Annals of Tropical Research 44 (1):46-60(2022)
https://doi.org/10.32945/atr4414.2022

Insect vectors transmission of phytoplasma to vegetables in Eastern Visayas Philippines

Lucia M. Borines[1*], Nickie D. Duero[1], Joy Adeline C. Nuñez[1], Rezel Sagarino-Borines[2], Sandra McDougall[3], and Reny G. Gerona[1]

ABSTRACT

Received: 08 January 2021 | Accepted: 14 January 2022

Phytoplasma diseases are found affecting vegetables in Eastern Visayas, Philippines. Identifying the insect vectors that transmit phytoplasma in the field is necessary for the management of this disease. This study was conducted to identify the vectors of phytoplasma affecting bitter gourd, loofah, and string beans. Insects associated with these vegetables that had shown phytoplasma symptoms in the field were collected, mass-reared, and the progenies of mass reared insects were used for transmission to healthy host plants. Phytoplasma detection was done through PCR and nest PCR assays. Phytoplasma was positively transmitted to healthy bitter gourd plants by a cicadellid leafhopper, *Hishimonus* sp. and brown planthopper, *Ricania speculum*. The ~ 1.25Kb phytoplasma-specific band was amplified in these insects. The phytoplasmas transmitted by the *Hishimonus* sp. and *R. speculum* produced two different types of symptoms to the bitter gourd, and rDNA sequence analysis separated them into two clusters which confirmed that the phytoplasmas belong to two different strains. Although the *Aphis gossypii* and *Aphis craccivora* also produced symptoms in bitter gourd and string beans, and the pathogen was detected in the insects, whether they vectored phytoplasma to their respective hosts needs further study, since the symptoms produced were quite different from the ones produced by the cicadellid leafhopper and *Ricania speculum*.

Keywords: *Hishimonus sp. Ricania speculum* bitter gourd witches broom

[1]Department of Pest Management, Visayas State University, Baybay City, Leyte Philippines
[2]Caraga State University, Ampayon, Agusan del Norte
[3]New South Wales Department of Primary Industries (DPI), Yanco Agricultural Institute, NSW, Australia

***Corresponding Author.** Address: Department of Pest Management, Visayas State University, Baybay City, Leyte Philippines; Email: lucyborines@vsu.edu.ph

INTRODUCTION

Phytoplasmas are phloem-limited phytobacteria that lack cell walls and are mainly transmitted by insect vectors in a persistent manner (Weintraub and Beanland 2006). Rojas-Martinez (2009) reported that phytoplasmas are transmitted by phloem-feeding insect vectors most commonly belonging to Homoptera of the families: Cicadellidae, Cixiidae, Psyllidae, Cercopidae, Delphacidae, Derbidae, Menoplidae, and Flatidae. Weintraub and Beanland (2006) listed 92 insect species that transmit phytoplasma diseases, primarily leafhoppers, planthoppers, and psyllids. An additional five confirmed vector species and some previously known vectors with new phytoplasma associations were reported by Weintraub (2007).

The identification of insect vectors that transmit phytoplasma diseases is essential for their management. Beets phytoplasma was reported to be transmitted by the leafhopper, *Orosius orientalis (*Mirzaie et al 2007). Lettuce phytoplasma is transmitted by leafhoppers *Macrosteles* sp. (Borth et al 2006) and *Neoaliturus fenestratus (*Salehi et al 2006). Khan et al (2003) positively amplified phytoplasma affecting alfalfa from two leafhopper species identified as *Austroagallia avicula* and *Empoasca* sp. which indicates that these insects are potential vectors of Alfalfa phytoplasma. Mirzaie et al (2007) reported that *Orosius orientalis* transmits the garden beet witches' broom. Sugarcane white leaf disease is transmitted by the leafhopper *Yamatotettix flavovittatus* (Hanboonson et al 2006). *Hishimonus phycitis* was reported to transmit phytoplasma to citrus (Salehi et al 2007).

Phytoplasma diseases are becoming more common in vegetables in the Eastern Visayas region of the Philippines, specifically in bitter gourd (*Momordica charantia*), loofah (*Luffa cylindrica*), and string beans (*Vigna unguiculata sesquipedalis)*. Phytoplasmas have also been observed in "Baguio" beans (*Phaseolus vulgaris*), cucumber (*Cucumis sativus*), and tomatoes (*Solanum lycopersicum*) (Borines et al 2020). Typical symptoms include reduced size of new leaves and the plant showing "witches' broom" or bunched apical growth symptoms. Fruit, if produced, is much reduced in size or absent, thereby severely affecting yield.

The insect vectors that lead to the spread of phytoplasma diseases need to be identified for their management, however, knowledge on insect vectors of vegetable phytoplasmas is lacking in the Philippines, particularly for bitter gourd, loofah, and string beans. This insect transmission study aims to identify the insect vectors of the phytoplasmas affecting bitter gourd, loofah, and string bean.

