Annals of Tropical Research 42 (2):1-20(2020)
© VSU, Leyte, Philippines

Detection and molecular characterization of phytoplasma affecting vegetables in Eastern Visayas, Philippines

Lucia M. Borines[1*], Joy Adeline C. Nuñez[1], Nickie D. Duero[1], Rezel S. Borines[2] and Reny G. Gerona[1]

Submitted: 13 June 2019 | Accepted: 9 June 2020

ABSTRACT

Phytoplasma-like diseases were observed affecting bitter gourd, Loofah, string bean, "Baguio" bean, cucumber, and tomato in Eastern Visayas, Philippines. The infected vegetables commonly show little leaf/witches' broom symptoms. The study aimed to detect and confirm phytoplasma's presence in these vegetables through PCR and nest PCR assays using universal primers, electron microscopy, and 16srDNA sequence analysis. Loofah little leaf had the highest prevalence (50% of the surveyed farms), followed by bitter gourd (45%) and string beans (31%). The disease had an approximate mean incidence of 27% for bitter gourd, 38.0% for Loofah, and 42.5% for string bean, in farms where plants showed infections. Electron micrographs of bitter gourd and Loofah samples showed phytoplasma cells in the phloem sieve tubes. Nest PCR assays using R16F2n/R16R2 primer linked to phytoplasma 16srDNA amplified a ~1.25Kb band in the majority of DNA samples. rDNA sequence analysis using Blastn showed that phytoplasmas in bitter gourd, Loofah, and one cucumber samples shared 98-99% identity with Loofah's reference gene phytoplasma clones. More than one phytoplasma strain infected the vegetables based on Rsa1 enzyme digestion and phylogenetic analysis.

Keywords: witches broom, PCR, bitter gourd rDNA sequence

INTRODUCTION

Phytoplasma is an ultramicroscopic, wall-less specialized bacterium belonging to the Mollicutes that resides in the plants' phloem. Little leaf, witches' broom phyllody, and virescence are some of the visible symptoms caused by this pathogen in plants (Bertaccini 2007). Phytoplasma diseases pose a severe threat to

[1]Department of Pest Management, Visayas State University, Baybay City-6521, Leyte, Philippines
[2]Caraga State University, Ampayon, Butuan City, Agusan del Norte

***Corresponding Author.** Address: Department of Pest Management, Visayas State University, Baybay City-6521, Leyte, Philippines; Email: lucyborines@vsu.edu.ph
DOI: 10.32945/atr4221.2020

agriculture (Bertaccini et al 2014). The reduction in the size of leaves and fruits of affected plants eventually leads to reduced yield due to stunted growth and production of smaller fruits.

Unlike fungal and bacterial diseases, phytoplasma could not be detected using common clinical examination protocols in the laboratory. Phytoplasma diseases were once thought to be caused by viruses (Bertaccini & Duduk 2009) because the type of symptoms exhibited by infected plants resemble those of virus diseases. Molecular detection of the pathogen is cheaper than electron microscopy and is commonly performed through PCR and nested PCR using universal phytoplasma primers (Kummert & Rufflard 1997, Gundersen & Lee 1996). Differentiation of strains at the molecular level can also be achieved using RFLP and rDNA sequence analysis (Bertaccini 2007, Schneider et al 1997, Montano et al 2007, Montano et al 2000, Khan et al 2003, Lee et al 1998).

In Mexico, Lebsky et al (2007) detected phytoplasma from the yellows-type disease in tomato through PCR analysis. In Brazil, Montano et al (2000; 2001; 2006; 2007) detected phytoplasma from the cucurbit *Luffa cylindrical, Cucurbita moschata,* diseased chayote, and hibiscus, respectively showing witches' broom symptom. Singh and Singh (2000) detected phytoplasma from chili little leaf in India. Almomani and Almuaikel (2014) detected phytoplasma from tomato, pepper, and squash in Jordan. AL-Saleh & Amer (2014) characterized the phytoplasma associated with fava bean in Saudi Arabia. Samuel et al (2014) detected phytoplasma from pea, watermelon, tomato, amaranthus, and bottle gourd. It was also detected in witches' broom of bitter gourd in Vietnam by Nang et al (2014). Habili et al (2014) detected phytoplasma from a bitter gourd using generic primers, as well as in Loofah, bamboo, and coconut specimens from the Philippines.

Phytoplasma disease symptoms were first seen as early as 2012 in bitter gourd showing little leaf and little fruits in few vegetable farms in Leyte Philippines but formal study on it started in 2015. These little leaf symptoms were observed later in Loofah, stringbean, "Baguio" bean cucumber, and tomato. Knowledge of phytoplasma diseases is insufficient, particularly the ones affecting vegetables, hence the need to detect the presence of this emerging disease. The causal organism needs to be characterized so that specific programs for its prevention and control can be formulated. This study was conducted to detect and characterize phytoplasma diseases affecting vegetables in Leyte using PCR and nest-PCR assays and rDNA gene sequencing.

MATERIALS AND METHODS

Survey and Collection of Disease Specimens

Vegetable areas in Eastern Visayas were surveyed for phytoplasma disease symptoms. A total of 16 municipalities and 5 cities in Eastern Visayas were surveyed, but the disease was observed mostly in Leyte. Bitter gourd and Loofah were not commonly planted in Samar during the survey. The disease prevalence (Percentage of farms where the disease has been recorded over the total number of surveyed farms) and the mean disease incidence (% infection) per farm was determined. Diseased specimens were brought to the Plant Disease Diagnostic Laboratory of Visayas State University for DNA extraction and phytoplasma detection.

Total Genomic DNA Extraction

Leaf and stem tissues of vegetables that showed little leaf/witches' broom symptoms were subjected to total DNA extraction. An optimized extraction protocol for phytoplasma used by Ahrens and Seemüller (1992) was slightly modified. In this method, 0.5g of shoots from each vegetable sample was 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 10min. The tissue was then ground with a pestle and the homogenate was centrifuged at 5000rpm for 5min. The supernatant was transferred into a clean 2mL tube and further centrifuged at 13000rpm for 30min. The pellet was dissolved in 750µL warm 2% CTAB (20g L^{-1} CTAB, 100mM Tris-HCl pH8.0, 1.4M NaCl, 2% PVP-10, 20mM EDTA pH8.0) and incubated at 60°C for 30min. Each sample was purified with 900µL chloroform-isoamyl alcohol (24:1) and centrifuged at 12000rpm for 5min. Nucleic acids were precipitated with 600µL isopropanol before incubating at –20°C overnight. Samples were centrifuged at 12000rpm for 30min and washed with 70% ethanol. The pellets were air-dried and re-suspended in 100µL TE (10mM Tris-HCl, 1mM EDTA, pH8.0) buffer. Two further extractions, first with 100µL phenol: chloroform: isoamyl alcohol (25:24:1), and then 100µL chloroform followed. The upper aqueous layer was removed, and 300µL of absolute cold ethanol was added to precipitate the nucleic acids. DNAs were centrifuged at 13000rpm for 30min, washed with cold 300µL 70% ethanol, air-dried, and re-suspended in 10-40µL TE buffer. Two µL RNAse were added to each tube and incubated at 37°C for 30min. Nucleic acids were then stored at –20°C.

