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Isolation and identification of *Hibiscus Chlorotic Ringspot Virus* (HCRSV) infecting Gumamela (*Hibiscus rosasinensis*) in the Philippines

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Abstract

Gumamela (*Hibiscus rosa-sinensis L.*) is a malvaceous ornamental plant commonly grown in the Philippines. Virus-like symptoms such as chlorotic spots, mottling, vein banding and clearing, vein enation, chlorosis, rosetting, leaf distortion, flower abortion and distortion and stunting were observed on gumamela plants growing in the hibiscus breeding blocks at the Institute of Plant Breeding (IPB).. A new virus, *Hibiscus chlorotic ringspot virus* (HCRSV), a member of the genus *Carmovirus* was isolated and identified to be infecting gumamela plants by mechanical inoculation using the sap from symptomatic leaves. The virus isolate caused chlorotic lesions to *Chenopodium quinoa*, *C. amaranticolor*, *C. murale* and *Abelmoschus esculentus*. However, it did not induce any symptoms in *Gomphrena globosa*, *Nicotiana glutinosa*, *Physalis floridana*, *Datura stramonium*, *Lycopersicon esculentum* and *Capsicum annuum*. Gumamela plants (IPB Accessions No. 95 and 97 and the variety "Superstar") showing chlorotic ringspots and the symptomatic indicator plants tested positive for HCRSV by Enzyme-linked immunosorbent assay (ELISA). The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using primers that amplify a conserved region in the coat protein (CP) gene of HCRSV giving an amplification with a size of 557bp further confirmed the results. Nucleotide sequence analysis of the CP gene of the HCRSV Philippines showed 97.5 to 97.9% similarity to the HCRSV isolates of Iran, New Zealand and Singapore. It is most related to the Israel isolate with 98.1% identity and less similar with HCRSV-Taiwan with only 93% sequence identity. To our knowledge, this is the first report of HCRSV in gumamela in the Philippines.

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Introduction

Hibiscus (*Hibiscus rosa-sinensis L.*), locally known as gumamela is a malvaceous ornamental plant with many colors, sizes and shapes and often referred to as the "queen of the Filipino garden". It is grown mainly for landscaping and sometimes for its medicinal value as a laxative and a remedy for bronchitis, cough and fever. Some varieties are also edible as food garnish, jam, jelly and juice. Gumamela is also consumed as tea as an excellent source of antioxidants. Thus, it is noted for its health and wellness value. In the past, gumamela is just used as bleaching platforms of clothes. However, with the development of new hybrid varieties with different colors and flower forms, it becomes competitive with other cut flowers (Magdalita and Pimentel, 2013).

Like any other plant, gumamela plants are also prone to diseases mostly caused by plant viruses. Some of the plant viruses infecting gumamela plants include: *Tobacco mosaic virus* (TMV), *Cotton leaf curl virus* (CLCuV), *Eggplant mottled dwarf virus* (EMDV), *Hibiscus latent Singapore virus* (HLSV), *Hibiscus latent Fort Pierce virus* (HLFPV) and *Hibiscus chlorotic ringspot virus* (HCRSV) (Rajeshwari *et al*., 2005; Liu *et al*., 2004; De Stravis *et al*., 2008; Tang *et al*., 2008).

HCRSV is a member of the genus *Carmovirus* from the family *Tombusviridae* with positive single stranded RNA genome of 3911 nucleotides that encode for seven open reading frames (ORFs) (Huang *et al*., 2000). It causes severe symptoms in gumamela plants such as chlorotic spots or ringspots, mottling, vein banding, stunting and flower distortion and abortion. It can be spread mechanically and through vegetative propagation of infected plants but is not transmitted by seed or by the aphid *Myzus persicae* (Waterworth *et al*., 1976; Brunt and Spence, 2000). HCRSV naturally infecting *H. rosa-sinensis* was previously reported in Nigeria (Lana, 1974), the United States (Waterworth *et al*., 1976), Singapore (Wong *et al*., 1992), Australia, Thailand, the South Pacific Islands, El Salvador (Brunt *et al*., 2000), Taiwan (Li and Chang, 2002), New Zealand (Tang *et al*., 2008), Iran (Pourrahim *et al*., 2013) and Israel (Luria *et al*., 2013). Gumamela plants growing in the hibiscus breeding blocks at the Institute of Plant Breeding (IPB), University of the Philippines Los Baños (UPLB) compound were observed with various virus-like symptoms. A complex of viruses was suspected causing the disease wherein HCRSV is one of them, hence this study was conducted from May 2013 to June 2014. This study aims to investigate the i) symptomatology of the virus disease and ii) test the host range, serological reactions and molecular identification of the local HCRSV isolate.