MATERIALS AND METHODS

Collection and Mass Rearing of Insects Associated with Phytoplasma Symptoms

Insects associated with phytoplasma disease symptoms (chlorosis and little leaf), namely a cicadellid leafhopper, *Ricania speculum*, and *Aphis gossypii* from bitter gourd and loofah, and black aphids from string beans showing little leaf, were collected from vegetable farms in representative areas Baybay City, Ormoc City and Maasin City in the Eastern Visayas region of the Philippines. The brown cicadellid leafhopper, *R. speculum*, and *A. gossypii* were the insects suspected to be vector in bitter gourd and loofah phytoplasma, so they were used for insect transmission to bitter gourd. Whereas the black aphid (*A. craccivora*) was the insect suspected

to transmit the pathogen to the string bean. The collected insects were placed in mass-rearing cages with healthy bitter gourd plants, which were regularly replaced with new healthy plants. The plants that had been fed upon were regularly checked for phytoplasma disease symptoms, ie, chlorosis and little leaf/ witches broom. The insect species associated with the loofah and bitter gourd phytoplasma symptoms were commonly observed on both plants, so only bitter gourd was used as a healthy host for the transmission studies that involved cicadellid leafhopper, planthopper *R. speculum,* and green aphid *A. gossypii*. Mass-reared juvenile insects from the rearing cages where the plants remained healthy were transferred using an aspirator to small plastic containers with field-collected diseased host plants for a 24h acquisition feeding. After the feeding period 10-20 insects were transferred to healthy bitter gourd plants in the greenhouse for insect transmission. Ten healthy plants were used in the transmission study for each insect. As control, the mass-reared insects from plants which did not show phytoplasma symptoms were transferred directly to healthy host plants. The insects were removed from the cages one week after introduction and destroyed. Throughout the experiment each plant was covered entirely with tulle cloth to avoid escape and unwanted infestation. The test plants were observed for the development of symptoms typical of a phytoplasma disease.

Phytoplasma Detection from Insects

Total DNA extraction, PCR, and nest PCR assays

Phytoplasma detection was conducted on insects associated with phytoplasma symptoms of bitter gourd, loofah, and string bean, to confirm whether they harbored the pathogen. Detection through PCR and nested PCR assay was also done on insects that had positively introduced the pathogen to healthy host plants. During the PCR analysis and after the conduct of insect transmission studies, it was observed in one string bean field visit where the plants were showing phytoplasma-like symptoms, a green leafhopper, *Empoasca sp*. was associated with the diseased plants so samples of these insects were collected and included in the PCR analysis for the presence of the pathogen. However, no transmission study was conducted with this insect since the project was near termination.

An optimized extraction protocol for phytoplasma used by Ahrens and Seemüller (1992) was slightly modified. In this method, approximately 200mg of insects were soaked in a mortar containing 2mL phytoplasma grinding buffer (100mM K_2HPO_4, 31mM KH_2PO_4, 10% sucrose, 2% polyvinylpyrrolidone-10 (PVP-10), 10mM EDTA pH8.0) and kept at -4°C for 10mins. They were ground with a pestle, and the homogenate was centrifuged at 5000rpm for 5mins. The supernatant was transferred into a clean 2mL tube and further centrifuged at 13000rpm for 30mins. The resultant pellet was dissolved in 750µL warm 2% CTAB ($20gL^{-1}$ CTAB, 100mM Tris-HCl pH8.0, 1.4M NaCl, 2% PVP-10, 20mM EDTA pH 8.0) and incubated at 60°C for 30mins. Samples were purified with 900µL chloroform-isoamyl alcohol (24:1) and centrifuged at 12000rpm for 5mins. Nucleic acids were precipitated with 600µL isopropanol before incubating at -20°C overnight. Next day the samples were centrifuged at 12000rpm for 30mins then washed with 70% ethanol. The pellet was air-dried and re-suspended in 100µL TE (10mMTris-HCl, 1mM EDTA, pH 8.0) buffer.

Two further extractions were performed, first with 100µL phenol:chloroform:isoamyl alcohol (25:24:1) followed by 100µL chloroform. The upper aqueous layer was removed, and 300µL cold absolute ethanol was added to precipitate the nucleic acids. DNAs were centrifuged at 13000rpm for 30mins, washed with cold 300µL 70% ethanol, air-dried, and re-suspended in 10-40µL TE buffer, depending on the size of the DNA pellet. The DNA concentration in each isolation tube was measured through gel electrophoresis together with known concentration of λDNA for comparison. Next 2µL RNAse was added to each tube and incubated at 37°C for 30mins. Nucleic acids were stored at -20°C. A concentration of 50-100ng per uL DNA was used in the PCR analysis. The same protocol was used in extracting DNA from *Aphis craccivora*, the string beans aphid.