PCR and Nest PCR Assays

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 & 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 50ng µL^{-1} template DNA. PCR conditions were as follows: initial denaturation at 94° for 2min; 30 cycles of denaturation at 92° for 1min; annealing at 55° for 15s and extension 72° for 30s; and a final extension at 72° for 10min. The PCR products were subjected to agarose gel electrophoresis using 1% agarose, stained with GelRed (Biotium). The bands were viewed under UV transilluminator attached to an Alpha Digi-Doc Documentation System.

Electron Microscopy of Disease Specimens

Stem and leaf samples of the bitter gourd and Loofah showing witches' broom symptoms were submitted for transmission electron microscopy at the Research Institute for Tropical Medicine (RITM) Muntinlupa City, Philippines to confirm the presence of phytoplasma in the phloem cells of the tissues.

16SrDNA Sequence Analysis

Nest PCR products amplified by $R_{16}F_2n/R_{16}R_2$ primers of selected samples showing positive phytoplasma bands were sent for rRNA sequence analysis at the Philippine Genome Program, University of the Philippines, Diliman, Quezon City. Sequence alignment was performed using Blastn, and phylogenetic analysis of the different isolates was conducted using the Mega6 software (Tamura 2013). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980), and evolutionary relationships were inferred using the UPGMA method (Sneath & Sokal 1973).

RESULTS AND DISCUSSION

Symptoms of Phytoplasma Diseases in Affected Vegetables Prevalence and Incidence

Enumerated in Table 1 are the municipalities and cities in Eastern Visayas where phytoplasma disease symptoms were observed, together with the disease prevalence and incidence. Loofah phytoplasma had the highest prevalence (50% of the surveyed farms), followed by bitter gourd (45%) and string beans (31%). Stringbeans phytoplasma had the highest mean incidence per farm (42.5%), followed by Loofah (38%) and ampalaya. Cucumber phyllody and Baguio bean little leaf was found in one farm each, with 30% and 10% incidence, respectively. Tomato stunt was observed and taken from a single plant in a farm. PCR analysis were conducted in disease samples from the municipalities like Baybay, Inopacan, Hindang, Hilongos in Leyte and as far as Maasin City in Southern Leyte where phytoplasma disease was more common. The affected vegetables commonly exhibited little leaf and witches' broom symptoms and little fruit when able to bear.

The infected bitter gourd produced tiny leaves, with or without yellowing, small elongated fruits (shoestring symptom), and sometimes stem pitting (Figures 1a-1b). Little leaf was also the symptom of phytoplasma infection in Loofah (Figure 1c), string bean (Figure 1d), and "Baguio" bean (1e). In cucumber, some leaves turn tiny, but phyllody symptoms can be observed (Figure 1f). Tomato infected with phytoplasma becomes stunted with little leaves (Figure 1g).

Electron Microscopy of Infected Bitter gourd and Loofah

Electron microscopy results from the Research Institute of Tropical Medicine showed few phytoplasmas (red arrows) in the phloem sieve tubes of infected bitter gourd (Figure 2a) and Loofah samples confirming the presence of the pathogen (Figure 2b).

Table 1. Municipalities and cities in Eastern Visayas where phytoplasma diseases were found, the disease prevalence and mean disease incidence

Province/Municipality Surveyed For Vegetable Phytoplasma Disease	Municipalities Where Vegetable Phytoplasma Was Recorded	Disease Prevalence** (%) (%)	Mean Disease Incidence (%)
Leyte: Province	*Bittergourd Little Leaf*		
Abuyog, Alang-alang, Albuera, Baybay City, Capoocan, Inopacan, Hindang, Hilongos, Ormoc City, Palo, San Isidro, and Tacloban City	Baybay City, Inopacan, Hindang, Ormoc City and Abuyog in Leyte; Maasin City in Southern Leyte and Lavesares, Northern Samar	45	27
Southern Leyte: Province	*Loofah Little Leaf:*		
Anahawan, Bontoc, Maasin City, Malitbog, and Silago	Baybay City, Inopacan, and Hindang in Leyte and Maasin City, Malitbog, Anahawan and Silago in Southern Leyte	50	38
Samar: Province	*Stringbean Little Leaf:*		
Calbayog City and San Jorge	Hindang and Hilongos in Leyte and Calbayog City Samar	31	42.5
Northern Samar: Province	*Cucumber Phylllody:*		
Lavesares	Baybay City, Leyte	*	10.0
Biliran: Province	*Baguio Bean Little Leaf:*		30.0
Caibiran	Hilongos, Leyte	*	-
	Tomato Stunt:		
	Baybay City, Leyte	-	-

- Only one plant in one farm was documented
[*] Only one farm was observed showing the symptom
[**] Refers to percentage farms where the disease was observed over the total number of surveyed farms in Leyte

Molecular Detection and Characterization of the Pathogen

Initial PCR amplification results using the universal phytoplasma-specific primer P1/P7 by Deng and Hiruki (1991) on five infected bitter gourd and four Loofah DNA samples produced only faint bands of ~ 1.5Kb for loofah samples from VSU (PV), Inopacan (PI), and Hindang in Leyte (PHg). All bitter gourd samples and the Loofah sample from Maasin showed no bands (Figure 3). Nest PCR assay of the same samples using $R_{16}F_2n/R_{16}R_2$ primers, however, produced positive amplification (Figure 4). Bitter gourd samples collected from fields in VSU (AV2) and Guadalupe (AG), and from the screen house of the VSU Department of Pest Management (AS1 and AS2) as well as Loofah samples from VSU (PV), Inopacan (PI), and Hindang (Phg) showed the ~1.25Kb amplicon which is the expected phytoplasma-specific band based on the report of Lee et al (1993, 1995).