Materials and method

Collection of virus infected samples

Leaf samples were collected from gumamela plants showing virus symptoms such as chlorotic spots or ringspots, mottling, mosaic, vein banding, vein clearing, vein enation and thickening, leaf chlorosis, leaf cupping, leaf deformation, rosetting, stunting and flower abortion in the hibiscus breeding blocks inside the IPB compound, University of the Philippines Los Baños (UPLB) (Fig 1; Table 1). Infected plant samples were also maintained in pots inside an insect proof greenhouse for observation and further tests.

Virus transmission and host range of HCRSV

Indicator and assay host plants from four families (*Chenopodiaceae, Amaranthaceae, Solanaceae* and *Malvaceae*) were mechanically inoculated using leaf sap from symptomatic gumamela plant. Thirteen plant species were used as test plants including *Chenopodium amaranticolor, C. quinoa, C. murale, Gomphrena globosa, Datura stramonium, D. metel, Physalis floridana, Nicotiana glutinosa, Solanum melongena L., Lycopersicon esculentum*, *Capsicum annuum*, *Abelmoschus esculentus* and *Hibiscus rosa-sinensis*. Seeds were sown in sterilized soil with coir dust and hog manure and were maintained in an insect proof greenhouse. Plants at the 2-3 leaf stage were mechanically inoculated using sap from homogenized symptomatic gumamela leaves in 1:10 dilution in 0.01M phosphate buffer pH7.4. Inoculated test plants were then kept and maintained in the greenhouse for 6-8 weeks for observation of

symptoms and further tests.

Serological Tests

For Dot Blot and Indirect Enzyme-linked immunosorbent assay (ELISA), the procedure was followed as previously described (Dolores and Pateña, (2009); Dolores *et al* (2013)). Tests for the presence of HCRSV were done in 2 replications using commercial antibodies (Agdia Inc., USA). Proper controls were used such as buffer and healthy gumamela extract from tissue cultured plants served as negative controls and HCRSV-infected gumamela leaf extract as the positive control. Color reaction was observed both in Dot blot and Indirect ELISA. The absorbance reading at 405 nM was also taken using a microplate reader (BioRad) in the Indirect ELISA.

RT-PCR Detection

Total plant RNAs of infected samples were extracted and later used for detection. For Reverse Transcription-Polymerase Reaction (RT-PCR), 2 sets of primers were used to amplify the coat protein gene (CP) of HCRSV) as previously described by Pourrahim *et al.* (2013) and Tang *et al.*(2008). The conserved region in the CP gene of the HCRSV was amplified by Reverse Transcription-Polymerase Reaction (RT-PCR) using the forward (5′-GGAACCCGTCCTGTTACTTC-3′) and reverse (5′-ATCACATCCACATCCCCTTC-3′) primers of Tang *et al.* (2008) obtaining 570 bp fragment while the other primers designed by Pourrahim *et al.* (2013) were also used to amplify the 1.3 kb CP gene fragment of the local HCRSV isolate. The assay was carried out with cDNA synthesis at 55°C for 30 min and an initial denaturation step at 94°C for 2 min followed by 40 cycles with each cycle consisting of the following steps: 1) denaturation at 94°C for 15 sec; 2) primer annealing at 55°C for 30 sec; and 3) primer extension at 68°C for 1 min. After completing the 40 cycles, it was followed by 1 cycle of final extension at 68°C for 5 min. Proper controls were provided in each run. PCR products were separated by agarose gel electrophoresis and the expected fragments were excised from the gel, cleaned and sent to the Singapore Sequencing Facility. Nucleotide sequence analysis was done to compare the HCRSV Philippine isolate with those of the other HCRSV isolates deposited in the GenBank, Maryland, USA.