Initial PCR analysis was done using the universal primer P_1/P_7 for Phytoplasma developed by Deng and Hiruki (1991), followed by a nested PCR using the primer $R_{16}F_2n/R_{16}R_2$ (Gundersen and Lee 1996, Lee et al 1995). Nest PCR reactions were carried out in a total volume of 25µL. Each reaction was composed of nuclease-free water, 2.5µL GoTaq buffer (GoTaq Green, Promega, Madison, USA), 0.5µL 10mM dNTPs (Vivantis), 1µL of 50nM each of forward primer ($R_{16}F_2n$) and reverse primer ($R_{16}R_2$), 0.5µL Taq Polymerase enzyme and 2µL of 50ngµL^{-1} template DNA. PCR conditions were as follows, initial denaturation at 94ºC for 2mins, 30 cycles of denaturation at 92ºC for 1min, annealing at 55ºC for 15s, and extension 72ºC for 30s, and a final extension at 72ºC for 10mins. The PCR products were subjected to agarose gel electrophoresis using 1% agarose gel, stained with Gel Red Stain® (Biotium). The bands were viewed under a UV trans-illuminator attached to an Alpha Digi-Doc Documentation System. The insects that produced positive transmission of phytoplasma to healthy bitter gourd and loofah and showed positive phytoplasma bands in the PCR analysis were considered as the phytoplasma vector of these host plants.

16SrDNA Sequence Analysis

Nest PCR products amplified by $R_{16}F_2n/R_{16}R_2$ primers (Gundersen and Lee 1996, Lee et al 1995) of selected samples showing positive phytoplasma bands were sent for RNA sequence analysis at the Philippine Genome Center, University of the Philippines, Diliman, Quezon City. Sequence analysis was conducted using BLASTn, and phylogenetic analysis of the different isolates was conducted using the Mega6 software. Evolutionary relationships of taxa, inferred using the unweighted pair group method with arithmetic mean (UPGMA) method (Sneath and Sokal 1973). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980). Evolutionary analyses were conducted in Mega6 (Tamura 2013).

RESULTS AND DISCUSSION

Insect Transmission

All the bitter gourd test plants exposed to the three phytoplasma infected insects (brown cicadellid leafhopper, *R. speculum,* and *A. gosypii*) exhibited the phytoplasma-like symptoms (Figure 1). However, there were slight differences in the symptoms

produced by these insects (Table 1). String bean aphid, *A. craccivora* also produced the little leaf symptom in its host plant. The inoculated plants were also subjected to PCR and nest PCR which showed positive results for phytoplasma.

Table 1. Phytoplasma insect transmission result for bitter gourd and string bean

Host Plant	Insect	Transmission Result	Symptom produced
1. Bitter gourd	Cicadellid leafhopper (*Hishimonus* sp.)	+	typical little or very tiny leaves/witches broom symptom
2. Bitter gourd	Planthopper *Ricania speculum*	+	reduced leaf size but not too tiny leaves and leaves are not thickened, with chlorosis
3. Bitter gourd	*Green aphid Aphis gossypii*	+	reduced leaf size, thickened leaves with chlorosis
4. String bean	Black aphid *Aphis craccivora*	+	little leaf/witches broom symptom

The cicadellid leafhopper with light brown and dark brown markings on the wings transmitted the phytoplasma that produced the typical little leaf with witches' broom symptoms in bitter gourd. Affected plants produce very tiny pale green leaves, witches' broom and shoestring symptoms, and generalized stunting (Figure 1a), but no foliar chlorosis was evident. The cicadellid leafhopper was small, approximately 5mm for the adult (Figure 1b) and 2mm for the nymph (Figure 1c). Figure 1d is a close-up photo of the insect viewed under a USB microscope. It was morphologically identified as one of the *Hishimonus* species and closely resembles *Hishimonus phycitis* Distant, a cicadellid leafhopper associated with citrus described by Du and Dai (2019). *Hishimonus* spp. are described by Du and Dai (2019) as small species, 3.0–5.0mm in length. Coloration usually greenish or yellow with legs stramineous, usually with symmetrical brown markings dorsally on the head, pronotum, and forewing, forewings silvery-white with dark brown mottling, and usually with a large brown semicircular spot at mid-length of commissural margin forming, when the wings at rest, a conspicuous median diamond-shaped spot with that of the opposite wing.

The leaves of plants introduced with the brown planthopper, *R. speculum* (Figure 2a) were also reduced in size (but not very tiny) and did not exhibit the typical witches broom symptoms but showed foliage chlorosis. No thickening of the leaves was observed (Figure 2b). On the other hand, the bitter gourd plants introduced with the green aphid, *A. gossypii,* produced tiny leaves and chlorosis. Unlike the chlorosis exhibited by plants introduced with *R. speculum*, the leaves were thickened (Figure 3). The thickening of the leaves with yellowing is also a characteristic symptom of "Namamarako" disease caused by the cucurbit aphid-borne yellow virus (CABYV). The namamarako-infected leaves, however, although thickened usually do not turn very tiny as was observed in the plants under this study. Yellowing and tiny leaf size are definitely symptoms of phytoplasma and thickening of the leaves may also be

due to phytoplasma infection. The juvenile progenies of mass-reared field-collected aphids used in the transmission studies were assumed free from any pathogen. If phytoplasma did not cause the thickening of the leaves, it could also have been due to the direct effect of aphid feeding. Nang et al (2014) positively detected phytoplasma in bitter gourd little leaf in Myanmar that showed small, yellowish green, thickened and puckered leaves, and the internodes were also thickened.

