台灣農業研究 (J. Taiwan Agric. Res.) 61(2):132–143 (2012)

Complete Nucleotide Sequence of RNA2 of *Cucurbit chlorotic yellows virus* Isolated from Melons in Taiwan and Its Comparisons with Currently Existing Isolates[1]

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Abstract

Lin, Y. T, J. Y. Liao, C. H. Tsai, and T. C. Deng. 2012. Complete nucleotide sequence of RNA2 of *Cucurbit chlorotic yellows virus* isolated from melons in Taiwan and its comparisons with currently existing isolates. J. Taiwan Agric. Res. 61:132−143.

The nucleotides of genomic RNA2 of *Cucurbit chlorotic yellows virus* (CCYV) isolates CCYV-Erlun (JN126045) and CCYV-Yilan (JN126046) collected from Taiwan were completely sequenced. In addition to 5’- and 3’- terminal untranslated regions, a total of 8041 bp in length encoding genes of *p4.9* (132 nt), *HSP70h* (1671 nt), *p6* (165 nt), *p59* (1554 nt), *p9* (240 nt), *CP* (753 nt), *CPm* (1425 nt), and *p26* (642 nt) were determined by reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of 5' complementary DNA ends (5’-RACE). The CP gene-targeted primers for RT-PCR would be useful for improving the sensitivity and accuracy of CCYV detection. Comparative analyses of the nucleotide sequences of CCYV isolates with those of various geographic origins including Japan, Taiwan, China, and Sudan revealed that the identities are as high as 99% to 100% in respective genes of *HSP70h*, *CP*, *CPm* and entire RNA2. Entropy plotting demonstrated the variability of nucleotide sequences among two Taiwan isolates and an initial Japan isolate had only 12 divergent nt in a total of 8041 nt of RNA2, and the nucleotide sequence variance located mainly in the 5’- and 3’- terminal regions. The remarkable homogeneity and very close cluster of all CCYV nucleotide sequences available for the phylogenetic analyses indicate that all the currently existing CCYV isolates in the world were originated from the same source.

Key words: CCYV, RT-PCR, Phylogenetic analysis, Entropy plot.

Introduction

Cucurbit chlorotic yellows virus (CCYV) belongs to genus *Crinivirus* and family *Closteroviridae*, emerged in 2004 as a new disease in Japan (Gyoutoku *et al*. 2008). Usually, serious yellowing is the typical symptom of CCYV-infected plants and that causes damages to the yield and quality of cucurbit fruit production. CCYV can be transmitted by the B-type or Q-type tobacco whitefly (*Bemisia tabaci* L.) with semi-persistent manner (Gyoutoku 2008; Huang *et al*. 2010). Host range tested by inoculation with viruliferous *B. tabaci*, CCYV could infect

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[1] Contribution No. 2679 from Taiwan Agricultural Research Institute (TARI), Council of Agriculture. Accepted: May 2, 2012.

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plants of *Asteraceae*, *Chenopodiaceae*, *Convolvulaceae*, *Cucurbitaceace*, *Fabaceae*, and *Solanaceae* (Okuda *et al*. 2010). The name of CCYV was designated by Gyoutoku et al. (2009), but the nomenclature was formally set up with the evidence of entire CCYV genomic sequences and their organizations were determined by Okuda et al. (2010). The initial Japanese CCYV isolate consisted of two positive sense single-stranded RNAs, namely RNA1 and RNA2. RNA1 possesses four open reading frames (ORFs) encoding for papain-like protease, methyltransferase, RNA helicase 1 and RNA-dependent RNA polymerase (RdRp), respectively. RNA2 possesses eight ORFs encoding for hypothetical 4.9-kDa protein (p4.9), 70-kDa heat-shock protein (HSP70h), hypothetical 6-kDa protein (p6), hypothetical 59-kDa protein (p59), hypothetical 9-kDa protein (p9), coat protein (CP), minor coat protein (CPm) and hypothetical 26-kDa protein (p26) as shown at the top of Fig. 3 (Okuda *et al*. 2010).

