Isolation and Identification of a Begomovirus Associated With the Leaf Curl Disease of Gumamela (*Hibiscus rosa-sinensis*) in the Philippines

Lolita M. Dolores, Maricel C. Gonzales and Pablito M. Magdalita

Crop Science Cluster-Institute of Plant Breeding (CSC-IPB), College of Agriculture
University of the Philippines Los Baños (UPLB)
Laguna, Philippines

Corresponding author's email: lpmdolores {at} gmail.com

ABSTRACT--- ***Hibiscus rosa-sinensis, locally known as 'gumamela' were observed with virus symptoms of mosaic, leaf curl, cupping, enation, mottling, and stunting. The virus was transmitted by whiteflies and by grafting to different host plant species including: Gossypium hirsutum, Vigna unguiculata and Hibiscus rosa sinensis but not to Abelmoschus esculentus, Datura metel, D. stramonium, Solanum melongena , Lycopersicon esculentum, Nicotiana glutinosa and Physalis floridana . The thirty four (34) gumamela samples showed positive to the PCR amplification with begomovirus degenerate primers yielding an expected band size of ~1.5kb. Multiple sequence alignment of the obtained DNA-A fragments showed 97.0-99.0% similarities among isolates. Further sequence analysis of Philippine hibiscus leaf curl isolate with other hibiscus leaf curl viruses registered in the Genbank showed 98% nucleotide similarities with the Cotton leaf curl Multan virus.***

Keywords--- begomovirus; Cotton leaf curl Multan virus; Hibiscus rosa-sinensis; gumamela leaf curl

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1. INTRODUCTION

Hibiscus rosa-sinensis or gumamela, is a species of flowering plant in the family *Malvaceae*, a native to the East Asia (1). It is a widely grown ornamental plant throughout the tropics and subtropics and is also popular owing for its many uses. It is grown mainly for landscaping due to the various colors of its flowers and for its medicine and food value. In the Philippines, years ago, gumamela was known and used as platform for laundry but with the breeding of new varieties with large and colourful flowers, the gumamela become competitive with the other flowers (2). Its flowers and leaves were also used by kids as bubble toys. It is now being used by landscape architects as landscape materials. These beautiful flowers can now be seen in adorning big subdivisions, schools, parks and tourist areas. In addition, the hibiscus became popular plants for heath and wellness. The flowers are being used ingredient for making shampoo, dye and lotion; for making health drink and fritters. However, like most any other plant, gumamela is not exempted from being infected by various diseases including viruses. These viruses are widespread and their effects can be very unlikely. Some of the viruses that have been previously described infecting *Hibiscus rosa-sinensis* include, *Hibiscus chlorotic ringspot virus* (HCRSV, genus *Carmovirus*), *Hibiscus latent ringspot virus* (HLRSV, genus *Nepovirus*), *Hibiscus yellow mosaic virus* (genus *Tobamovirus*), *Eggplant mottled dwarf virus* (EMDV, genus *Nucleorhabdovirus*), and *Okra mosaic virus* (OkMV, genus *Tymovirus*) (3). Another virus of the begomovirus group (family Geminiviridae) has also been identified affecting gumamela in other countries . The virus caused the leaf curl disease of *Hibiscus* as reported in Pakistan (4), India (5), and China (6) among other countries where hibiscus is widely grown. The disease could cause great yield losses due to reduced flowering and stunting of plants. The causal virus was transmitted by whiteflies (*Bemisia tabaci*) in a persistent manner indicative of a *begomovirus* infection. Moreover, *Hibiscus* or gumamela is a natural host of the *begomovirus Cotton leaf curl virus* (CLCuV). CLCuV belongs to the genus *Begomovirus* of the family *Geminiviridae* with a monopartite genome and a betasatelite (DNA-β). The virus is exclusively transmitted by whiteflies (*Bemisia tabaci* Genn.) in a persistent manner (5). CLCuV-infected plants show cupping or curling of the leaves, vein enation and clearing, mottling,

mosaic, malformation of the flowers and stunting. In the Philippines, the nature of the leaf curl disease of gumamela has not yet been studied thoroughly. This study aims to isolate and identify the causal virus of the disease causing leaf curl in gumamela (*Hibiscus rosa-sinensis*).