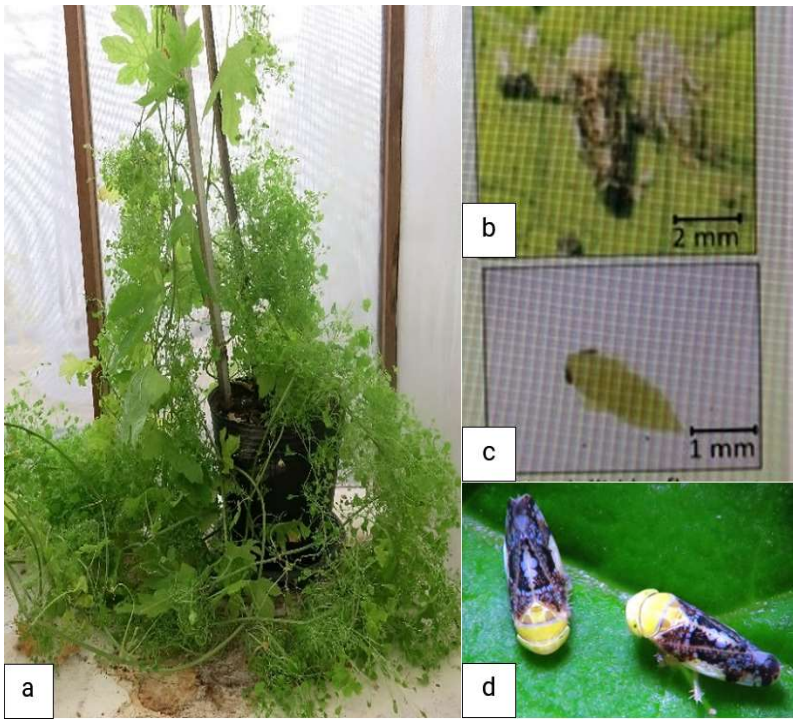

Figure 1. The typical witches broom symptoms of bitter gourd introduced with the insect vector identified as *Hishimonus* sp. (a), close up of the brown cicadellid leafhopper adult (b) and nymph (c) vectors using an ordinary camera and close up of the insect using a USB Digital microscope (d)

Since reports on insect vectors of phytoplasmas to host plants were mostly identified as leafhoppers, planthoppers and psyllids (Rojas-Martinez 2009, Weintraub and Beanland 2006, Weintraub 2007), even if the pathogen was detected in the aphids, we could not conclude that this insect is really a vector of phytoplasma. Cainelli et al (2007) had detected the pathogen in aphids but, according to their research, the aphids did not transmit phytoplasma to apple plants.

On the transmission study on string beans using *A. craccivora,* results showed that the test plants introduced with *A. craccivora* also showed little leaf symptoms (Figure 4). As with bitter gourd phytoplasma, there was also the need to confirm the phytoplasma transmission to string beans by *A. craccivora*.

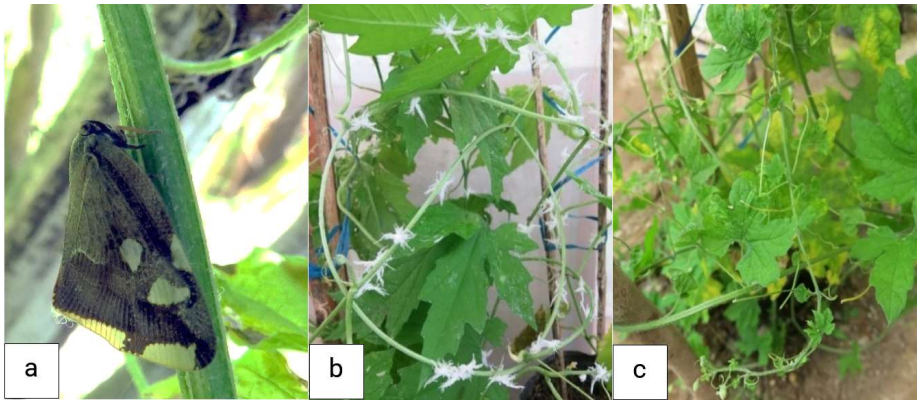

Figure 2. *Ricania speculum* adult (a) nymphs (b) and phytoplasma-infected bitter gourd showing reduced leaf size and yellowing symptoms (c)

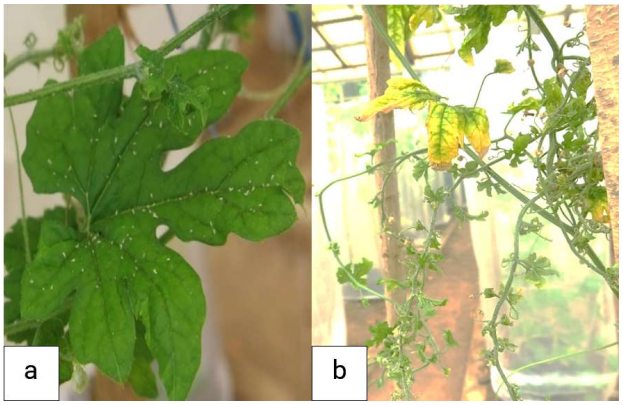

Figure 3. Bitter gourd in the screenhouse introduced with *Aphis gossypii* (a) positively showing tiny and thickened leaves and yellowing symptoms (b)