CCYV was first found on melon (*Cucumis melo* L.) in Kumamoto Prefecture Kyushu, Japan in August 2004 (Gyoutoku *et al*. 2008), then it was also detected on cucumber (*Cucumis sativus* L.) grown in plastic houses in Saga Prefecture (Furuta *et al.* 2008). Till the winter of 2004, CCYV had spread to Miyazaki Prefecture and induced severe yellows disease of cucumber and melon (Kuno *et al*. 2008). From 2005 to 2007, CCYV was prevalent on cucurbit crops, especially cucumbers and melons, and the epidemics were widespread to the whole island of Kyushu in 2008 (Gyoutoku 2008). In September of 2008 the plague disseminated over 1000 km to the eastern Gunma in Kanto region and then spread to neighboring prefectures of Saitama, Tochigi, and Ibaraki in Honshu, Japan (Kuwabara *et al*. 2009; Okuda 2010). In this case, isolates of CCYV occurred in Gunma (Honshu) and those in Kumamoto (Kyushu) were considered to be derived from the same source (Kuwabara *et al*. 2009). CCYV could be distributed long distances within a short time due to the outsourced seedlings which carried CCYV-infected plants or viruliferous whitefly during the transportation. After invasive CCYV was established, transmission by *B. tabaci* accelerated the dissemination of the epidemics. When CCYV was emerged in Kyushu, the epidemics also scattered across the strait to Ehime Prefecture, Shikoku, and in 2009, spread to the Hiroshima and Kochi Prefectures (Okuda 2010).

Outside Japan, in October 2007, symptoms of CCYV infection were found on Hami melon (*C. melo* cv. Xuelihong) cultivated under tunnel in Nanhui, Shanghai, China and then the disease broke out around Shanghai area in 2009. The nucleotide sequence of heat shock protein 70-kDa homologue gene (*HSP70h*) of Shanghai origin CCYV (GenBank Accession No. HQ148667) had 100% identity with Japan isolate (Zeng *et al*. 2011). Gu *et al*. (2011) also reported that CCYV had occurred on plants of melon, watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] and cucumber grown in plastic houses in Shanghai area in 2008, and then spread over 13,000 ha and became prevalent in Shanghai, Ningbo (Zhejiang Province), and Shouguang (Shandong Province). The nucleotide sequence of cucumber isolate collected in Ningbo had 99.7% (*CP*), 99.9% (*CPm*) and 99.9% (*HSP70h*) nucleotide identities with the respective genes of Japanese isolate. First case of CCYV in Taiwan was discovered in 2009, in Lunbei County of Yunlin Prefecture (Tseng *et al.* 2009), then the epidemics have been found in melon, oriental pickling melon (*C. melo* L. var. *conomon*), cucumber, watermelon, pumpkin (*Cucurbita moschata* Duchesne), wax gourd (*Benincasa hispida* Thunb.), luffa (*Luffa cylindrica* Mill.), and bottle gourd [*Lagenaria siceraria* (Molina) Standl.] in all regions of Taiwan (Huang *et al*. 2010; Deng 2011). The RNA2 partial nucleotide sequence (GenBank Accession No. JF502222) of CCYV isolated from muskmelon in Taiwan shared 99% identity with Japanese isolate (GenBank Accession No. AB523789) (Li 2009). Recently, the presence of CCYV affecting melon and cucumber plants in Khartoum, Sudan was reported as the first case of CCYV outside Eastern Asia; however, the Sudanese CCYV nucleotide sequence had only negligible differences from Asian isolates (Hamed *et al*. 2011).

In this study, we have completed the sequencing of the full-length nucleotide of RNA2 of CCYV isolates collected from cucurbits in Taiwan. According to these data and comparing with those obtained from other currently existing isolates of CCYV in the world, phylogenetic relationships were analyzed and the genomic divergence was revealed by Entropy (Hx) plotting. In addition, a pair of primers targeting coat protein gene (*CP*) for RT-PCR shown to be useful for improving the CCYV detection is discussed.