Results and discussion

Symptomatology and Host Range

Pure HCRSV isolate was successfully obtained through single lesion isolation including two serial passages to indicator host, *C. quinoa*. The pure isolate was used to inoculate healthy gumamela plants and different indicator host plants grown in the greenhouse. Inoculated gumamela plants exhibited chlorotic spots surrounded by a yellowish halo 4 weeks after inoculation (Fig 3a-b). These infected plants eventually developed vein banding and/or vein clearing as well as leaf chlorosis (Fig 3c-d). As the disease progressed, the leaves become irregular and the plant become stunted and failed to produce flowers. Previous studies reported that HCRSV-infected plants exhibit leaf symptoms that ranged from generalized mottle to chlorotic ringspots and vein banding patterns as well as severe stunting and distortion of leaves and flowers (Lana, 1974; Waterworth *et al.*, 1976; Wong *et al.*, 1992; Li and Chang 2002; Tang *et al.*, 2008; Luria *et al.*, 2013; Pourrahim *et al.*, 2013). On the other hand, 6 out of the 13 inoculated host plants (46.15%) showed symptoms typical of HCRSV infection 1 to 2 weeks after inoculation (Table 2). *C. quinoa, C. amaranticolor and C. murale* (Fig 2) displayed chlorotic local lesions or spots while *A. esculentus* showed mild chlorotic ringspot and yellowing of leaves. Earlier reports stated that HCRSV causes chlorotic local lesions in *C. quinoa* and *C. amaranticolor* and the two are the most useful hosts for studying the virus other than *H. cannabinus* or kenaf (Waterworth *et al.*, 1976; Tang *et al.*, 2008; Pourrhaim *et al.*, 2013). *C. murale* which is also a member of the *Chenopodiaceae* family was also found susceptible to the virus. *A. esculentus*, a member of the *Malvaceae* family together with *A. manihot, H. rosa sinensis*, *H. cannabinus, Alcea rosea* and *Gossypium hirsutum* were also reported vulnerable to the disease (Waterworth *et al.*, 1976; Brunt and Spence; 2000; Pourrahim *et al.*, 2013). Since it is

mechanically transmitted to gumamela, it should be taken with caution especially hibiscus is propagated by grafting. For example, HCRSV infected scions once grafted or joined with healthy rootstocks, the whole assembly could become infected. Similarly, once an infected plant is propagated by cuttings, all reproduced plants are infected, hence the need for virus indexing before mass propagation.

Table 1. List of gumamela plant samples tested for HCRSV infection.

Gumamela Sample Accession/Crosses/Variety	Symptoms
BGBxLoleng	Vein banding
Ledivina Cariño	Chlorotic spots
MCxNTG	Leaf cupping, mottling, vein banding
MCxLL	Vein banding, chlorosis
Spotted Pink	Chlorosis, vein banding
MC	Vein clearing
Acc. 35	Vein banding
Acc.177xMarcela	Leaf cupping, rosetting
MarcelaxCastillo	Leaf cupping, vein banding
Good-tiger	Leaf cupping, vein clearing
Tarantella	Chlorotic spots
Acc. 95	Vein clearing
Acc. 97	Vein banding, leaf cupping, rosetting
Acc. 201	Chlorotic spots
Acc. 104	Leaf cupping, vein enation
MDCxTahitia Princess	Vein banding, vein enation
Acc.58	Vein banding, mottling
PSOxBGB	Leaf cupping and distortion, vein banding
Marcela	Chlorotic patches
Acc. 106	Leaf cupping, vein banding
MBC	Vein clearing, rolling of leaves
Acc.27	Chlorotic to necrotic spots
Fuchsia Pink	Vein clearing
St. Bridget Light	Vein clearing, darkening of primary veins
Laguna Hybrid	Chlorotic patches
MRMxDR	Leaf cupping and curling
MM	Vein clearing
MCxGC	No symptom
Superstar	Vein clearing, leaf necrosis
Reddy or Not	Mosaic, mottling, vein and blade necrosis

Table 2. List of indicator and host plants mechanically inoculated with the HCRSV isolate.

Test plants	Family	Symptoms
Chenopodium amaranticolor	Chenopodiaceae	Chlorotic local lesion
Chenopodium quinoa	Chenopodiaceae	Chlorotic local lesion
Chenopodium murale	Chenopodiaceae	Chlorotic local lesion
Gomphrena globosa	Amaranthaceae	No symptom
Nicotiana glutinosa	Solanaceae	No symptom
Physialis floridana	Solanaceae	No symptom
Lycopersicon esculentum	Solanaceae	No symptom
Capsicum annuum	Solanaceae	No symptom
Datura stramonium	Solanaceae	No symptom
Datura metel	Solanaceae	Chlorotic spots
Solanum melongena L.	Solanaceae	Chlorotic spots
Abelmoschus esculentus L.	Malvaceae	Mild chlorotic ringspot and yellowing
Hibiscus rosa-sinensis	Malvaceae	Chlorotic spots with yellowish halos