Figure 4. String bean introduced with *Aphis craccivora* exhibiting the little leaf symptom

Detection of Phytoplasma from Arthropod Vectors

Phytoplasma detection from the suspected insect vectors through PCR and nest PCR assays were conducted using the universal primer P_1/P_7 and $R_{16}F_2n/R_{16}R_2$ to confirm that these insects harbored phytoplasma in their bodies. A phytoplasma-specific band of ~1.25Kb was positively amplified from the leafhopper *Hishimonus* sp. that had positively transmitted the pathogen to the bitter gourd, causing the typical witches broom symptoms in healthy plants in the screen house (Figure 5). No band was initially detected from field-collected *R. speculum* (Rn) DNA in the initial PCR run. The phytoplasma-specific band was also amplified from *A. gossypii* DNAs (AGA and AGP), which confirmed that aphids carry the pathogen in their bodies. Phytoplasma bands were also amplified from infected host plant DNAs such as loofah (PH) and bitter gourd (AG, AV1, and AV2) DNAs.

The nest PCR assay was repeated with the *R. speculum* DNA sample (Rn), cicadellid leafhopper (CLH) together with the string bean aphid (SBA), and green leafhopper *Empoasca* sp. (Esp) DNA since it was also reported to transmit loofah phytoplasma to plants (Khan et al 2003) and this insect was observed in a string bean field that showed phytoplasma-like infections.

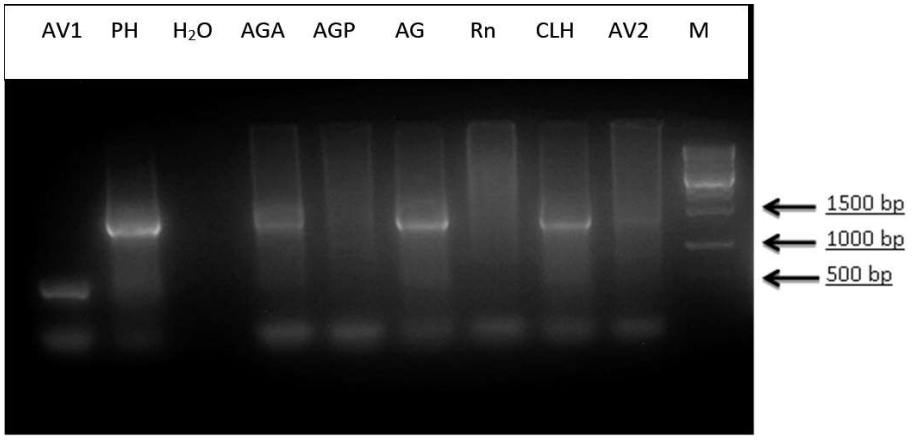

Figure 5. Nest PCR amplification results from insects used in transmission study. green aphids *Aphis gossypii* from bitter gourd (AGA) and loofah (AGP), *Ricania speculum* (Rn) and cicadellid leafhopper *Hishinomus* sp. (CLH). Positive phytoplasma band amplifications were obtained from the green aphid (AGA). Amplifications were also obtained from field-infected loofah (PH) and bitter gourd (AG, AV1, and AV2) that served as controls

The PCR products were subjected to gel electrophoresis together with amplified phytoplasma from infected plant DNAs of loofah little leaf (Pi), Baguio bean little leaf (BB), bitter gourd little leaf (AL2), and healthy bitter gourd DNA (C) (Figure 6). The ~1.25Kb band was amplified from the cicadellid leafhopper and *R. speculum,* which transmitted the disease to the bitter gourd in the screen house. The same band was also detected from string bean aphid (SBA), which transmitted the disease to string beans. The cicadellid leafhopper (CLH) and *R. speculum* (Rsp) phytoplasma DNA was again amplified in another PCR run (Figure 7). No band was detected from *Empoasca* sp in either of the two PCR analyses.

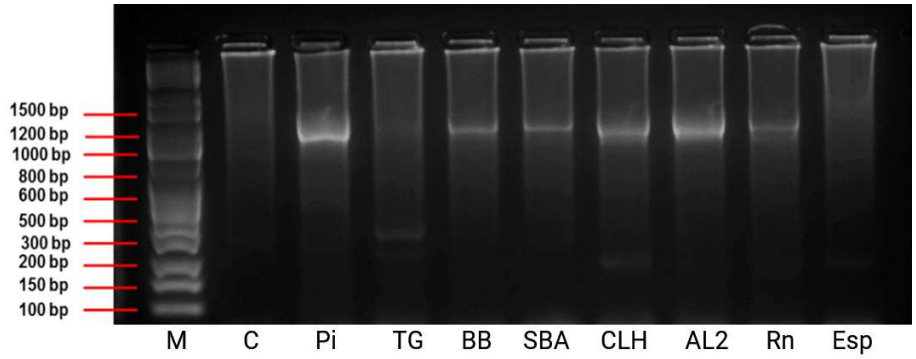

Figure 6. Positive nest PCR amplification of phytoplasma-specific band from insects, namely: string bean aphid (SBA), cicadellid leafhopper (CLH) and *Ricania speculum* (Rn) together with host plants, loofah little leaf (Pi), Baguio bean little leaf (BB), bitter gourd little leaf (AL2) and healthy bitter gourd (C). M is 1 Kb ladder). No amplification was obtained from *Empoasca* sp. (Esp)