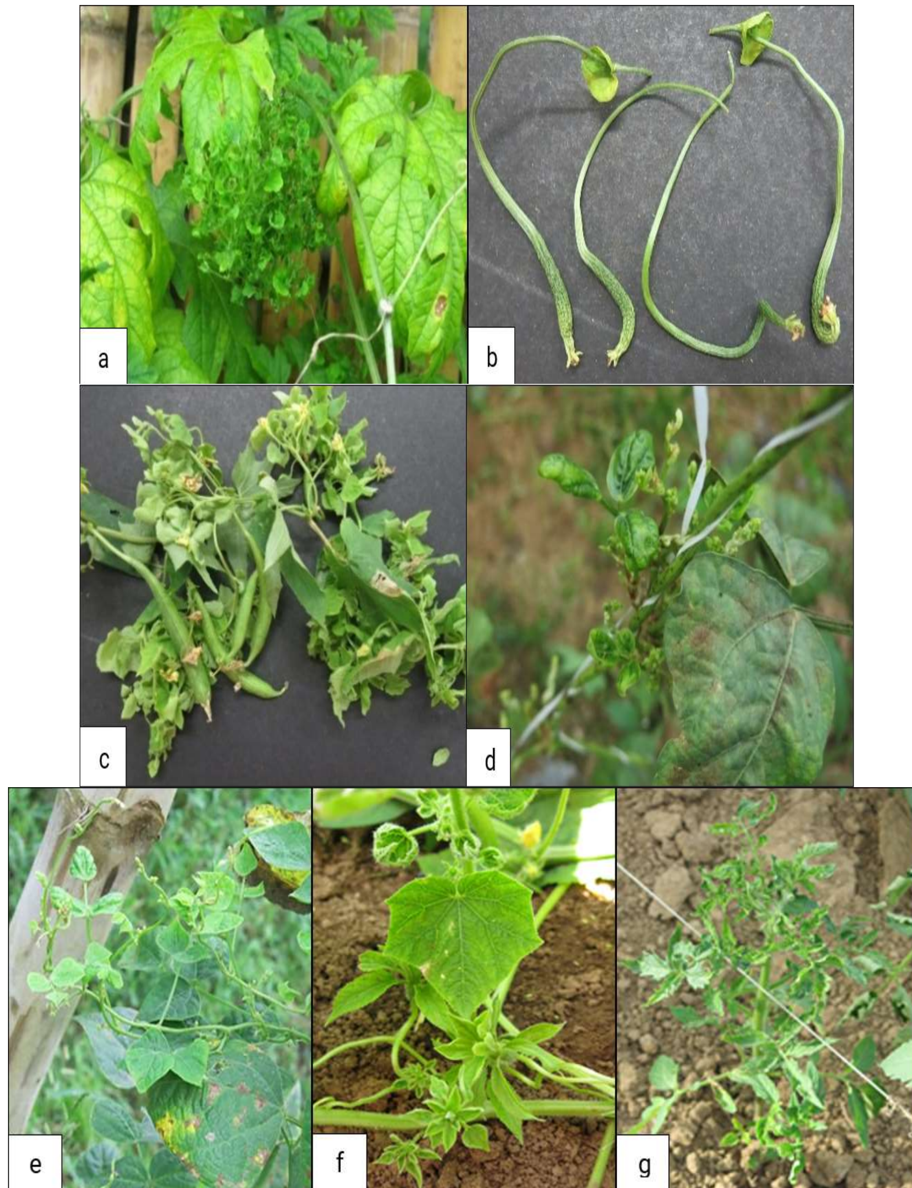

Figure 1. Phytoplasma symptoms on (a), bitter gourd leaves, b) bitter gourd fruit (c), Loofah leaf and fruit (d), string bean, "Baguio" bean (e), cucumber (f) and tomato (g)

Detection and molecular characterization of phytoplasma

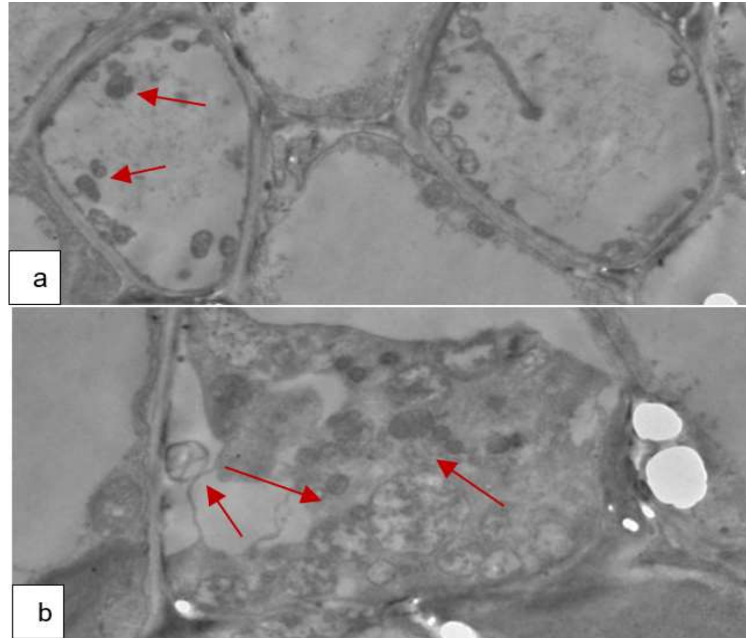

Figure 2. Electron micrographs of bitter gourd (a) and Loofah (b) affected with leaf disease showing pleomorphic phytoplasmas (arrows) in phloem cells

Interestingly, another field-collected bitter gourd DNA sample from VSU (AV1) produced a much shorter amplicon size (~500bp), which is still within the reported band sizes of phytoplasmas affecting different crops by Duleep et al (2014). Similarly, Montano et al (2007) also reported different phytoplasma band sizes of ~0.5Kb, 0.8Kb, and ~1.2Kb. This initially suggests that at least two different phytoplasma strains affecting bitter gourd exist in the Eastern Visayas region. The Loofah from Maasin City (PM) did not produce the expected band.

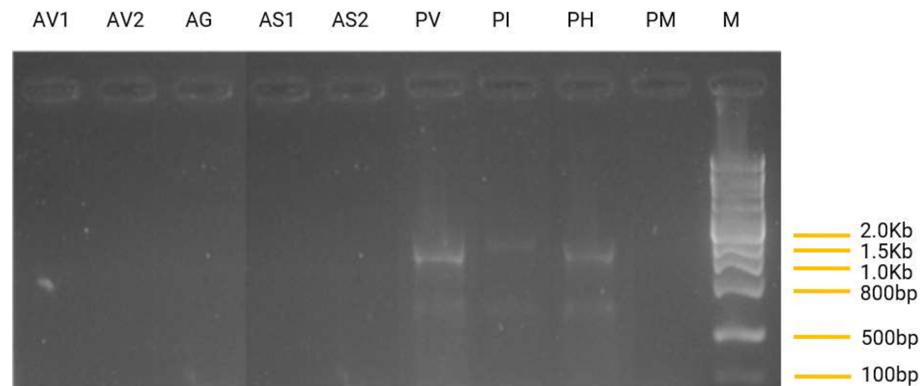

Figure 3. Initial phytoplasma amplification result using P1/P2 primer showing faint bands for a few samples. (AV1, AV2, AG, AS1, & AS2 are bitter gourd leaf samples) and PV, PI, PHg and PM were infected Loofah leaf samples. M (DNA ladder Vivantis)

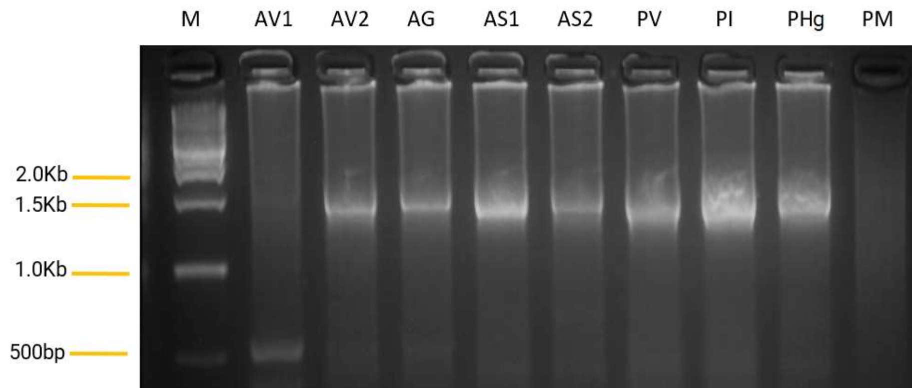

Figure 4. Nest PCR amplicons using $R_{16}F_2n/R_{16}R_2$ primers of bitter gourd (AV1, AV2, AG, AS2) and Loofah (PV,PI, PHg, PM) little leaf samples. M (1Kb DNA ladder Vivantis)