The *Solanaceae* family was not described as natural hosts of HCRSV thus *N. glutinosa, P. floridana, D. stramonium, L. esculentum and C. annuum* did not displayed any virus symptoms. However, inoculated *D. metel* and *S. melongena,* both members of the *Solanaceae* family exhibited chlorotic spots. This could be due to other factors such as the presence of insect pests, abiotic factors and the environmental condition when the experiment was conducted. On the other hand, inoculated *G. globosa* which is reported as a susceptible host of HCRSV, failed to develop symptoms. Tang *et al.* (2008) also did not observe any symptoms in *G. globosa* when it is inoculated with HCRSV-infected plant sap. In other studies, HCRSV also induce local lesions in *Cyamopsis tetragonoloba, Dolichus biflorus* and *Phaseolus vulgaris* which are all members of the Fabaceae family (Brunt and Spence, 2000).

Serological detection

HCRSV was detected by both Dot-blot and Indirect ELISA using leaf extracts of the field collected gumamela plants and the infected test plants used in the transmission test. Indirect ELISA was used to assay the collected gumamela leaf samples in the field. Out of the 30 samples tested, only 3 samples (10%) showed positive response to the HCRSV antibody namely IPB accessions 95 and 97 and the Superstar (Table 3). Superstar displayed the highest HCRSV titer (1.482) indicating its susceptibility to the virus compared to the other varieties. On the other hand, symptomatic *C. amaranticolor, C. quinoa, C. murale,* and *A. esculentus* displayed the positive yellow color reaction when tested for HCRSV infection using Dot Blot ELISA. Previous studies used DAS-ELISA to detect HCRSV in infected hibiscus plants to confirm virus infection. Other than gumamela, HCRSV was also detected in symptomatic *C. amaranticolor, C. quinoa* and *Phaseolus vulgaris* (Li and Chang, 2002; Tang *et al*., 2008) using ELISA.

Table 3. List of gumamela plant samples tested for HCRSV infection by ELISA.

Gumamela Accession/Crosses/Variety	Sample Symptoms	Absorbance Value (at 405nm)
BGBxLoleng	Vein banding	0.1665
Ledivina Cariñno	Chlorotic spots	0.1685
MCxNTG	Leaf cupping, mottling, vein banding	0.1665
MCxLL 2010	Vein banding, chlorosis	0.3305
Spotted Pink	Chlorosis, vein banding	0.2530
MC	Vein clearing	0.1735
Acc.35	Vein banding	0.1795
Acc.177xMarcela	Leaf cupping, rosetting	0.1825
MarcelaxCastillo	Leaf cupping, vein banding	0.2590
Good-tiger	Leaf cupping, vein clearing	0.1715
Turantella	Chlorotic spots	0.2135
Acc. 95	Vein clearing	0.6315
Acc. 97	Vein banding, leaf cupping, rosetting	0.4400
Acc. 201	Chlorotic spots	0.2545
Acc. 104	Leaf cupping, vein enation	0.1840
MDCxTahitian Princess	Vein banding, vein enation	0.2730
Acc.58	Vein banding, mottling	0.2610
PSOxBGB	Leaf cupping and distortion, vein banding	0.1970
Marcela	Chlorotic patches	0.1915
Acc. 106	Leaf cupping, vein banding	0.2550
MBC	Vein clearing, rolling of leaves	0.2650
Acc.27	Chlorotic to necrotic spots	0.2585
Fuchsia Pink	Vein clearing	0.1890
St. Bridget Light	Vein clearing, darkening of primary veins	0.2505
Laguna Hybrid	Chlorotic patches	0.1955
MDM-MRMxDR	Leaf cupping and curling	0.1990
MM	Vein clearing	0.2290
MCxGC	No symptom	0.2200
Superstar	Vein clearing, leaf necrosis	1.482
Reddy or Not	Mosaic, mottling, vein and blade necrosis	0.297

*Positive check = 1.0425 Negative check = 0.1750.

Table 4. Percent identities of CP gene of Los Banos isolate from the Philippines aligned with 5 HCRSV in Genbank (NCBI) using Clustal Omega.