2. METHODOLOGY

2.1 Collection of gumamela (*Hibiscus rosa-sinensis*) leaf curl disease samples

Gumamela cuttings showing typical leaf curl virus symptoms like cupping/curling, vein banding, vein clearing, vein enation, mottling/mosaic, chlorosis, and stunting were collected at the hibiscus breeding block of the Crop Science Cluster, Institute of Plant Breeding College of Agriculture UP Los Banos (UPLB). (Table 1; Fig 1) in March, 2013. A total of 34 plant samples were maintained in pots inside the greenhouse for observation. Leaf samples were also collected for DNA extraction and for further tests.

Figure 1. Some of the gumamela plants showing typical virus symptoms (a) vein clearing/banding; (b) yellowing/chlorosis and stunting; (c) vein enation; (d) leaf cupping; (e) rosetting and leaf curling; and (f) little leaf.

Table 1. List of gumamela plant samples exhibiting the leaf curl disease symptoms

Gumamela Sample No.	Symptoms
5	Vein clearing, vein banding, mild cupping
14	Mild cupping, mild mosaic
30	Cupping, mild chlorosis, rosetting, vein enation
32	Mild cupping, mosaic
36	Mild cupping, mottling
37	Cupping, mild mottling, mosaic
38	Cupping, little leaf, vein banding
48	Mild chlorosis, slightly crampled with mild vein banding, vein enation
53	Mild curling, mild vein clearing, vein banding, vein enation
55	Erect, mild vein banding, vein enation
64	Yellow patches, dark green background, cupping
72	Mild cupping, vein banding
75	Cupping, erect, mild vein banding, mottling
77	Mild cupping, vein banding, few chlorotic spots
85	Cupping, vein clearing, mottling
87	Stunting, mild vein banding, mild curling
101	Vein banding/ clearing, mild cupping
102	Vein clearing, cupping
103	Vein clearing, mild vein enation
104	Vein banding, chlorotic spots, mild vein enation
134	Stunting, little leaf, cupping
144	Mild cupping, chlorosis, vein banding
145	Cupping, vein banding
147	Vein banding, mild cupping
148	Mild cupping, chlorotic spots, vein banding
149	Cupping, chlorosis
150	Vein banding, cupping
151	Cupping, little leaf, vein banding, mottling
152	Cupping, vein banding
156	Cupping, vein enation
160	Cupping, vein enation
161	Cupping, stunting, little leaf
162	Rosetting, cupping
164	Mild cupping, vein banding
169	Mild chlorosis, cupping, vein banding
170	Cupping, chlorotic spots
172	Little leaf, mild cupping, vein enaton
175	Cupping, vein clearing
183	Stunting, little leaf, mild cupping
184	Cupping, vein enation

2.1 Virus Transmission Tests

Transmission tests using mechanical, graft-inoculations and the use of whiteflies were carried out using series of inoculations from virus infected plants to healthy gumamela. The resulting inoculum was transferred to the different host plants species including: *Solanum melongena L.* (eggplant), *Abelmoschus esculentus* (okra), *Gossypium hirsutum* (cotton), *Vigna unguiculata* (cowpea), *V. sesquipedalis* (pole sitao) and *Phaseolus vulgaris* (snapbean), *Chenopodium amaranticolor, C. quinoa, Datura metel, D. stramonium, Nicotiana glutinosa, Lycopersicon esculentum and Physalis*

floridana to determine its host range. Infected gumamela plants along with the test plants were maintained inside net cages with whiteflies to allow them to feed on the plants. Inoculated plants were kept and maintained for observation and further tests.