The nest PCR assay was repeated for samples AV1, AV2, and AG with another bitter gourd sample AL (Figure 5). The expected ~1.25Kb band was again amplified for samples AV2, AG, and AL, and the ~500bp band was produced from sample AV1, confirming that it was different from the other bitter gourd samples. Another nest PCR assay was also conducted on Loofah DNAs, PV, PI, PH, and PM, together with string bean, "Baguio" bean, tomato, and cucumber showing phytoplasma symptoms, as well as an associated insect collected from the field, ie, *Ricania speculum* nymphs (Figure 6). The DNA isolates from the infected Loofah samples from VSU, Inopacan, and Hindang Leyte (PV, PI & PHg), showed that the ~1.25Kb band was positively amplified. However, a shorter band (<1.0Kb) was amplified from the Loofah isolate from Maasin City (PM). This suggests that there is also more than one strain of phytoplasma affecting Loofah. No band was amplified from the infected string bean DNA (SB), but the ~1.25Kb band was amplified from "Baguio" bean (BB) and cucumber from VSU (CV). A faint, shorter DNA fragment (~300bp) was amplified from tomato showing little leaf and stunting symptoms (TG).

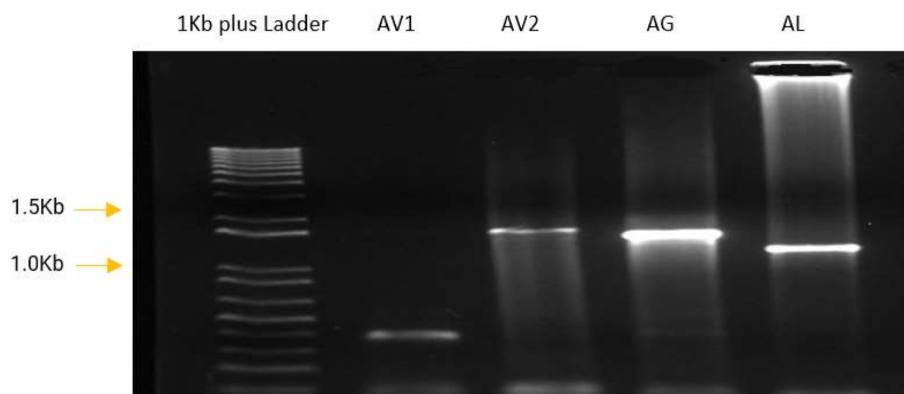

Figure 5. Nest PCR amplicons using $R_{16}F_2n/R_{16}R_2$ primers from bitter gourd little leaf from different places in Eastern Visayas (AV1, AV2, AG & AL) amplified using phytoplasma-specific primers $R_{16}F_2n/R_{16}R_2$

Detection and molecular characterization of phytoplasma

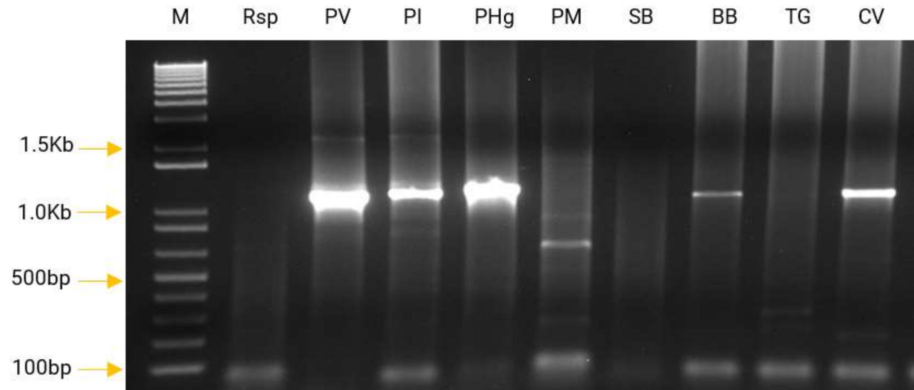

Figure 6. Nest PCR amplification results from collected suspected insect vector *Ricania speculum* (Rsp), field-collected infected Loofah (PV, PI, PHg & PM), string bean (SB), "Baguio" Bean ((BB), Tomato (TG), and cucumber (CV) using R16F2n/R16R2 Phytoplasma-specific primers. M=(100bp+ DNA ladder)

When selected DNA amplicons of some samples using $R16F_2n/R16R_2$ primer were subjected to restriction enzyme digestion using Rsa1 enzyme, two-band patterns were observed among the bitter gourd samples and three with Loofah samples (Figure 7). This further confirmed the existence of more than one strain of phytoplasma affecting bitter gourd and Loofah.

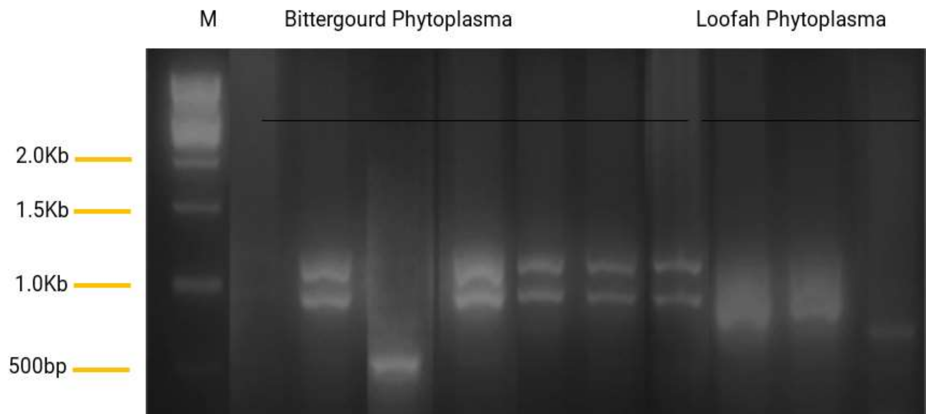

Figure 7. Restriction digests of R16F2N/R2 amplicons using Rsa1 enzyme showing two different band patterns for infected bitter gourd DNAs and three-band patterns for infected Loofah DNAs. M (1Kb DNA ladder Vivantis)

rDNA Sequence Analysis

Results of rDNA sequence analysis of the isolates is shown in (Tables 2, 3, 4 & 5). Only three bitter gourds with ~1.239bp amplicons had sequence results, ie, AG, AL and AV2. All three isolates were confirmed to be phytoplasma DNAs. Isolate AG

showed 98-99% homology to Loofah witches' broom phytoplasma rRNA and Trna-ile (AF353090.1, AF086621.2, AF248956.1, AB667970.1 & Y17055.1) and 97% similar to Stylosanthes little leaf phytoplasma (AJ289192.2), Malaysian periwinkle virescence and yellow dwarf phytoplasma (EU371934.2 & EU498727.1), oil palm phytoplasma (EU498728.1), and *Candidatus Phytoplasma trifolii* Tomato-Zac (KX092011.1) (Table 2).

