. This close genetic relationship suggests that these two populations, despite originating from different host plants (horse gram and Sunn hemp), share significant genetic similarities. The proximity of their genetic sequences indicates that they may have similar genetic characteristics or evolutionary origins.

The TN-CBE-RG (OR883922) and TN-VAMBAN-RG (OR883924) populations, collected from red gram crops in Tamil Nadu, have shown 100 % match with the Indian clone named TNCaC. This result indicates that the genetic sequences of these populations are identical to those of the TNCaC clone, highlighting their close genetic relationship.

The genetic sequence OR884078 from the black gram isolate named TN-VAMBAN-BG was found to be a 100 % match with the sequence from the groundnut population isolate named TN-CBE-GN, also from Tamil Nadu. This perfect match indicates that the genetic material from the TN-VAMBAN-BG isolate was identical to that of the TN-CBE-GN isolate. This close genetic relationship suggests that the pathogens affecting black gram and groundnut in this region have highly similar genetic profiles, pointing to a possible shared origin or closely related evolutionary pathways.

Phytoplasma disease-transmitting leafhopper species were identified on various leguminous crops through molecular analysis. The crops studied include red gram, green gram, black gram, horse gram, Bengal gram, cowpea, soybean and faba bean. Several leafhopper species were molecularly characterised, with their corresponding GenBank accession numbers provided in Table 2 for the different leguminous crops.

Virtual restriction fragment length polymorphism analysis

Virtual RFLP analyses were conducted on 16S rRNA sequences from various phytoplasma isolates obtained from different leguminous crops (Table 3), including red gram, green gram, black gram, horse gram, Bengal gram, cowpea, soybean and faba bean (Fig. 5). The RFLP patterns of isolates from red gram, green gram, black gram and horse gram were found to resemble those of '*Candidatus P. aurantifolia*', which was part of the 16Sr group II, subgroup D. Conversely, the sequences from Bengal gram, cowpea, soybean and faba bean isolates matched those of '*Candidatus P. asteris*', belonging to 16Sr group II, subgroups B and AE. The 16S rDNA sequence of the query showed 98.83 % identity with the '*Candidatus P. asteris*' reference strain (GenBank accession: AP006628 and DQ286953), with a similarity coefficient of 1.00. This study identifies two major phytoplasma species, '*Candidatus P. aurantifolia*' and '*Candidatus P. asteris*', as the causative agents of significant phytoplasma diseases affecting pulse crops in India.

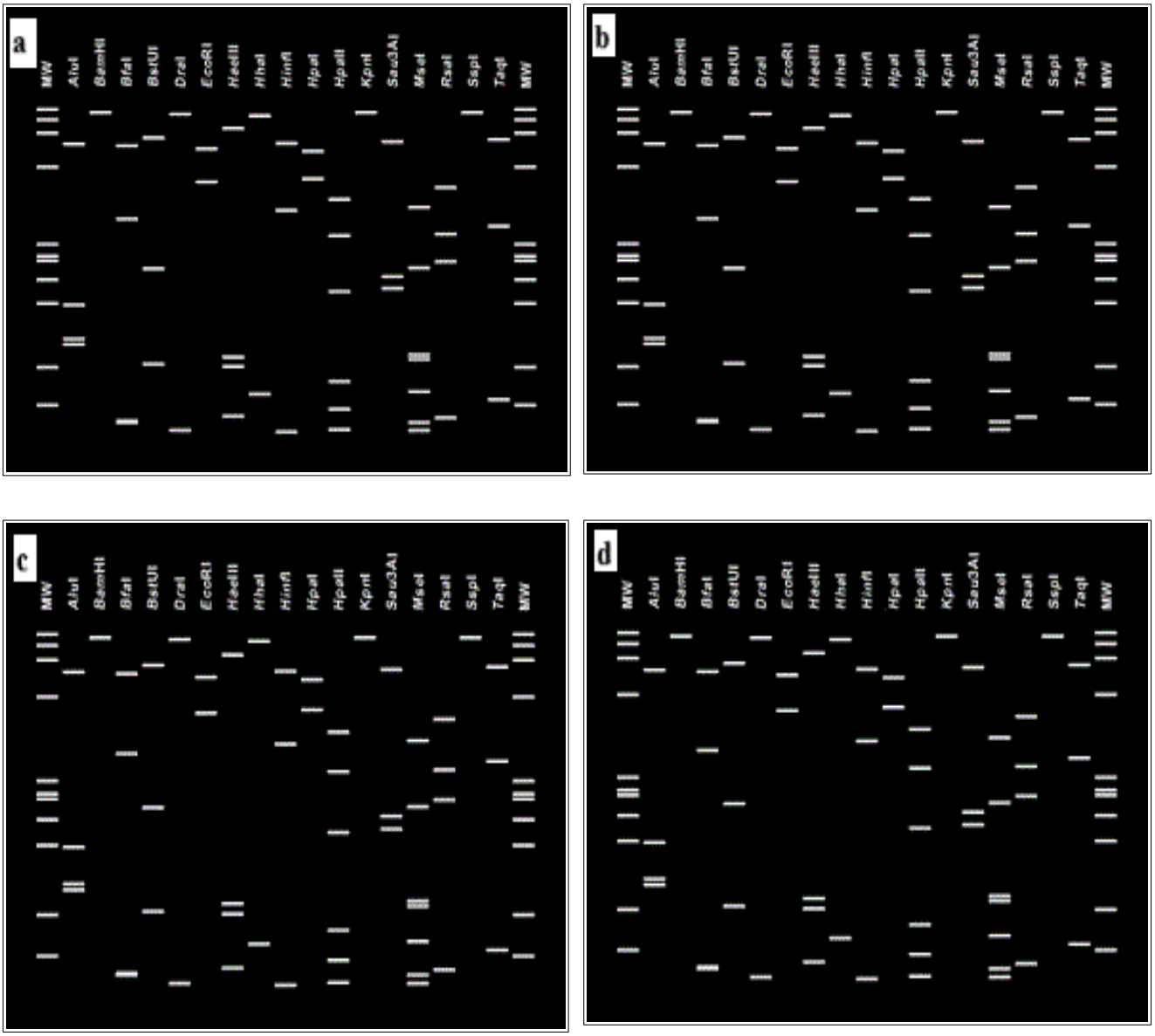
Table 2. List of crops affected by phytoplasma disease and their associated vectors