2.2 DNA extraction and Virus detection

Total DNAs of infected plants were extracted using the Dellaporta method (7). DNA samples from test plants (*G. hirsutum* and *V. unguiculata*) were also extracted to confirm virus infection. Detection of the leaf curl virus was carried out by PCR using degenerate primers for begomovirus detection that amplifies the ~1.5kb DNA-A fragment of product (including the 5' of C1 gene, intergenic region, the V2 gene and the 5' of CP gene) (8; 9). Proper controls were provided in each run wherein the total DNA of a tissue-cultured gumamela (healthy) provided by the Fruit and Ornamental Breeding Laboratory of IPB-CSC, was used as negative control.

2.3 Sequence Analysis and Phylogenetic Relationship

PCR products were sent to AITbiotech Pte Ltd (Singapore) for sequencing. Multiple sequence alignment of the obtained sequences was done using the BioEdit program. The sequences were also compared to other plant begomoviruses in the GenBank usng the BLAST program. A phylogenetic tree was also constructed using the MEGA5.2 program with 1000 bootstrap value.

Table 2. List of begomoviruses used for comparison of the obtained DNA-A fragments.

Virus Name	Abbreviation	GenBank Accession No.
Cotton leaf curl Burewala virus	CLCuBV	JF416947
Cotton leaf curl Burewala virus isolate C-32	CLCuBV C-32	HF549181
Cotton leaf curl Burewala virus India isolate	CLCuBV India	GQ247893
Cotton leaf curl Multan virus isolate D4	CLCuMV D4	KF413618
Cotton leaf curl Multan virus isolate GX01	CLCuMV GX01	JQ317603
Cotton leaf curl Multan virus	CLCuMV	GQ503175
Cotton leaf curl Multan virus isolate Okra08	CLCuMV Okra08	JX286664
Okra leaf curl virus	OLCV	GQ245760
Pepper leaf curl virus	PepLCV	AF414287
Squash leaf curl Philippine virus	SLCPV	AB085793
Squash leaf curl virus isolate 1-1	SLCV 1-1	JF746195
Squash leaf curl virus isolate P133	SLCV P133	EU487041
Squash leaf curl China virus isolate SLCCNV	SLCCNV	JN587811
Squash leaf curl China virus	SLCCV	HM566112
Squash leaf curl virus isolate Wg1	SLCV Wg1	EU310406
Tomato leaf curl virus Laguna isolate	ToLCV Lag	AB307731
Tomato leaf curl virus Los Baños isolate	ToLCV LB	AB377113

3. RESULTS AND DISCUSSION

3.1 Transmission Test

The leaf curl disease was successfully transmitted to healthy gumamela by grafting and whiteflies (*Bemisia tabaci* Gen.) but not by sap inoculation. This finding is also similar with the previous data obtained for the Philippine tomato leaf curl virus (ToLCV-Ph) and Squash leaf curl china virus (SLCCV) that were transmitted exclusively by whiteflies in the Philippines (10; 11). Whiteflies transmission to cotton and cowpea showed leaf cupping and curling, vein enation, mottling and mosaic a month after inoculation (Fig 2). However, okra which is a known host of begomoviruses (4; 12) failed to exhibit any symptoms. The other host plants including *V. sesquipedalis* (pole sitao) and *Phaseolus vulgaris* (snapbean), *Chenopodium amaranticolor, C. quinoa, Datura metel, D. stramonium, Nicotiana glutinosa, Lycopersicon esculentum and Physalis floridana* did not exhibit any symptom of virus infection upon inoculation of the leaf curl virus. It is interesting to note that cotton and cowpea which were not previously reported hosts of ToLCV-Ph and SLCCV, were positively identified hosts of this gumamela leaf curl isolate. On the other hand, the other plant species , including *D. stramonium, N. glutinosa and L esculentum* which were found hosts of ToLCV-PH and SLCCV showed negative results in this study. These host plant species were also found susceptible to a begomovirus causing the *Hibiscus* leafcurl disease in India (5).