Bitter gourd isolate AL is 98% homologous to most Loofah phytoplasma rRNA and tRNA-ile gene accessions (AF353090.1, AF248956.1, AB667970.1 & AF086621.2) and 96% homologous to stylosanthes little leaf phytoplasma (AJ289192.2), oil palm phytoplasma (EU498728.1), Malaysian yellow dwarf coconut and periwinkle virescence phytoplasma (EU498727.1 & EU371934.2), and *Candidatus Phytoplasma trifolii* of tomato (KY321932.1).

Bitter gourd isolate AV2, on the other hand, is only 94-96% similar to Loofah witches' broom phytoplasma rRNA and tRNA-ile genes (AF353090.1, AF086621.2, AF248956.1 & AB667970.1) and 94% similar to stylosanthes little leaf phytoplasma (AJ289192.2), oil palm phytoplasma (EU498728.1), Malaysian yellow dwarf, and periwinkle virescence phytoplasma (EU371934.2) and 93% similar to *Cucumis sativus* phyllody phytoplasma (KR633068.1).

The phytoplasma from baguio bean (*Phaseolus vulgaris*) showed 94-95% similarity to Loofah phytoplasma genes, stylosanthes little leaf phytoplasma, Malaysian periwinkle virescence, and yellow dwarf coconut phytoplasma, oil palm phytoplasma, and *Candidatus phytoplasma ulmi* (GU125723.1). Although no sequence result was produced from the string bean phytoplasma, it could be similar to the Baguio bean phytoplasma.

Three Loofah phytoplasma isolates were sequenced, namely PHg, PI, and PV. All three were 99% similar to Loofah witches' broom phytoplasma genes, 97% similar to stylosanthes little leaf phytoplasma, oil palm phytoplasma, Malaysian yellow dwarf coconut and periwinkle virescence phytoplasmas, and *Candidatus Phytoplasma trifolii* (Table 3). The tomato phytoplasma, on the other hand, (TG) is 96% similar to the same set of reference genes of phytoplasmas (Table 4).

The cucumber phytoplasma (CV) is 98% similar to most Loofah phytoplasma gene accessions and 96% similar to Stylosanthes little leaf phytoplasma, oil palm phytoplasma, Malaysian yellow dwarf coconut and Malaysian periwinkle virescence phytoplasmas, and *Candidatus Phytoplasma trifolii* (Table 3).

Multiple Sequence Alignment of 16srDNA sequences of eight phytoplasma samples (TG, BB, AL, AV2, PI, PV, AG, & PHg) together with five phytoplasma taxa controls (AF353090.1, AF248956.1, L33764.1, AF086621.2, AB667970.1) and outgroup (rice rbcl) is shown in Figure 8. Alignment annotation from 180 to 270 nucleotide bases showed strong evidence of common haplotype pattern among the phytoplasma isolates, which is signified by high consensus value (90%) indicated by the red-colored nucleotides. The blue-colored nucleotides indicate a low consensus value of 50%, while the black-colored nucleotides have neutral consensus. The figure showed that the tomato phytoplasma (TG) is the most homologous to the consensus sequence, while the sample AV2 is most divergent.

The phylogenetic dendrogram of fourteen nucleotide sequences, including eight phytoplasma isolates and 6 reference genes from the Genbank, was analyzed (Figure 9). The evolutionary relationship of taxa was inferred using the UPGMA method (Sneath & Sokal 1973). The optimal tree with the sum of branch length=2.70641489 is shown.

Table 2. Percent (%) nucleotide sequence similarities of the phytoplasma isolates from bitter gourd (AG, AL & AV2) with the top 10 homologous hits by BLASTn analysis

Phytoplasma Isolates Description	Genbank Accession No.	Host	Country	% Nucleotide sequence identity		
				AG	AL	AV2
Candidatus Phytoplasma luffae	AF353090.1	Loofah	Taiwan	99%	98%	96%
Candidatus Phytoplasma luffae	AF248956.1	Loofah	Taiwan	99%	98%	96%
Candidatus Phytoplasma luffae	AB667970.1	Loofah	Taiwan	99%	98%	96%
Candidatus Phytoplasma luffae	AF086621.2	Loofah	Taiwan	99%	98%	96%
Candidatus Phytoplasma sp.	Y17055.1	"not mentioned"	Australia	97%	96%	94%
Stylosanthes little leaf phytoplasma	AJ289192.2	*Stylosanthes*	Australia	97%	96%	94%
Malaysian periwinkle virescence phytoplasma	EU371934.2	*Catharanthus roseus*	Malaysia	97%	96%	94%
Malaysian yellow dwarf coconut phytoplasma	EU498727.1	coconut palm	Malaysia	97%	96%	94%
Oil palm phytoplasma	EU498728.1	oil palm	Malaysia	97%	96%	94%
Candidatus Phytoplasma trifolii	KX092011.1	tomato	Mexico	97%		
Candidatus Phytoplasma trifolii	KY321932.1	*Capsicum annuum*	Turkey		95%	
Cucumis sativus' phyllody phytoplasma Yazd	KR633068.1	Cucumber	Iran			93%

Table 3. Percent (%) nucleotide sequence similarities of the phytoplasma isolates from Loofah (PHg, PI & PV) with the top 10 homologous hits by BLASTn analysis

Phytoplasma Isolates Description	Genbank Accession No.	Host	Country	% Nucleotide sequence identity		
				PHg	PI	PV
Candidatus Phytoplasma luffae	AF353090.1	Loofah	Taiwan	99%	99%	99%
Candidatus Phytoplasma luffae	AF248956.1	Loofah	Taiwan	99%	99%	99%
Candidatus Phytoplasma luffae	AB667970.1	Loofah	Taiwan	99%	99%	99%
Candidatus Phytoplasma luffae	AF086621.2	Loofah	Taiwan	99%	99%	99%
Candidatus Phytoplasma sp.	Y17055.1	*	Australia	97%	98%	98%
Stylosanthes little leaf phytoplasma	AJ289192.2	*Stylosanthes*	Australia	97%	98%	98%
Malaysian periwinkle virescence phytoplasma	EU371934.2	*Catharanthus roseus*	Malaysia	97%	97%	97%
Malaysian yellow dwarf coconut phytoplasma	EU498727.1	coconut palm	Malaysia	97%	97%	97%
Oil palm phytoplasma	EU498728.1	oil palm	Malaysia	97%	97%	97%
Candidatus Phytoplasma trifolii	KY321932.1	*Capsicum annuum*	Turkey	97%	97%	97%

*not mentioned

Table 4. Percent (%) nucleotide sequence similarities of the phytoplasma isolates from baguio bean (BB) and cucumber (CV) with the top 10 homologous hits by BLASTn analysis