Materials and Methods

Source of the virus

In the routine survey of cucurbit viral diseases, isolate of CCYV-Erlun was collected from muskmelon cultivated in open field of Erlun, Yunlin Prefecture (Lin *et al*. 2010) and CCYV-Yilan was collected from oriental type melon (*C. melo* L. var. *albidia* Makino) grown in the screen house of Jhuangwei, Yilan Prefecture (Deng *et al*. 2010). Leaves or fruits of plants with chlorosis and yellowing symptoms were sampled for indirect ELISA to confirm they were free from other viruses, including *Cucurbit aphid-borne yellows virus*, *Cucumber green mottle mosaic virus*, *Cucumber mosaic virus*, *Melon yellow spot virus*, *Papaya ringspot virus*, *Squash leaf curl virus*, *Watermelon silver mottle virus*, and *Zucchini yellow mosaic virus*. Moreover, an approximate 500-bp amplified fragment produced by RT-PCR using crinivirus-specific primer CCYV-F/R (Gyoutoku et al. 2009) was used to confirm the status of CCYV-infection in CCYV-Erlun and CCYV-Yilan. Both isolates were used as the source of CCYV for the entire nucleotide sequence of RNA2.

Design of specific primers for CCYV detection and full-length sequencing

With reference to the database of NCBI, U.S. National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), the complete RNA2 nucleotide sequence of the initial Japan CCYV isolate (GenBank accession no. AB523789) (Okuda *et al*. 2010) was adopted to design specific primers for the RT-PCR amplification and full-length sequencing. By use of CLC Main Workbench v 5.71, a pair of primers CCYV-jF2 (5'-ACTGACAATAAACAAAACGATGAT-3') and CCYV-jR2 (5'-TCTTTGCTCTCCATTATTTACTAC-3') were designed to amplify a 733-bp fragment of the coat protein gene (*CP*). The other primers used in this study for RT-PCR were listed in Table 1.

Total RNA extraction and full genome amplification

Total RNA was extracted and purified from 100 mg of fresh leaf (CCYV-Erlun) or fruit (CCYV-Yilan) tissues using RNA extraction kit (Plant Total RNA Extraction Miniprep System, Viogene, CA, US) with the manufactures' instructions.

Primer pairs listed in Table 1 were adopted to amplify respective gene regions, such as CCYV-F/RNA2-HSP-P59-R for a 2.4 kb region spanning *HSP70h*−*p59* gene, RNA2-P59-CP-F/RNA2-CP-R1 for a 2.45 kb region spanning *p59*−*CP* gene , CCYV-jF2/jR2 for a 733 bp region of *CP* gene, and CCYV-mcp-F/CCYV-RNA2-R for a 2.2 kb region spanning *CPm*−3'end. A mixture of 1 μL RNA, 4 μL primer (0.5 μM), 17 μL Diethylpyrocarbonate-treat-

Table 1. Nucleotide sequence and target site of primers used for RT-PCR and sequencing

Name	Sequence (5'−3')	Target site ^{z}	Reference
RNA2-race0	GAGAAACCACGATCAATTCC	*Hsp70h*	Okuda *et al*. 2010
RNA2-race1	ACTCCGCCACATATAATGGT	*Hsp70h*	Okuda *et al*. 2010
RNA2-race2	CCAACCCATCTCTTCAAATC	*Hsp70h*	Okuda *et al*. 2010
RNA2-CP-R1	CTTTCAGAGCTTGGCACTTC	*CP*	Okuda *et al*. 2010
CCYV-F	AGTGCGTATGTCAATGG	*Hsp70h*	Gyoutoku *et al*. 2009
CCYV-R	GATAATCCTTCGCAGTG	*Hsp70h*	Gyoutoku *et al*. 2009
RNA2-HSP-P59-R	GATGCTGTGGGAATGTC	*Hsp70h*	This study
RNA2-P59-CP-F	GTGGAACCGAACCTGTTA	*p59*	This study
CCYV-jF	AGGCGGTACTACTTTATGCGTCTT	*CP*	This study
CCYV-jF2	ACTGACAATAAACAAAACGATGAT	*CP*	This study
CCYV-jR2	TCTTTGCTCTCCATTATTTACTAC	*CP*	This study
CCYV-mcp-F	TTCAAATAAAAATCGGTGTTCCTG	*CPm*	This study
CCYV-mcp-R	TATCGAAAAGCGCATGAGACTGTA	*CPm*	This study
CCYV-RNA2-R	GGCCTAGCTATGCTACTAACTA	3'-end of RNA2	This study