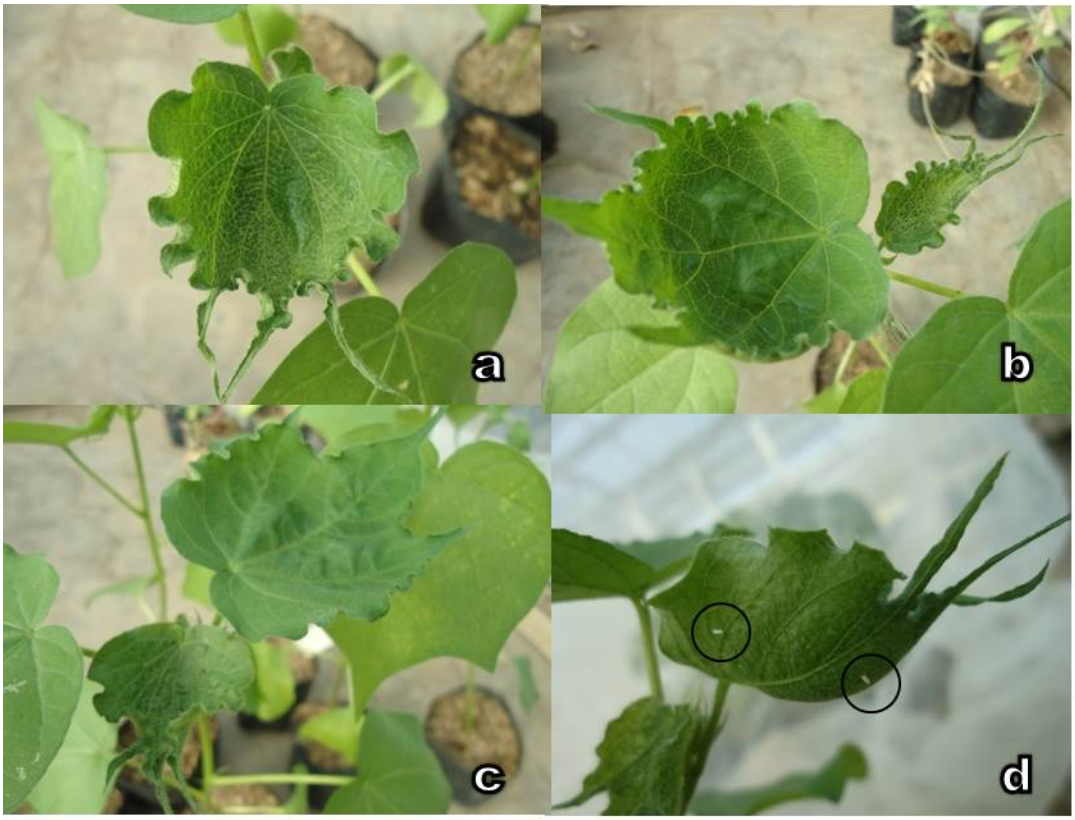

Figure 2. Cotton plants inoculated with the leaf curl virus by whitefly transmission (a-c) mild mottling, leaf curling/cupping and leaf distortion (d) whiteflies feeding on the cotton plant (encircled).

3.2 Detection by PCR

Thirty four (34) out of the 40 collected gumamela samples showed the expected band of ~1.5kb after PCR amplification using degenerate primers for begomoviruses (Fig 3; Table 3). Most of these samples exhibited cupping, enation, vein banding and vein clearing leaf symptoms. Whitefly inoculated tests plants were also subjected to PCR wherein the cotton and cowpea plants also displayed the expected band size (Fig 4). PCR has been efficiently used in detecting the presence of begomoviruses *in Hibiscus spp*. (4; 6; 5;13).. Amplification of the 1.5 kb DNA fragment from the leaf curl infected plants, thus, confirmed infection by a begomovirus.