Phytoplasma Isolates Description	Genbank Accession No.	Host	Country	% Nucleotide sequence identity	
				BB	CV
Candidatus Phytoplasma luffae	AF353090.1	Loofah	Taiwan	95%	98%
Candidatus Phytoplasma luffae	AF248956.1	Loofah	Taiwan	95%	98%
Candidatus Phytoplasma luffae	AB667970.1	Loofah	Taiwan	95%	98%
Candidatus Phytoplasma luffae	AF086621.2	Loofah	Taiwan	95%	98%
Candidatus Phytoplasma sp.	Y17055.1	*	Australia	95%	96%
Stylosanthes little leaf phytoplasma	AJ289192.2	*Stylosanthes*	Australia	95%	96%
Malaysian periwinkle virescence phytoplasma	EU371934.2	*Catharanthus roseus*	Malaysia	95%	96%
Malaysian yellow dwarf coconut phytoplasma	EU498727.1	coconut palm	Malaysia	95%	96%
Oil palm phytoplasma	EU498728.1	oil palm	Malaysia	95%	96%
Candidatus Phytoplasma ulmi	GU125723.1	*Rubus idaeus*	Poland	95%	
Candidatus Phytoplasma trifolii	KY321932.1	*Capsicum annuum*	Turkey	97%	96%

*not mentioned

Table 5. Percent (%) nucleotide sequence similarities of the phytoplasma isolate from tomato (TG) with the top 10 homologous hits by BLASTn analysis

Phytoplasma Isolates Description	Genbank Accession No.	Host	Country	% Nucleotide sequence identity
Candidatus Phytoplasma luffae	AF353090.1	Loofah	Taiwan	96%
Candidatus Phytoplasma luffae	AF248956.1	Loofah	Taiwan	96%
Candidatus Phytoplasma luffae	AB667970.1	Loofah	Taiwan	96%
Candidatus Phytoplasma luffae	AF086621.2	Loofah	Taiwan	96%
Candidatus Phytoplasma luffae	L33764.1	*Catharanthus roseus*	*	96%
Cape St. Paul wilt phytoplasma	KF419286.1	Coconut	Cote d'Ivoire	96%
Cape St. Paul wilt phytoplasma	KF387570.1	Coconut	Cote d'Ivoire	96%
Cape St. Paul wilt phytoplasma	KF364359.1	Coconut	Cote d'Ivoire	96%
Cape St. Paul wilt phytoplasma	JQ868442.1	Coconut	Ghana	96%
Cape St. Paul wilt phytoplasma	Y13912.1	Coconut	*	96%

*not mentioned

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 187 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al 2013).

The phylogenetic tree showed that bitter gourd phytoplasma samples AL, AV2, AG, and sponge gourd samples PI, PV, and PHG clustered together and were found to be very related (divergence value=0) and are quite distant from "Baguio" bean (BB) and Tomato phytoplasmas. Tomato stunt phytoplasma (TG) clustered together with Baguio bean phytoplasma and most of the reference phytoplasmas. Results of the partial sequence analysis of 16SrDNA confirmed that phytoplasma caused the little leaf/witches' broom symptoms in bitter gourd, Loofah, Baguio bean, and tomato. Sequence analysis also revealed slight differences in their rRNA sequence confirming the existence of different phytoplasma strains infecting these vegetables in Eastern Visayas.

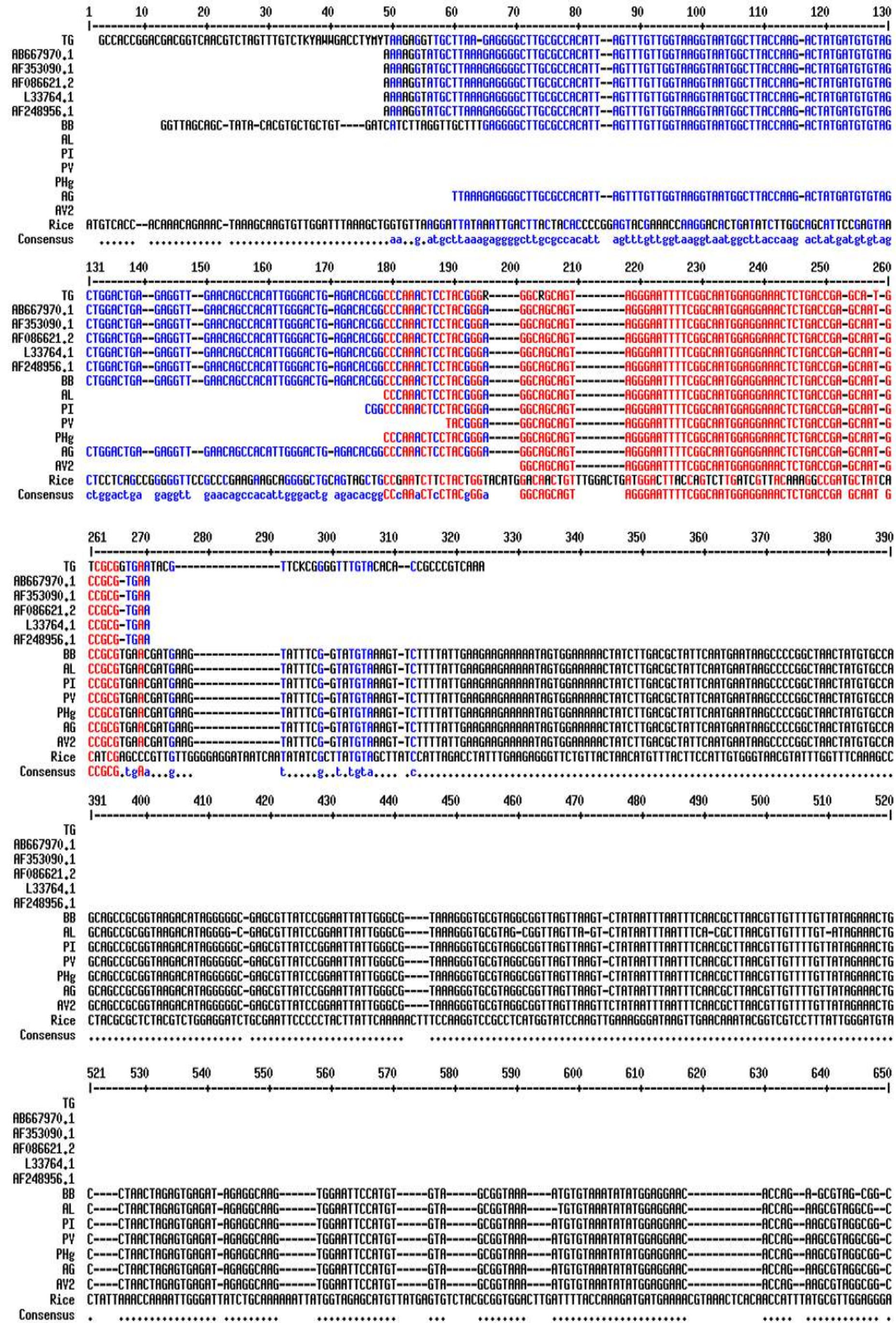

Figure 8. Multiple Sequence Alignment of eight 16srDNA sequences of eight phytoplasma samples (TG, BB, AL, AV2, PI, PV, AG, PHg) together with five phytoplasma taxa controls (AF353090.1, AF248956.1, L33764.1, AF086621.2, AB667970.1) and outgroup (rice rbcl)