^{z} *Hsp70h*: Heat shock protein 70-kDa homologue; *CP*: coat protein; *p59*: hypothetical 59-kDa protein; *CPm*: minor coat protein.

ed distilled water (DEPC-DDW), and AccuPower® RT-PCR PreMix (Bioneer, Daejeon, Korea), was placed into MultiGene Thermo Cycler TC-9600 (Labnet, Edison, NJ, USA) for amplification. The reactions were conducted at 70℃ for 5 min, 10℃ for 10 min, and 94℃ for 2 min, then repeated with 35 cycles of 94℃ for 60 s, 50℃ or 54℃ for 60 s, 70℃ for 60 s or 150 s before the final run at 70℃ for 20 min.

As nucleotide sequence of 5'-end, 5'-RACE system (5´ Rapid Amplification of cDNA Ends; Invitrogen, Carlsbad, CA, USA) was adopted to amplify the unknown fragments (Frohman *et al*. 1988).

Full length sequencing of RNA2

The small amount of gel DNA recovery kit (Gel Advanced Extraction Miniprep System; Viogene, CA, US) was used to recover and purify RT-PCR products which were then cloned to yT&A vector and ECOS101 competent cells (Yeastern Biotech, Taiwan). The plasmid DNA of transformed clones were extracted and analyzed for sizes of embedded fragments to confirm the expected nucleotides that were cloned before the nucleotides of DNA fragments embedded in these recombinant plasmids were sequenced by the Mission Technology Co., Taiwan. All the sequenced data of each fragment were integrated into a coherent RNA2 full-length nucleotide sequence.

Analysis of phylogenetic relationships

By means of alignment in ClustalX2 (http://www.clustal.org/), genomic nucleotide sequences of heat shock protein 70-kDa homologue (*HSP70h*), coat protein (*CP*), minor coat protein (*CPm*), and full-length or partial RNA2 of CCYV-Erlun and CCYV-Yilan were ccompared with other isolates of CCYV listed in Table 2. The other related criniviruses, such as *Lettuce chlorosis virus* (LCV) (Salem *et al*. 2009) and *Cucurbit yellow stunting disorder virus* (CYSDV) (Aguilar *et al*. 2003) were included for comparative analyses and *Beet yellows virus* (BYV) (Agranovsky *et al*. 1994) was set as an out-group control. Phylogenetic dendrograms were generated by neighborhood-joining method (MEGA4: Molecular Evolutionary Genetics Analysis, http://www.megasoftware.net/) and checked with Bootstrap by 1000 replicates. The variability of nucleotide sequence of CCYV RNA2 between a Japan isolate (AB523789) and two Taiwan isolates (JN126045, JN126046) were searched by BioEdit which resulted in generating Entropy (Hx) plots (Hall 1999).

Table 2. List of all CCYV RNA2-associated nucleotides available in the database of NCBI GenBank