Host	Scientific name	GenBank accession number	Vector	GenBank accession number
Red gram	*Cajanus cajan*	OR883922 OR883924	*Orosius albicinctus*	PQ721018
Black gram	*Vigna mungo*	OR884077 OR884078	*Orosius albicinctus*	PQ721008
Green gram	*Vigna radiata*	OR880916	*Orosius albicinctus*	PQ721014
Horse gram	*Macrotyloma uniflorum*	PQ314866	*Hishimonus phycitis*	PQ721011
Bengal gram	*Cicer arietinum*	OR884060 OR883928	*Orosius orientalis*	PQ686238
Cowpea	*Vigna unguiculata*	PQ305642	*Orosius albicinctus*	PQ721013
Soybean	*Glycine max*	PQ699255	*Orosius orientalis* *Amrasca biguttula biguttula*	- PQ721010
Faba bean	*Vicia faba*	PP626699	*Hishimonus phycitis*	PQ721009

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Table 3. List of leguminous crops infected by phytoplasma disease with representatives of phytoplasma groups 16SrI and 16SrII

Host	Scientific name	Phytoplasma species	Group name	Reference strain's accession number
Red gram	*Cajanus cajan*	*Candidatus* Phytoplasma aurantifolia	16Sr group II, subgroup D	JF781309
Black gram	*Vigna mungo*	*Candidatus* Phytoplasma aurantifolia	16Sr group II, subgroup D	EU099568
Green gram	*Vigna radiata*	*Candidatus* Phytoplasma aurantifolia	16Sr group II, subgroup D	JF781309
Horse gram	*Macrotyloma uniflorum*	*Candidatus* Phytoplasma aurantifolia	16Sr group II, subgroup D	EU099568
Bengal gram	*Cicer arietinum*	*Candidatus* Phytoplasma asteris	16Sr group I, subgroup B	AP006628
Cowpea	*Vigna unguiculata*	*Candidatus* Phytoplasma asteris	16Sr group I, subgroup B	AP006628
Soybean	*Glycine max*	*Candidatus* Phytoplasma asteris	16Sr group I, subgroup B	DQ286953
Faba bean	*Vicia faba*	*Candidatus* Phytoplasma asteris	16Sr group I, subgroup AE	DQ286953