HCRSV isolate	Taiwan	New Zealand	Singapore	Philippines	Iran	Israel
Taiwan	ID	93.6%	94.1%	93.0%	92.7%	93.4%
New Zealand	93.6%	ID	99.5%	97.9%	97.9%	98.8%
Singapore	94.1%	99.5%	ID	97.5%	97.5%	98.4%
Philippines	93.0%	97.9%	97.5%	ID	97.9%	98.1%
Iran	92.7%	97.9%	97.5%	97.9%	ID	97.9%
Israel	93.4%	98.8%	98.4%	98.1%	97.9%	ID

NCBI isolates:

Taiwan – AY546635.1 Hibiscus chlorotic ringspot virus isolate TW-Ch coat protein (CP) gene, complete cds

New Zealand - EU554660.1 Hibiscus chlorotic ringspot virus isolate NZ coat protein (CP) gene, partial cds

Singapore - X86448.2 Hibiscus chlorotic ringspot virus genomic RNA

Iran – JX865593.1 Hibiscus chlorotic ringspot virus isolate Iran coat protein (CP) gene, complete cds

Israel - KC876666.1 Hibiscus chlorotic ringspot virus isolate HCRSV-Is complete genome.

HCRSV Detection by RT-PCR

A conserved region in the CP gene of the HCRSV was amplified by RT-PCR with the total RNA extracted from the inoculated gumamela plants and the symptomatic indicator host plants, *Chenopodium quinoa* and *C. amaranticolor* using the primers of Tang *et al.* (2008). All of the inoculated gumamela plants except for one (sample #7) showed the expected ~557 bp DNA product thereby further confirming the presence of the virus. This jibes with the results when naturally infected gumamela plants were tested for HCRSV infection using RT-PCR.

Fig. 1. Different types of virus-like symptoms observed in gumamela plants in the Hibiscus Breeding Blocks at the Institute of Plant Breeding, Crop Science Cluster, College of Agriculture, UP Los Baños (UPLB) (a) vein clearing and thickening; (b) vein banding; (c) vein enation; (d) rosetting; (e) leaf chlorosis; (f) chlorotic spots; (g) narrowing of leaves (little leaf); (h) leaf cupping; (h-arrow) flower abortion; (i) flower variegation; and (j) flower distortion; (k-l) healthy gumamela flower and plant.

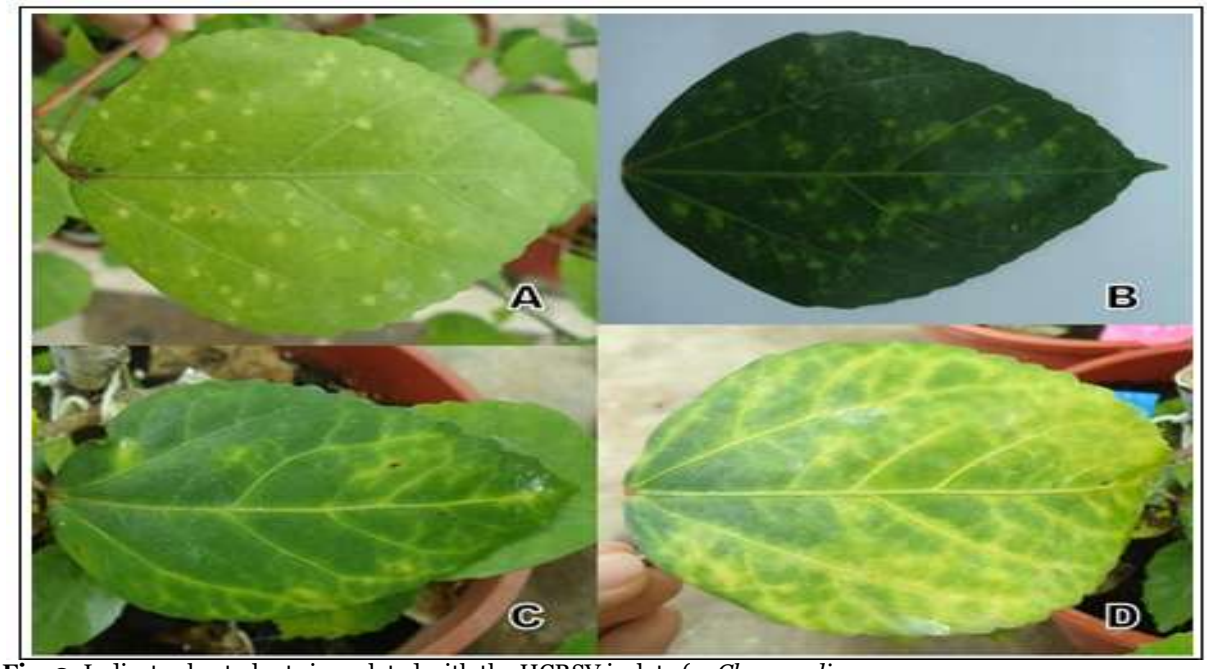

Fig. 2. Indicator host plants inoculated with the HCRSV isolate (a**.** *Chenopodium murale*; b. *C. amaranticolor*; c. *C. quinoa* – showing chlorotic spots; and d. *C. quinoa* – showing chlorotic ringspots).