Accession	Gene/Length	Host	Location/Isolate	Date	Reference
AB523789	Complete RNA2/8041 bp	Melon	Japan: Kumamoto/Initial isolate	2004	Okuda *et al*. 2010
JF502222	Partial RNA2/6632 bp	Muskmelon	Taiwan: Yunlin/CCYV-TW	Jun-2009	Li 2010
JN126046	Complete RNA2/8041 bp	Oriential melon	Taiwan: Yilan/CCYV-Yilan	Aug-2010	Deng *et al*. in press
JN126045	Complete RNA2/8041 bp	Muskmelon	Taiwan: Erlun /CCYV-Erlun	Feb-2011	Lin *et al*. 2011
JF807055	*HSP70h*/295 bp	Melon	Sudan/CCYV-3P	May-2009	Hamed *et al*. 2011
HQ148667	*HSP70h*/462 bp	Melon	China/CCYV-shjd	Oct-2009	Zeng *et al*. 2011
GU721111	*Hsp70h*/462 bp	*B. tabaci*	China: Shouguang/CCYV-btc	Nov-2009	Gu *et al*. 2011
GU721109	*Hsp70h*/636 bp	Melon	China: Ningbo/CCYV-nbm	Oct-2009	Gu *et al*. 2011
GU721107	*Hsp70h*/636 bp	Melon	China: Shouguang/CCYV-sgm	Oct-2009	Gu *et al*. 2011
GU721105	*Hsp70h*/636 bp	Melon	China: Shanghai/CCYV-sgm	Oct-2009	Gu *et al*. 2011
HM581659	Complete *Hsp70h*/1671 bp	Cucumber	China: Ninbo/CCYV-nbc	Oct-2009	Gu *et al*. 2011
GU721110	*HSP70h/*636 bp	Cucumber	China: Ninbo/CCYV-nbc	Oct-2009	Gu *et al*. 2011
GU721108	*HSP70h*/636 bp	Watermelon	China: Ninbo/CCYV-nbw	Oct-2009	Gu *et al*. 2011
GU721106	*HSP70h*/636 bp	Watermelon	China: Shouguang/CCYV-sgw	Oct-2009	Gu *et al*. 2011
AB457591	*HSP62h*/472 bp	Melon	Japan: Kumamoto/	2004	Gyoutoku *et al*. 2009
JF807053	*CP*/750 bp	Melon	Sudan/CCYV	Jun-2010	Hamed *et al*. 2011
HM581658	*CP*/753 bp	Cucumber	China: Ninbo/CCYV-nbc	Oct-2009	Gu *et al*. 2011
HM581657	Complete *CPm*/1425 bp	Cucumber	China: Ninbo/CCYV-nbc	Oct-2009	Gu *et al*. 2011

Results

Specific primers for CCYV detection

A 733 bp nucleotide fragment was produced with primers CCYV-jF2 and CCYV-jR2 designed based on CCYV coat protein gene (CP) for RT-PCR. When compared with CCYV-F/R (for *HSP70h*) for RT-PCR amplification, both pairs had equivalent sensitivity to detect up to 10^{-4} dilution of the saps of CCYV-infected melon fruit; however, CCYV-jF2/jR2 had a negative reaction with the health control in contrast to CCYV-F/R which had a nonspecific reaction as shown in Fig. 1.

Full length sequencing of RNA2

Full length sequencing of RNA2 of CCYV-Erlun and CCYV-Yilan were completed by RT-PCR and 5’-RACE. A total of 8041 bp including genes of *p4.9* (1035−1166 nt), *HSP70h* (1207−2877 nt), *p6* (2878−3042 nt), *p59* (3036−4589 nt), *p9* (4571−4810 nt), *CP* (4941−5693 nt), *CPm* (5693−7117 nt), *p26* (7179−7820 nt), and 5’- and 3’-terminal untranslated regions were obtained and submitted to NCBI GenBank. The accession numbers were assigned as JN126045 for CCYV-Erlun and JN126046 for CCYV-Yilan.

Analysis of phylogenetic relationships

Alignment with the relevant nucleotide sequences in the NCBI GenBank, CCYV-Erlun (JN126045) and CCYV-Yilan (JN126046) both had > 99% nucleotide identities to other isolates (Tables 3−6) and their phylogenetic dendrograms were shown in Fig. 2. Results of comparative analyses of nucleotide sequences of CCYV isolates derived from various origins revealed the nucleotide identities as high as 99% to 100% in genes of *HSP70h* (Table 3), *CP* (Table 4) , *CPm* (Table 5) and RNA2 (Table 6). In contrast, they shared 70.1%, 63.6%, 50.6%, and58.2% identities with the above-mentioned corresponding gene sequences of *Cucurbit yellow stunting disorder virus* (CYSDV).

The variability of nucleotide sequences of CCYV RNA2 among a Japan isolate (AB523789) and two Taiwan isolates (JN126045, JN126046) were revealed by Entropy (Hx) plot as shown in Fig. 3. The divergence of nucleotide sequences mostly located in the 5’- and 3’-terminal untranslated regions; otherwise, very low entropies were observed in locations of *HSP70h* (1 nt), *CP* (2 nt) and *CPm* (2nt). Within two Taiwan indigenous isolates of CCYV, only three nucleotides were found differed from each other of entire RNA2 (8041nt) and they were scattered in *CP*, *CPm*, and 3’-terminal regions, respectively.