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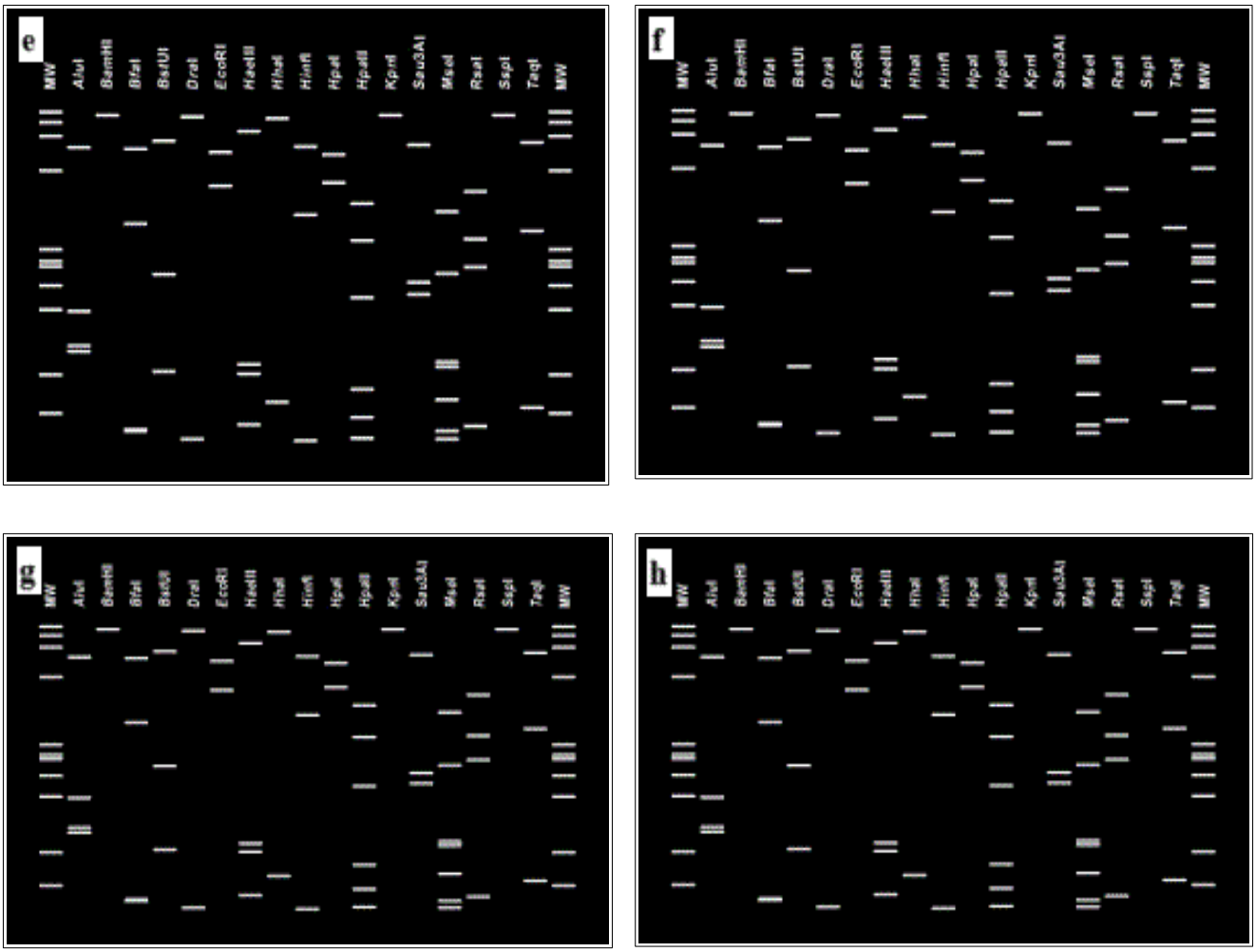


Fig. 5. Virtual restriction fragment length polymorphism (RFLP) profiles of phytoplasma DNA fragments (100-bp DNA ladder) from leguminous crops were generated using the *i*PhyClassifier program through *in silico* digestion. The profiles correspond to the 16Sr groups and include the following crops: a: red gram; b: black gram; c: green gram; d: horse gram; e: Bengal gram; f: cowpea; g: soybean.

Expression of phytoplasma disease symptoms

Phytoplasma infections were detected in eight different leguminous crops. These crops exhibited a range of symptoms including stunted growth, leaf chlorosis and distorted flowering. The severity of the symptoms varied depending on the crop species and the environmental conditions. These symptoms have been noted in several agricultural crops, including pulses like red gram (*C. cajan*) (Fig. 6a), black gram (*V. mungo*) (Fig. 6b), cowpea (*V. unguiculata*) (Fig. 6c), horse gram (*M. uniflorum*) (Fig. 6d), green gram (*V. radiata*) (Fig. 6e), Bengal gram (*C. arietinum*) (Fig. 6f), soybean (*G. max*) and faba bean (*V. faba*).

Discussion

Phytoplasma diseases pose a severe biotic threat to pulse production globally, causing devastating yield losses ranging from 40 % to 100 % depending on the crop growth stage at the time of infection. Transmitted primarily by phloem-feeding leafhopper vectors like *Orosius orientalis*, these wall-less, phloem-limited bacterial pathogens severely disrupt the host plant's hormonal balance. This disruption triggers distinct morphological deformities such as phyllody, witches' broom and little leaf symptoms. Because these abnormalities replace normal reproductive organs with vegetative structures, affected pulse crops suffer from complete or near-complete sterility, directly compromising seed formation and causing substantial economic and quantitative losses in major leguminous crops. The taxonomical characteristics of *O. albicinctus* described in our study align with the observations made by earlier researchers, providing strong support for our findings (32, 33). A notable feature of this species was the widely divergent aedeagal shafts with an apically expanded posteromedian lobe. Additionally, based on the male genital structures, *A. biguttula biguttula* conforms to the morphological descriptions reported by earlier researchers (34, 35). These consistent findings reinforce the morphological criteria used in identifying these leafhopper species.