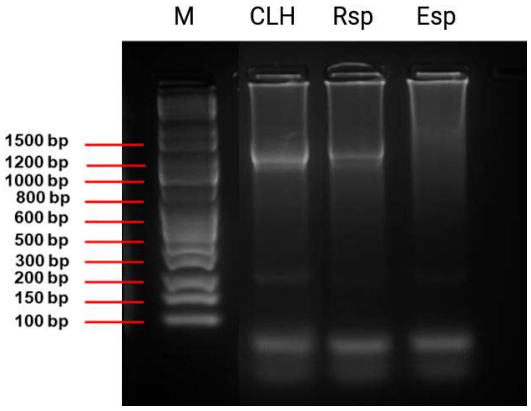

Figure 7. Positive re-amplification result from cicadellid leafhopper (CLH), *Ricania speculum* (Rsp) while no amplification was obtained from *Empoasca* sp. (Esp)

The $R_{16}F_2n/R_{16}R_2$ amplified products from insect vectors were subjected to gene sequencing to confirm these results. Table 2 shows the nucleotide sequences of the $R_{16}F_2n/R_{16}R_2$ amplified bands linked to the Phytoplasma 16SrDNA from the insect vectors analyzed using BLASTn. The Phytoplasma band amplified from the *Hishimonus* sp. (CLH) had 99% similarity to three loofah witches' broom phytoplasma genes in the GenBank, 97% similar to Stylosanthes little leaf phytoplasma, oil palm phytoplasma, Malaysian yellow dwarf coconut phytoplasma, and Malaysian periwinkle virescence phytoplasma and 96% similar to *Candidatus Phytoplasma trifolii*. Another *Hishimonus* sp. amplicon from the screen house (LC2) showed almost the same results.

Table 2. Top 10 BLASTn homologous hits of phytoplasma isolates from insect vectors

Phytoplasma Isolates	GenBank Accession	Host	Country	% Nucleotide Sequence Similarity			
				Hishimonus sp. (CLH)	*Hishimonus* sp. (LC2)	*Ricania* sp. (Rn)	*A. craccivor* (SBA)
Ca. Phytoplasma luffae	AF353090.1	Loofah	Taiwan	99	99	95	90
Ca. Phytoplasma luffae	AF248956.1	Loofah	Taiwan	99	99	95	90
Loofah witches'-broom	AB667970.1	Bitter gourd	Taiwan	99	99	94	90
Loofah witches'-broom	AF086621.2	Loofah	Taiwan	99	99	95	90
Phytoplasma sp. 16S rRNA gene	Y17055.1	Stylosanthes	Australia	97	97	93	88
Stylosanthes little leaf phytoplasma	AJ289192.2	Stylosanthes	Australia	97	97	92	88
Oil palm phytoplasma	EU498728.1	Oil palm	Malaysia	97	97	92	88
Malaysian yellow dwarf coconut phytoplasma	EU498727.1	coconut palm	Malaysia	97	97	92	88
Malaysian periwinkle virescence phytoplasma	EU371934.2	*C. roseus*	Malaysia	97	97	92	88
Ca. Phytoplasma trifolii	KY321932.1	Pepper	Turkey	96	97	-	-
Ca. Phytoplasma trifolii	KX092011.1	Tomato	Mexico	-		92	-
Fragaria multicipita' Phytoplasma	AF190225.1	Fragaria	Canada	-	-	-	87

However, the phytoplasma amplicon from *R. speculum* diverged slightly from most loofah phytoplasma genes and other controls (92-95% similar), and even more for the string bean aphid, which only shares 87-90% similarity to the controls. These results suggest that the strains of phytoplasma transmitted by *R. speculum* to bitter gourd and loofah in the field are different from those transmitted by the cicadellid leafhopper *Hishimonus* sp. based on the symptoms they produced in the bitter gourd host in this study. It corroborates well with the symptoms noted on the introduced plants, which varied between *Hishimonus* sp. that caused the very tiny leaves causing the typical witches broom symptoms, and *R. speculum*-transmitted phytoplasma just reduced the affected leaf's size a little together with leaf yellowing symptoms. The strains from string bean aphid *A. craccivora* are just 87–90% similar to the controls used, suggesting another strain of phytoplasma carried by the string bean aphid.

Figure 10 shows the multiple sequence alignment of phytoplasma amplicons from host plants, vectors, and controls showing a high consensus value of 90% from the 260 to 326 genomic nucleotide position indicated by the red- colored nucleotides. Few single nucleotide polymorphisms (SNPs) and indels were observed, with S1R16 (string bean phytoplasma) showing the most frequent variation other than the outgroup reference Rice.