Discussion

In case of CCYV infection, cucurbit plants always show the typical chlorotic yellowing symptoms on the leaves which affect the yield and fruit quality. The viral disease generally is untreatable with chemicals; thus, the strategy to prevent virus infection and technique to detect the causal agent in advance become relatively important. RT-PCR is a usual technique for RNA virus detection and the heat shock protein-like gene (*HSP70h*) is family-specific to members of *Closteroviridae* (Karasev 2000). Accordingly, primer pair (CCYV-F/R) targeting *HSP70h* (Okuda *et al*. 2010) were used for RT-PCR amplification in this study. However, the *HSP70h* gene in members of *Crinivirus* is highly conserved (Dolja *et al*. 2006) and that lacks of specificity to differentiate the species in this genus by RT-PCR using primers of CCYV-F/R. Other than *HSP70h* gene, a pair of CP-based primers (CCYV-jF2/jR2) were designed and used in RT-PCR to compare the efficiency of both tactics in CCYV detection. Results shown in Fig. 1 demonstrated the RT-PCR products made from CCYV-jF2/jR2 were comparatively denser and clearer than those from CCYV-F/R and as well, no noise interference with healthy control was observed. Consequently, primer pair CCYV-jF2/jR2 for RT-PCR is conducive to subsequent CCYV detection RT-PCR for higher sensitivity and accuracy.

Two isolates of *Cucurbit chlorotic yellows virus*, CCYV-Erlun and CCYV-Yilan, were recovered from melon plants in Taiwan, and their complete RNA2 nucleotide sequences of 8041 bp were determined. Regardless of geographic distributions (Japan, China, Taiwan, and Sudan) and host origins (melon, oriental melon, cucumber, and watermelon), all nucleotide sequences of CCYV isolates tested were closely clustered in phylogenetic tree as shown in Fig. 2 while they were significantly distinctive from other species of *Crinivirus*.

Although CCYV is prevalent in cucurbit production areas, there are only three complete nucleotide sequences of RNA2 available in NCBI GenBank database; they are two Taiwan isolates (JN126045, JN126046) and one Japan isolate (AB523789). By

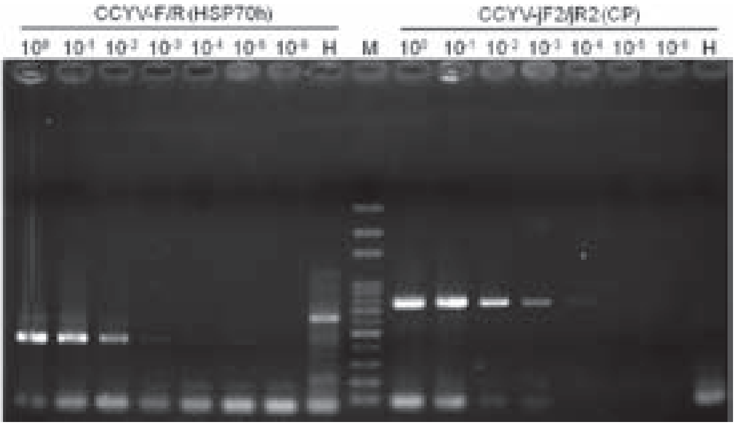


Fig. 1. Comparison of the sensitivity and specificity of the primers, CCYV-F/R (for *HSP70h*) and CCYV-jF2/jR2 (for *CP*) used in RT-PCR for the detection of *Cucurbit chlorotic yellows virus*. Both pairs had equivalent sensitivity to detect up to 10^{-4} dilution of the saps of infected melon fruit. CCYV-jF2/jR2 was negative to the health control in contrast to CCYV-F/R which had a nonspecific reaction to health control.