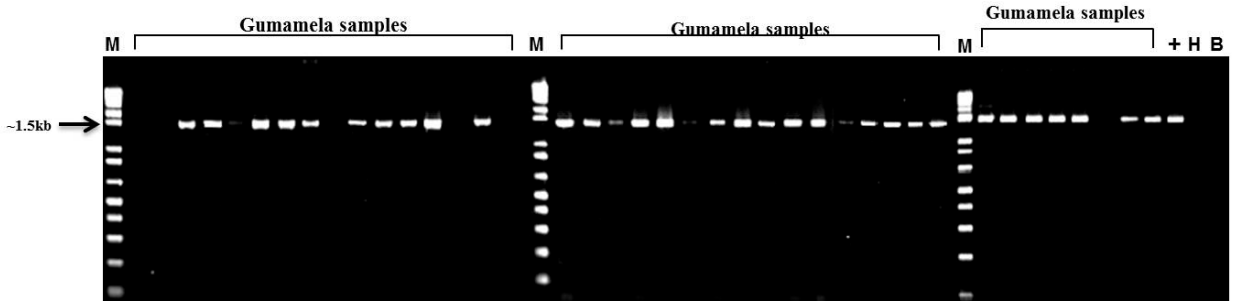

Figure 3. PCR amplification of gumamela leaf samples using degenerate primers showing the expected ~1.5kb product. (M - 1kb plus DNA ladder; + - Positive check; H – healthy check; and B – blank).

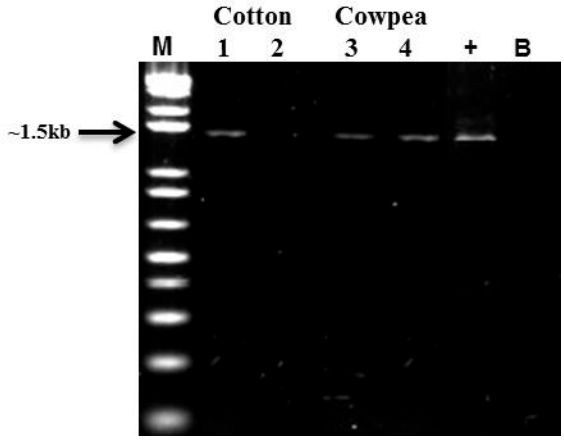

Figure 4. PCR amplification of cotton and cowpea leaf samples using degenerate primers showing the expected ~1.5kb product. (M - 1kb plus DNA ladder; (1) – cotton plant showing leaf cupping and leaf distortion; (2) – cotton plant with no symptoms; (3-4) – cowpea plants showing leaf cupping and mosaic; + - Positive check; and B – blank).

Table 3. List of gumamela plants showing positive reaction to PCR using degenerate primers.

Gumamela Sample No.	Symptoms	PCR Result
5	Vein clearing, vein banding, mild cupping	-
14	Mild cupping, mild mosaic	-
30	Cupping, mild chlorosis, rosetting, vein enation	+
32	Mild cupping, mosaic	+
36	Mild cupping, mottling	+
37	Cupping, mild mottling, mosaic	+
38	Cupping, little leaf, vein banding	+
48	Mild chlorosis, slightly crampled with mild vein banding, vein enation	+
53	Mild curling, mild vein clearing, vein banding, vein enation	-
55	Erect, mild vein banding, vein enation	+
64	Yellow patches, dark green background, cupping	+
72	Mild cupping, vein banding	+
75	Cupping, erect, mild vein banding, mottling	+
77	Mild cupping, vein banding, few chlorotic spots	-
85	Cupping, vein clearing, mottling	+
87	Stunting, mild vein banding, mild curling	-
101	Vein banding/ clearing, mild cupping	+
102	Vein clearing, cupping	+
103	Vein clearing, mild vein enation	+
104	Vein banding, chlorotic spots, mild vein enation	+
134	Stunting, little leaf, cupping	+
144	Mild cupping, chlorosis, vein banding	+
145	Cupping, vein banding	+
147	Vein banding, mild cupping	+
148	Mild cupping, chlorotic spots, vein banding	+
149	Cupping, chlorosis	+
150	Vein banding, cupping	+
151	Cupping, little leaf, vein banding, mottling	+
152	Cupping, vein banding	+
156	Cupping, vein enation	+
160	Cupping, vein enation	+
161	Cupping, stunting, little leaf	+
162	Rosetting, cupping	+
164	Mild cupping, vein banding	+
169	Mild chlorosis, cupping, vein banding	+
170	Cupping, chlorotic spots	+
172	Little leaf, mild cupping, vein enaton	+
175	Cupping, vein clearing	-
183	Stunting, little leaf, mild cupping	+
184	Cupping, vein enation	+