Figure 11, on the other hand, shows the phylogenetic dendrogram of phytoplasma nucleotide sequences from the insect vectors (Rn, CLH, LC2, and SBA), together with plants (TG [tomato], BB [Baguio bean], AG [bitter gourd], AL [bitter gourd], AV2 [bitter gourd], PHg [loofah], PI [oofah], PV [loofah]), and controls analyzed using UPGMA method. The data show that the strains were grouped into two main clusters. A tight cluster comprised of cicadellid leafhoppers (*Hishimonus* sp.) (CLHR16, LC2R16), string bean aphid (*A. craccivora*) (SBAR16), bitter gourd, and loofah phytoplasmas (ALR16, AV2R16, PHgR16, PIR16, and PVR16) was noted, which also represents the most recent type of strains. By contrast, the other group representing early lineages of Phytoplasma strains, including tomato stunt phytoplasma TG R16, *R. speculum* Rn R16 and Baguio bean phytoplasma BB R16, showed expansion probably by frequent nucleotide substitution events.

These overall results implied that cicadellid leafhopper, *Hishimonus* sp., and brown planthopper, *R. speculum* are confirmed vectors of two strains of phytoplasma based on their positive transmission to healthy bitter gourd hosts, positive detection through PCR and Nest-PCR, and rDNA sequencing and phylogenetic analysis. Athough the green aphid, *A. gossypii,* and black aphid transmission to bitter gourd and string bean also produced the phytoplasma-like little leaf symptoms and the pathogen was detected in the insect DNAs, further studies should be done to confirm their positive transmission of phytoplasma to host plants. Cainelli 2007 reported that aphids seem to acquire quite a high load of the apple phytoplasma but the concentration of the pathogen in these insects was lower when compared to the concentrations detected in psyllids which transmitted the disease to apples. They proposed that aphids don't play any relevant role in apple phytoplasma spreading.

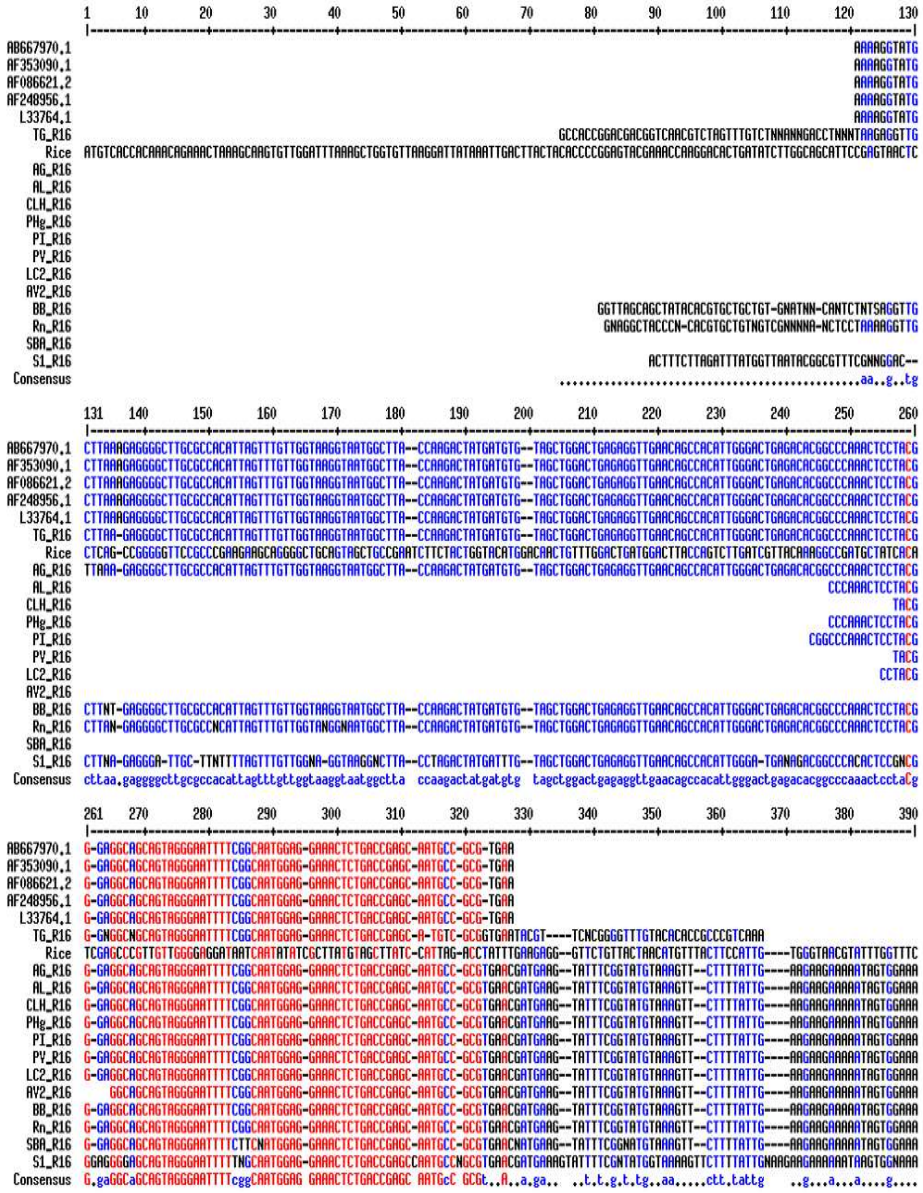

Figure 10. Alignment of the 19 nucleotide sequences of Phytoplasma-specific amplicons from host plants, insect vectors, and controls. (Multiple sequence alignment using MultAlin). Note: TG represents Phytoplasma rDNA from tomato, AG is Phytoplasma amplicon from *A. gossypii*, AL, and Av2, -Phytoplasma amplicon from infected Bitter gourd, CLH from cicadellid leafhopper (*Hishimonus* sp.), PH, PI, and PV−Phytoplasma from infected sponge gourd, LC2- from cicadellid leafhopper *Hishimonus* sp. from the screen house, Rn- from *R. speculum*, SBA from string bean aphid and S1 from infected string bean. The others are controls from the genebank