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Fig. 6. Expression of phytoplasma symptoms in agricultural crops. a: bushy leaves and malformed flowers in red gram; b: shoot and leaf proliferation in black gram; c: little leaf in cowpea; d: little leaf in horse gram; e: stunting and floral malformations in green gram; f: virescence and yellowing leaves in chickpea.

A previous study documented notable colour variations in *H. phycitis,* particularly in the median spot on its wings, emphasising its morphological diversity. According to earlier reports, *O. orientalis* can be identified by specific markings on its head, including two distinct spots at the front, two smaller spots near the posterior margin (36) and a pair of dark transverse stripes between the compound eyes that form a "V" shape in the centre (6). In India, *A. biguttula biguttula* and *H. phycitis* are identified as vectors of brinjal little leaf disease. The transmission capability of *H. phycitis* is well-established, whereas there is limited evidence regarding the transmission ability of *A. biguttula biguttula*.

BLAST analysis that *V. mungo* phytoplasma shares 99 % identity with 16SrII group '*Candidatus P. aurantifolia*', including strains from Myanmar associated with black gram witches' broom and phyllody (AB690304) (37). Further study supported these findings by showing the close genetic alignment of Indian *V. mungo* phytoplasmas within the '*Candidatus P. aurantifolia*' cluster, highlighting their regional and phylogenetic significance (38).

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Extensive diversity of phytoplasma groups affecting leguminous crops in India, with five distinct phytoplasma groups (16SrI, 16SrII, 16SrVI, 16SrXIV and 16SrIX) reported in major crops like *C. cajan*, *C. arietinum*, *G. max* and others (39). Among these groups, the 16SrII-D subgroup was notably prevalent across legume crops in Asia, suggesting its dominant role in phytoplasma-induced diseases in the region. This was consistent with earlier findings, indicating the widespread impact of this subgroup. The use of virtual RFLP analysis with restriction enzymes (BstUI, HaeIII, HpaII and MseI) further supported this finding, as the RFLP patterns of the phytoplasma strains from India closely matched those from Myanmar, specifically the blackgram witches' broom and phyllody phytoplasma attributed to '*Candidatus P. aurantifolia*' (40). These findings emphasise the molecular similarities between phytoplasma strains across neighbouring regions, reinforcing the need for collaborative and region-wide efforts in monitoring and managing these plant pathogens.

The symptoms of red gram phytoplasma disease observed in this study, namely phyllody, stunted growth and leaf yellowing, were consistent with previous findings and are indicative of phytoplasma infections (9). Phytoplasma diseases affecting Bengal gram have been reported in various regions, including India (7), Pakistan (10), Sudan (12) and Oman (13), with phyllody being the most distinct symptom, though its presentation may vary. In Pakistan, the leafhopper *O. orientalis* was identified as the vector for chickpea phyllody phytoplasma (10). Observations of phytoplasma disease symptoms in green gram during this study were consistent with reports from India, Thailand and Australia (41), but additional symptoms, such as pod splitting and germination of mature seeds within pods, were noted in Pakistan (42).

Conclusion

The study signifies a crucial advancement in the molecular epidemiology and management of pulse-infecting phytoplasmas in Southern India. This comprehensive four-year investigation (2021–25) provides critical baseline data on the spatial distribution, vector associations and genetic diversity of phytoplasma infections affecting leguminous crops across Tamil Nadu. The study conclusively links specific symptomatologies, such as phyllody and bushy growth, to two distinct phytoplasma strains: '*Candidatus Phytoplasma aurantifolia*' and '*Candidatus Phytoplasma asteris*', establishing a clear host-strain dichotomy among different legume varieties. Furthermore, by robustly identifying *Orosius albicinctus*, *Orosius orientalis*, *Hishimonus phycitis* and *Amrasca biguttula biguttula* through integrated morphological and *mtCOI* gene analysis, this research mapping lays a vital foundation for vector-ecology studies. While experimental transmission electron microscopy or feeding assays are still required to fully transition these species from "putative" to "confirmed" vectors, their high field correlation marks them as primary targets for pest management. Ultimately, these insights into disease prevalence and strain distribution are indispensable for designing targeted, region-specific integrated pest and disease management (IPDM) strategies to safeguard legume cultivation and enhance food security in Southern India.

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Authors' contributions

VS and LK wrote the manuscript. GS and MM designed the article and contributed to its revision. MS analysed the data. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest

Authors do not have any conflict of interests to declare.

Ethical issues

None

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Gemini AI and Grammarly to improve English language clarity, grammar check. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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