The symptomatic *C. amaranticolor* and *C. quinoa* also displayed the expected band size (Fig 4) which also validates the presence of HCRSV. HCRSV was also amplified in symptomatic *P. vulgaris, G. hirsutum* and *Alcea rosea* using specific primers (Pourrahim *et al.*, 2013). Furthermore, the specific primers designed by Pourrahim *et al.* (2013), Hb-F (5'-GGTT(C/T)TTT(A/G)TCACTGCCTGAT-3') and Hb-R (5'-GGAGGAAAG-3') were also able to amplify the 1.3 kb fragments of symptomatic gumamela plants (Fig 5). This further confimed the presence of HCRSV in gumamela plants with chlorotic ringspot symptom.

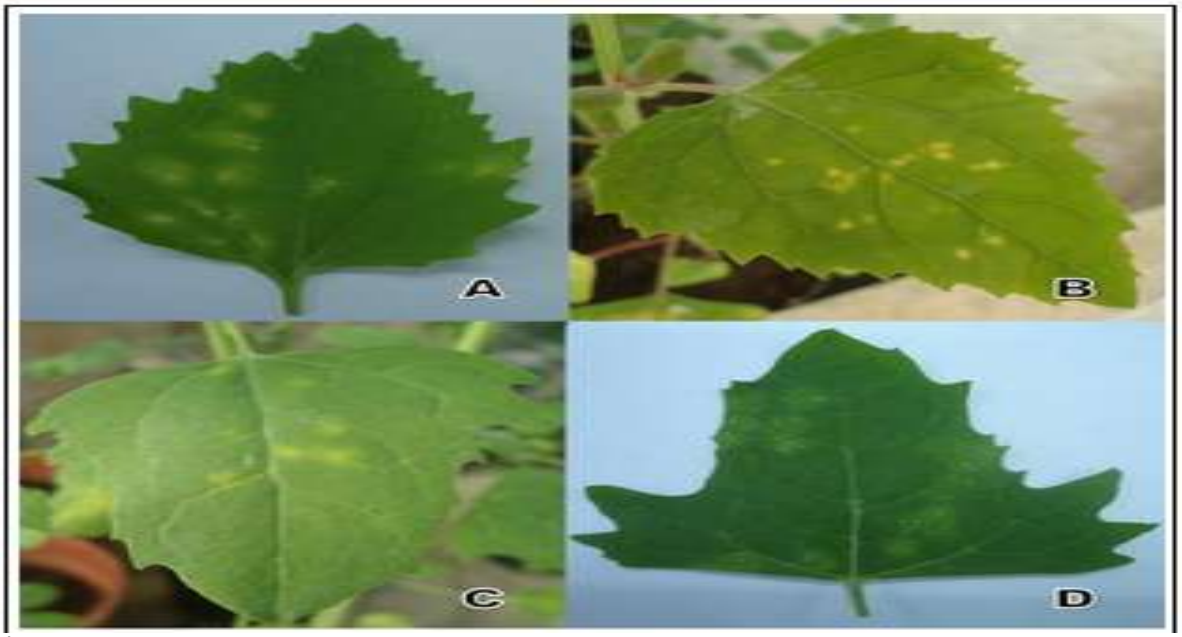

Fig. 3. Inoculated gumamela plants exhibiting typical HCRSV symptoms (**a.** chlorotic spots; **b.** chlorotic spots with yellowish halo; **c.** vein clearing; and **d.** yellowing or chlorosis).

Sequencing and phylogenetic analysis of the HCRSV Philippine isolate

Nucleotide sequence identity among HCRSV isolates from the GenBank including HCRSV isolates from Taiwan, New Zealand, Singapore, Iran and Israel showed 92.7-99.5% similarities. However, comparative sequence analysis have shown 98.1% identity with Israel, 97.5-97.9 % with Singapore, Iran and New Zealand and, only 93 % identity with the Taiwan isolate (Table 4).