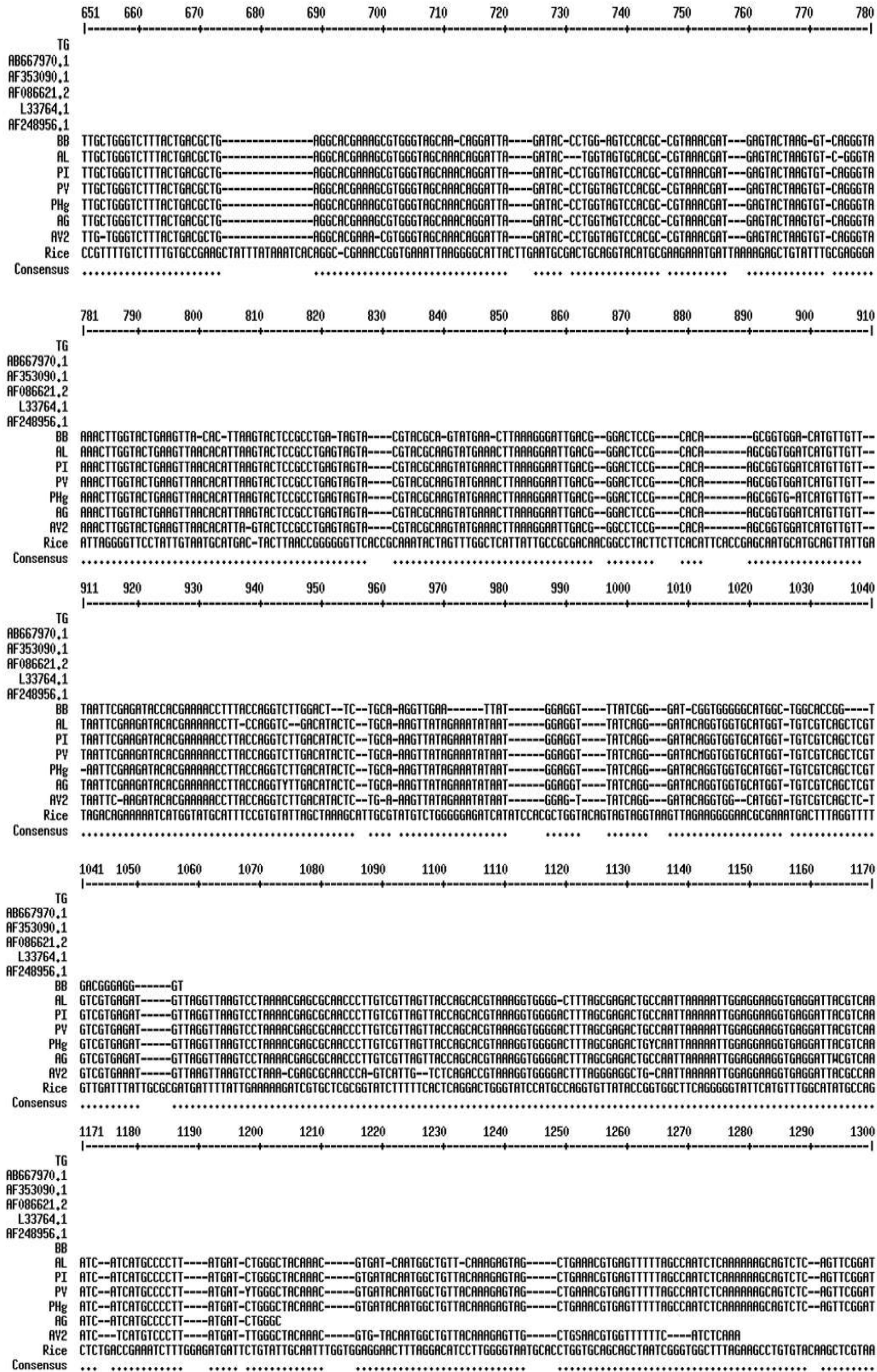

Figure 8 continued

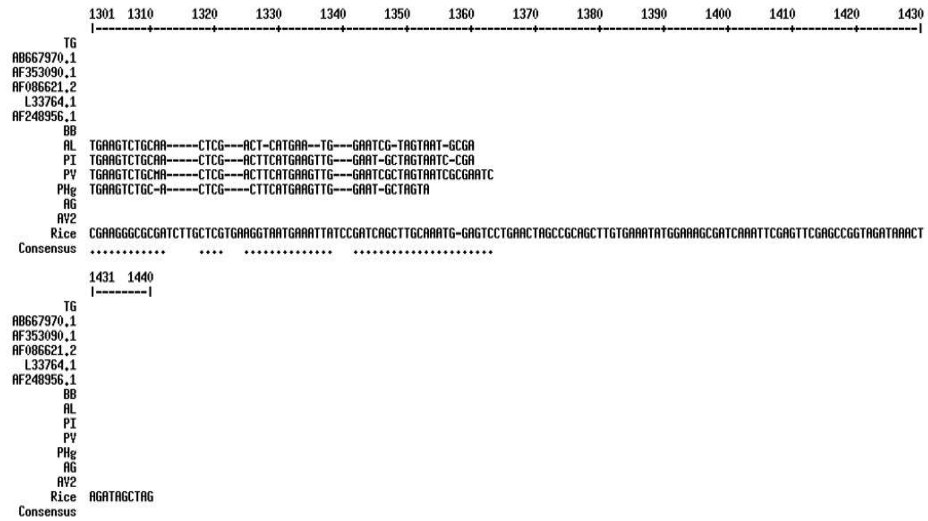

Figure 8 continued

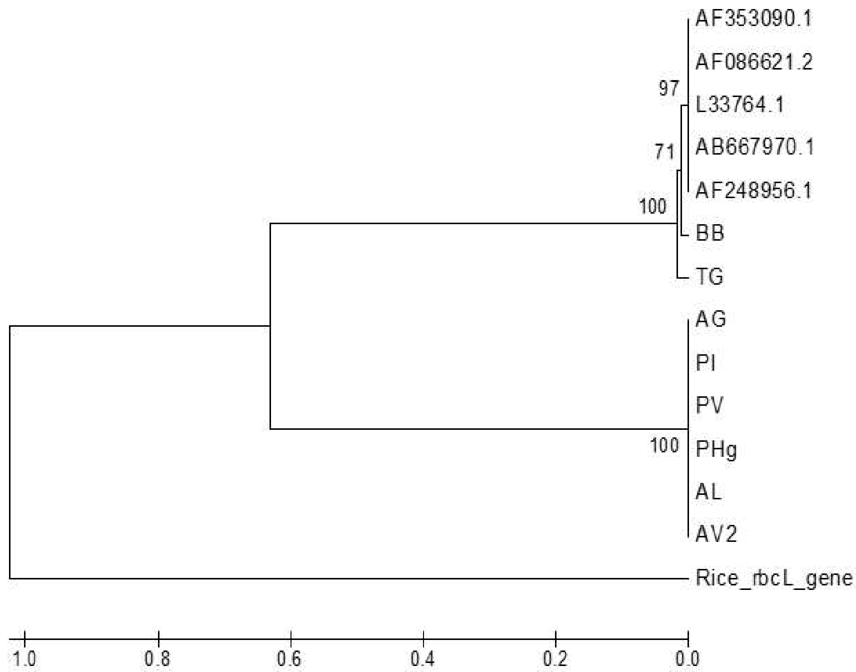

Figure 9. Phylogenetic dendrogram of fourteen phytoplasma nucleotide sequences, (three from bitter gourd (AG, Al, & AV2), three from Loofah, one "Baguio" bean, and one tomato, with 6 reference genes (Genebank accessions: AF353090.1, AF086621.2, L33764.1, AB667970.1, and AF248956.1) analyzed using UPGMA method