3.3 Sequence Analysis

Multiple sequence alignment of the obtained DNA-A fragments revealed 97.0-99.0% similarities suggesting that there are no differences among isolates and that they are closely related to each other. On the other hand, sequence comparison of these sequences to other begomoviruses registered in the GenBank showed that the leaf curl virus infecting gumamela is highly related to the CLCuMV and CLCuBV with 98-99% and 95-96% nucleotide identities,

respectively (Table 4). CLCuV was already reported infecting hibiscus in other countries like Pakistan (4), India (5), and China (6). Furthermore, phylogenetic tree analysis indicates that the putative virus clustered with the CLCuV (Fig 5).

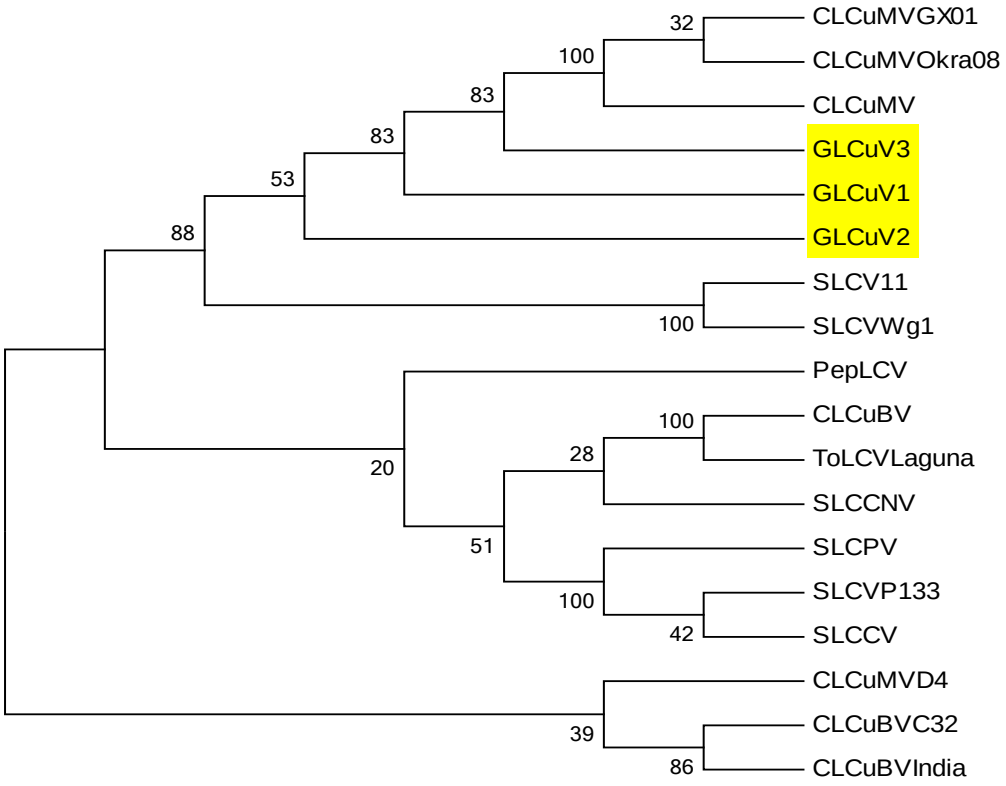

Figure 5. Phylogenetic tree showing the predicted relationship of the Gumamela leaf curl virus-Philippines (GLCuV) to other begomoviruses in the GenBank. Bootstrap percentage was carried out in 1000 replicates.