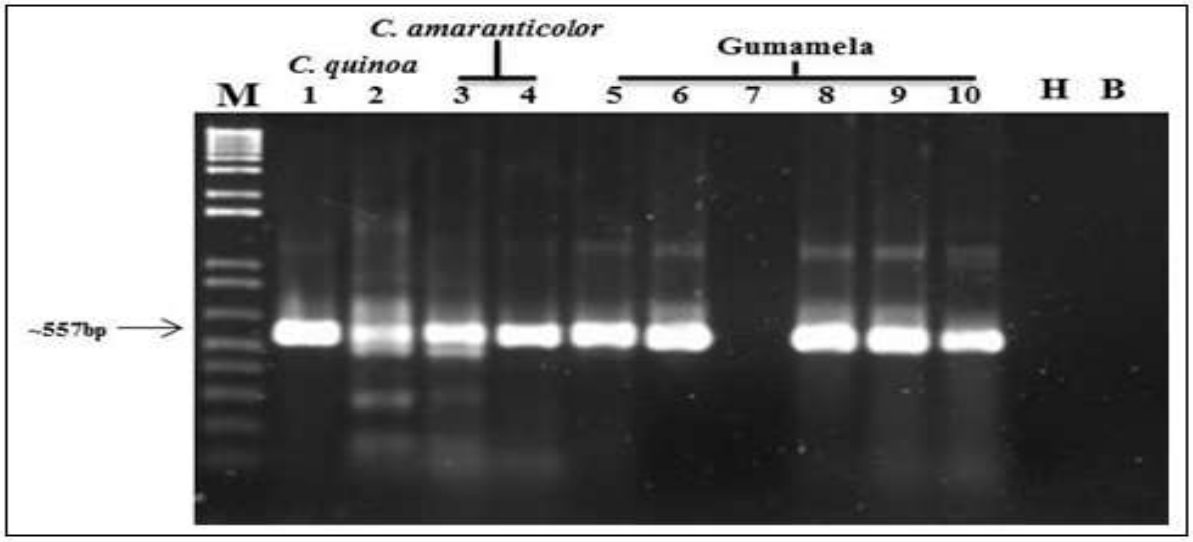

Fig. 4. Amplified conserved region in the HCRSV-CP gene (~557bp) obtained by RT-PCR from total plant RNA of gumamela plants and indicator host plants, *Chenopodium quinoa* and *C. amaranticolor* (M – 1kb plus DNA marker; H – total RNA from healthy gumamela plant; B – blank).

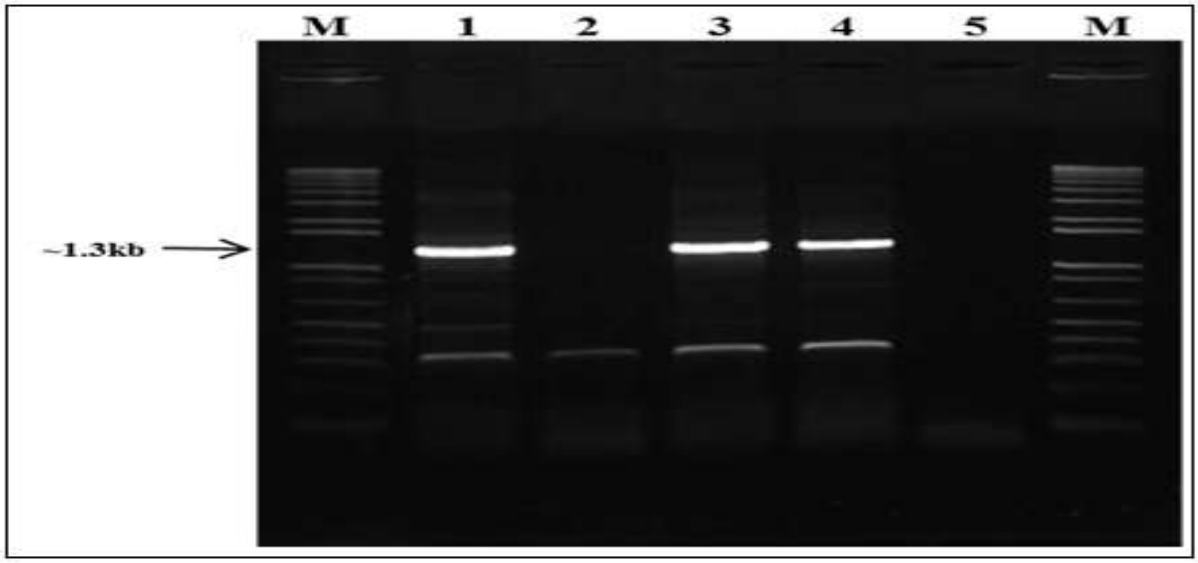

Fig. 5. Amplified DNA product of the HCRSV sequence region flanking the CP gene (~1.3kb) obtained by RT-PCR from total plant RNA of gumamela plants. (M – 1kb plus DNA marker; 1-4 – symptomatic gumamela plants; 5 – blank).