CONCLUSION, IMPLICATION, AND RECOMMENDATION

The study confirms that bitter gourd, Loofah, string bean, “Baguio” bean, cucumber, and tomato showing little leaf, witches' broom, and phyllody symptoms in Eastern Visayas Philippines are caused by phytoplasma. More than one strain of phytoplasma is infecting these vegetables based on nest PCR assay, rsa1 enzyme digestion, and rDNA sequence analysis. The specific phytoplasma strains affecting these vegetables need to be confirmed in future studies. Further studies on their control are also deemed necessary. The disease is getting more common, thereby implying the need for immediate mitigation of the problem. Vegetable growers must be educated on the disease's occurrence and must be familiarized with the symptoms and dissemination methods.

ACKNOWLEDGMENT

The authors wish to acknowledge the Philippine Council for Agriculture Aquatic and Natural Resources Research and Development (PCAARRD) through the former and current executive directors Dr. Patricio S. Faylon and Dr. Reynaldo V. Ebora respectively, and the Australian Centre for International Agricultural Research (ACIAR) for the research funds through Dr. Sandra McDougall and Dr. Gordon Rogers. Special thanks are also given to the late Dr. Jocelyn E. Eusebio, Ms. Leilani D. Pelegrina, and other staff of the PCAARRD's Crop Research Division for all the support given to the project. Special thanks are also given to Dr. Len Tesoriero of New South Wales Department of Trade and Industries, Sydney, Australia, for his contribution to this work.

REFERENCES

Ahrens U and Seemuller E. 1992. Detection of DNA of plant pathogenic mycoplasma like organisms by a polymerase chain reaction amplifies a sequence of the 16S rRNA gene. *Phytopathology* 82(8):828-832

Almomani F and Almuaikel N. 2014. Molecular detection of phytoplasma from different infected hosts in Jordan based on the PCR amplification of 16srDNA sequences. Proceedings of the 3rd International Conference on Medical, Biological, and Pharmaceutical Sciences (ICMBPS'2014) (pp115-117), March 19-20, 2014 Abu Dhabi, UAE

Al-Saleh MA and Amer MA. 2014. Molecular characterization of the 16sRII group of a phytoplasma associated with faba bean (Vicia faba) in Saudi Arabia. *The Journal of Animal & Plant Sciences* 24(1):221-228

Bertaccini A. 2007. Phytoplasmas: diversity, taxonomy, and epidemiology. *Frontieres in Bioscience: a journal and virtual library* 12:673-689

Bertaccini A, Duduk B, Paltrinieri S & Contaldo N. 2014. Phytoplasmas and Phytoplasma diseases: a severe threat to agriculture. *American Journal of Plant Sciences* 5(12):1763-1788

Bertaccini A and Duduk B. 2009. Phytoplasma and phytoplasma diseases: A review of recent research. *Phytopathologia Mediterranea* 48:355-378

Deng S and Hiruki C. 1991. Genetic relatedness between two noncultureable mycoplasma-like organisms reaveled by nucliec acid hybridization and polymerase chain reaction. *Phytopathology* 81(12):1475-1479

Duleep KS, Reddy MK, Jalali S & Reddy HC. 2014. Sensitive detection of phytoplasmal infection in vegetable cby *secA* gene. *Current Biotica* 8(2):165-173

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39(4):783-791

Gundersen DE and Lee IM. 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea* 35(3):144-151

Habili N, Wu Q, Sta. Cruz FC, Huan KH, Jormasiea J, Lanisat NB & Randles JW. 2014. ASEAN Regional Diagnostic Network: Results of a survey on the detection of viruses and virus-like pathogens in selected ASEAN countries. 11th Australasian Plant Virology Workshop. Plant virology in the 'omics' era Handbook

Khan AJ, Botti S, Al-subhi AM, Zaidi MA, Altosaar I, Alma A & Bertaccini A. 2003. Molecular characterization of the 16S rRNA gene of phytoplasmas detected in two leafhopper species associated with alfalfa plants infected with witches' broom in Oman. *Phytopathologia Mediterranea* 42(3):257-267

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16(2):111-120

Kummert J and Rufflard G. 1997. A Preliminary Report on the Detection of Phytoplasmas by PCR. *Biochemica* 1:19-22

Lebsky V, Gonzalez J, Astorga G, Conejo Y & Poghosyan A. 2007. Detection of phytoplasmas in mixed Infection with Begomoviruses: A case study of tomato and pepper in mexico. *Bulletin of Insectology* 64 (Supplement): S55-S56

Lee IM, Bertaccini A, Vibio M & Gundersen DE. 1995. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. *Phytopathology* 85(6):728-735

Lee IM, Gundersen RD, Davis RE & Bartoszyk IM. 1998. Revised classification scheme of phytoplasmas based on RFLP analysis of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology* 48(4):1153-1169

Montano HG, Davis RE, Dally EL, Pimentel JP & Brioso PST. 2000. Identification and phylogenetic analysis of a new phytoplasma from diseased chayote in Brazil. *Plant Disease* 84(4):429-436

Montano HG, Davis RE, Dally EL, Hogenhouts S, Pimentel JP & Brioso PST. 2001. '*Candidatus* Phytoplasma brasiliense', a new phytoplasma taxon associated with hibiscus witches' broom disease. *International Journal of Systematic and Evolutionary Microbiology* 51:1109-1118

Montano HG, Brioso PST, Pimentel JP, Figuerido DV & Cunha JO Jr. 2006. *Cucurbita moschata*, new phytoplasma host in Brazil. *Journal of Plant Pathology* 88:226-226

Montano HG, Brioso PST, Cunha JO Jr, Figuerido DV & Pimentel JP. 2007. First report of group 16SrIII phytoplasma in loofah (*Luffa cylindrica*). *Bulletin of Insectology* 60(2):277-278

Nang KKW, Young HK & Hee YJ. 2014. Bitter Gourd Little Leaf Disease-Associated to '*Candidatus* Phytoplasma asteris'. *Tropical Plant Pathology* 39(1):082-088

Saitou N and Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4(4):406-425

Samuel DK, Reddy MK, Jalali S & Reddy HC. 2014. Sensitive detection of phytoplasmal infection in vegetable crops by *secA* gene. *Current Biotica* 8(2):165-173

Schneider B, Gibb KS & Seemuller E. 1997. Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. *Microbiology* 143(10):3381-3389

Singh D and Singh SJ. 2000. Chilli little leaf - a new phytoplasma disease in India. *Indian Phytopathological Society* 53(3):309-310

Sneath PHA and Sokal RR. 1973. The estimation of taxonomic resemblance. In Kennedy D, Park RB (eds) *Numerical Taxonomy* (pp129-132). The principles and Practice of Numerical Classification. Freeman, San Francisco

Tamura K, Stecher G, Peterson D, Filipski A & Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30(12):2725-2729