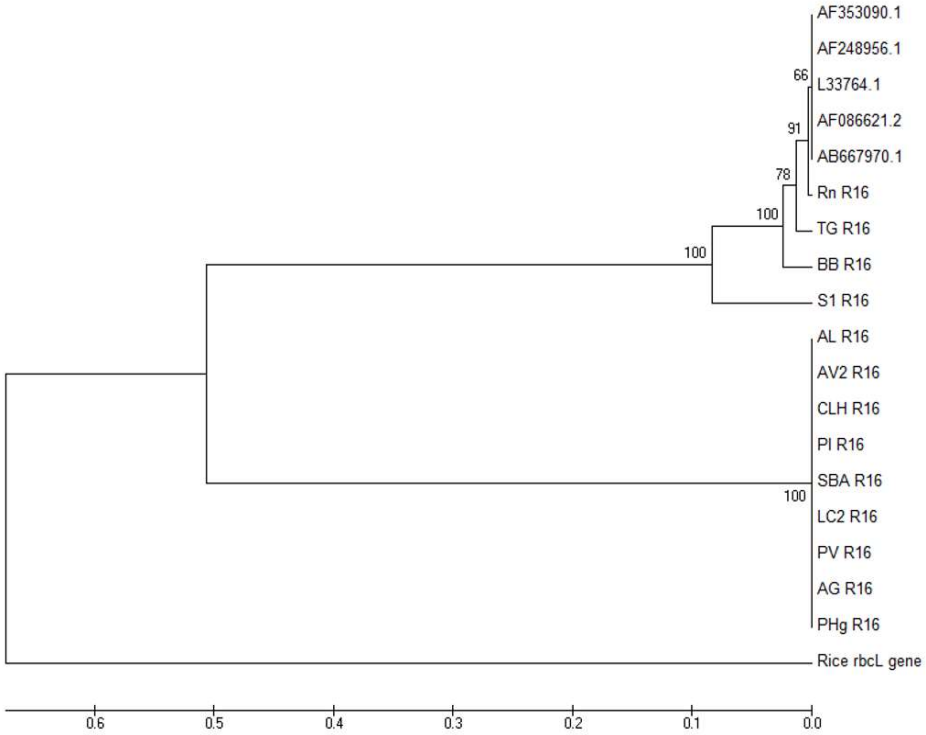

Figure 11. Phylogenetic dendrogram of nineteen phytoplasma nucleotide sequences from host plants (tomato-TG, Baguio bean-BB, bitter gourd -AG, AL, and AV2, loofah -PHg, PI, PV), insect vectors (Rn, CLH, LC2 and SBA) and the rest are controls

CONCLUSION, IMPLICATIONS AND RECOMMENDATIONS

Phytoplasma was positively transmitted to healthy bitter gourd plants by a cicadellied leafhopper, *Hishimonus sp*. and brown planthopper *R. speculum*. The *Hishimonus sp.* produced the typical little leaf/witches broom symptoms, while *R. speculum* transmitted the yellows-type phytoplasma disease symptoms. Phytoplasma was positively detected in these insects through PCR and nest-PCR assay. These results confirmed that these insects are vectors of two different strains of phytoplasma to bitter gourd. Although the green aphid, *A. gossypii* transmission produced the little leaf and chlorosis symptoms, and the pathogen was positively detected in the insect, whether or not the green aphid vectored phytoplasma to the test plants needs further study since the symptoms produced were quite different from that of the symptoms produced by the cicadellid leafhopper (*Hishimonus* sp.) and only one literature was encountered which reported that aphids are able to transmit phytoplasma. The same is true for *A. craccivora,* further study is needed to prove whether it transmits the little leaf phytoplasma to string bean. Further study also needs to be conducted on the possibility of phytoplasma transmission by *Empoasca* sp. to string bean, bitter gourd and loofah, or whether it transmits a viral pathogen instead.

ACKNOWLEDGMENT

The authors wish to acknowledge and thank the Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development (PCAARRD) for the research funds. Dr. Gordon Rogers of Applied Horticultural Research in Sydney, Australia, Dr. Patricio S. Faylon, who helped in the materialization of this research, and the late Dr. Jocelyn Eusebio, former director of the Crop Research Division of PCAARRD, and Ms. Leilani Pelegrina for the various support they rendered to the project. We want to acknowledge also Dr. Marjohn Niño's help in interpreting the DNA sequence and phylogenetic data.

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