Phylogenetic tree displayed at least 3 subgroups, the Philippines and Iran were grouped with the Israel isolate, the New Zealand isolate with the Singapore isolate, and the Taiwan isolate having the least sequence identity among all isolates, was by itself (Fig 6).

Further studies should be done to fully characterize

the virus at molecular level. In an earlier study, the complete nucleotide sequence of HCRSV was determined and the genome organization was characterized (Huang *et al.*, 2000). This knowledge could be used for the development of virus resistant gumamela plants in the country.

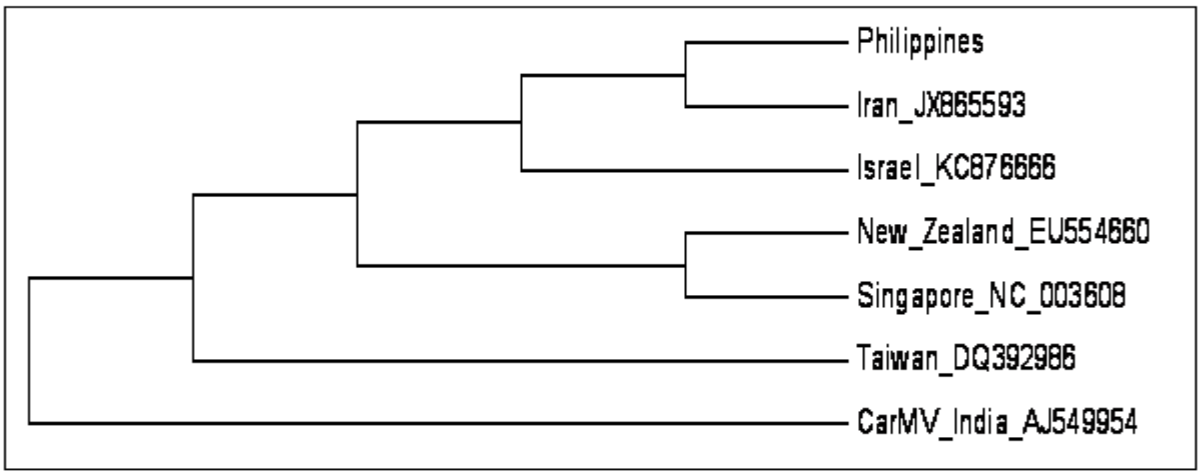

Fig. 6. Phylogenetic relationship of the CP nucleotide sequence of the Philippines isolate with five other HCRSV sequences available in GenBank. The tree was constructed by the NJ algorithm implemented by Mega 6. Bootstrap values (1000 replicates) are given at the branch model. Carnation mottle virus AJ549954 was used as the outgroup.

Summary and conclusion

Hibiscus chlorotic ringspot virus causes abnormalities in gumamela by rendering the plant unattractive and by reducing the flower production and quality. Symptoms observed in gumamela plants growing in the hibiscus breeding blocks in IPB compound and in mechanically inoculated plants resembled those of plants infected by HCRSV (Li *et al.*, 2002; Tang *et al.*, 2008). Diagnostic host plants, *C. amaranticolor and C. quinoa* exhibited the expected chlorotic local lesions (Tang *et al.*,, 2008) as well as *C. murale* and *A. esculentus*. HCRSV infection was further confirmed both by serological and PCR methods. Serological test indicated that HCRSV tested positive using HCRSV antiserum (Agdia, Inc., USA) with an absorbance of 405 nM. Using PCR, amplification product of 1.3 kb fragment was obtained which corresponded to the reported size of the CP of this virus.

Based on the results of the transmission test, ELISA and RT-PCR the virus was identified as HCRSV, a member of genus *Carmovirus* in the family *Tombusviridae*. This study establishes information about the symptomatology and host range of HCRSV confirmed by serological and PCR detection methods. Nucleotide sequence analysis also indicated that the HCRSV Philippine isolate is similar to HCRSV isolate of Iran, New Zealand and Singapore , less similar with the Taiwan isolate but mostly identical with the isolate of Israel having 98.1 % similarities. In our knowledge this is the first report of HCRSV in the Philippines.

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