Table 4. Nucleotide sequence identities of the obtained DNA-A fragments to other begomoviruses in the GenBank.

Gumamela isolate	G1	G2	G3	G4
Gumamela 1 (G1)				
Gumamela 2 (G2)	96.8			
Gumamela 3 (G3)	96.8	98.7		
Gumamela 4 (G4)	97.0	99.0	98.7	
GenBank Sequences				
CLCuBV	95.0	96.0	96.0	96.0
CLCuBV C-32	96.0	96.0	96.0	96.0
CLCuBV India	95.0	95.0	95.0	95.0
CLCuMV	98.0	99.0	99.0	99.0
CLCuMV D4	98.0	99.0	99.0	99.0
CLCuMV GX01	98.0	99.0	99.0	99.0
CLCuMV Okra08	98.0	99.0	99.0	99.0
OLCV	81.0	81.0	81.0	81.0
PepLCV	75.0	75.0	75.0	75.0
SLCPV	79.0	79.0	79.0	79.0
SLCV 1-1	78.0	79.0	79.0	78.0
SLCV P133	80.0	81.0	80.0	81.0
SLCCNV	76.0	78.0	78.0	78.0
SLCCV	75.0	77.0	77.0	77.0
SLCV Wg1	75.0	79.0	79.0	79.0
ToLCV Lag	83.0	83.0	83.0	83.0
ToLCV LB	83.0	83.0	83.0	83.0

4. SUMMARY and CONCLUSIONS

The results of transmission and PCR tests showed that a begomovirus is present in gumamela plants with leaf curl symptoms. The symptoms produced on gumamela ranging from leaf curling, vein banding, mottling, cupping, vein enations and stunting are typical symptoms induced by begomoviruses. Similarly, successful transmission of the leaf curl isolate to healthy gumamela seedlings and other test plants was achieved using whiteflies and graft inoculation tests but not by sap inoculation, a general characteristic of the genus Begomovirus in the family Geminiviridae (14; 15). Amplification of 1.5 DNA fragment using begomovirus detection primers further confirmed the presence of a begomovirus on leaf curl infected gumamela. Sequence analysis of DNA-A fragment and multiple sequence alignment revealed its close relationship with the cotton leaf curl viruses, the Cotton Leaf Curl Multan Virus (CLCuMV) and Cotton Leaf Curl Burewala Virus (CLCuBV).

Based on the results of transmission, PCR and sequence analysis of DNA-A fragment of the putative virus, a begomovirus which is closely related to the cotton leaf curl virus (CLCuV) is the causal pathogen associated with the leaf curl disease of gumamela in the Philippines. The cotton leaf curl virus which has been detected and identified on *Hibiscus spp* in other countries like India, Pakistan and China, has also been reported to be associated with a beta satellite. This finding has not been sought yet for our gumamela leaf curl virus isolate and should likewise be considered for a follow up experiments in order to understand the true identity of the virus isolate. Data obtained from the current study would show the impact that it can create on the epidemiology of the leaf curl disease considering its whitefly mode of transmission and the range of host affected. The present results showed that gumamela is an alternate host of the CLCuMV and its

presence on cotton, gumamela and cowpea can be a threat to other economically important crops like ornamentals, vegetables and ultimately to the cotton industry.

Future studies should focus more on the biology of the virus and its vector, and genetic characterization of the Cotton leaf curl virus (CLCuV) isolate and its satellites. Such information would greatly contribute to the establishment of an appropriate breeding strategies for resistance against the CLCuV in the Philippines.

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