Table 3. The nucleotide sequence identities of heat shock protein 70-kDa homologue gene (*Hsp70h*) among currently existing *Cucurbit chlorotic yellows virus* (CCYV) isolates

Accession no.of nucleotide ^{z}		Nucleotide sequence identity (%)																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
AB523789	1																	
HQ148667	2	100																
JN126045	3	100	100															
JN126046	4	100	100	100														
JF502222	5	100	100	100	100													
AB457591	6	100	100	100	100	100												
GU721111	7	100	100	100	100	100	100											
JF807055	8	100	100	100	100	100	100	100										
HM581659	9	100	100	100	100	100	100	100	100									
GU721108	10	100	100	100	100	100	100	100	100	100								
GU721110	11	100	100	100	100	100	100	100	100	100	100							
GU721105	12	100	100	100	100	100	100	100	100	100	100	100						
GU721109	13	100	100	100	100	100	100	100	100	100	100	100	100					
GU721107	14	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6				
GU721106	15	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	100			
FJ380119 ^{y}	16	74.9	74.9	74.9	74.9	74.9	74.9	74.9	74.9	74.9	74.9	74.9	74.9	74.9	74.5	74.5		
FJ492808 ^{x}	17	70.1	70.1	70.1	70.1	70.1	70.1	70.1	70.1	70.1	70.1	70.1	70.1	70.1	70.5	70.5	71.5	
NC_001598 ^{w}	18	45.2	45.2	45.2	45.2	45.2	45.2	45.2	45.2	45.2	45.2	45.2	45.2	45.2	45.5	45.5	45.9	44.9

[z] Details of CCYV nucleotide sequences are listed in Table 2.

[y] FJ380119: Complete RNA2 sequence (8556 bp) of *Lettuce chlorosis virus* (LCV), *Crinivirus*, isolated from *Lactuca sativa* in California, USA (Salem *et al*. 2009) .

[x] FJ492808: Complete RNA2 sequence (7976 bp) of *Cucurbit yellow stunting disorder virus* (CYSDV), *Crinivirus*, isolated from melon in Arizona, USA. in October, 2006.

[w] NC_001598: Complete genome (15480 bp ss-RNA) of *Beet yellows virus* (BYV), *Closterovirus*, isolated from Ukrainian, Russia before 1993 (Agranovsky *et al*. 1994).

Table 4. Coat protein gene (*CP*) nucleotide sequence identities of currently existing *Cucurbit chlorotic yellows virus* (CCYV) isolates

		Nucleotide sequence identity (%)							
Accession no. of nucleotide[z]		1	2	3	4	5	6	7	8
JN126046 (Taiwan: Yilan, Melon)	1								
HM581658 (China: Ningbo, Cucumber)	2	99.8							
JN126045 (Taiwan: Erlun, Muskmelon)	3	99.8	99.7						
JF807053 (Sudan, Melon)	4	99.6	99.4	99.7					
AB523789 (Japan: Kumamoto, Melon)	5	99.8	99.7	99.7	99.4				
JF502222 (Taiwan: Yunlin, Muskmelon)	6	99.8	99.7	99.7	99.4	99.7			
FJ380119 (LCV, *Crinivirus*)	7	73.8	73.7	73.7	73.4	74.0	73.8		
FJ492808 (CYSDV, *Crinivirus*)	8	63.7	63.6	63.6	63.3	63.7	63.7	62.6	
NC_001598 (BYV, *Closterovirus*)	9	36.5	36.6	36.6	36.5	36.5	36.5	35.6	34.1

[z] Details of CCYV nucleotide sequences are listed in Tables 2 and 3.

Table 5. Minor coat protein gene (*CPm*) nucleotide sequence identities of currently existing *Cucurbit chlorotic yellows virus* (CCYV) isolates

		Nucleotide sequence identity (%)						
Accession No. of Nucleotide[z]		1	2	3	4	5	6	7
AB523789 (Japan: Kumamoto, Melon)	1							
JF502222 (Taiwan: Yunlin, Muskmelon)	2	100.0						
JN126045 (Taiwan: Erlun, Muskmelon)	3	99.9	99.9					
HM581657 (China: Ningbo, Cucumber)	4	99.9	99.9	100.0				
JN126046 (Taiwan: Yilan, Melon)	5	99.8	99.8	99.9	99.9			
FJ380119 (LCV, *Crinivirus*)	6	64.7	64.7	64.7	64.7	64.6		
FJ492808 (CYSDV, *Crinivirus*)	7	50.8	50.8	50.7	50.7	50.7	50.6	
NC_001598 (BYV, *Closterovirus*)	8	34.0	34.0	33.9	33.9	33.9	33.0	32.0

[z] Details of CCYV nucleotide sequences are listed in Tables 2 and 3.

Table 6. Complete genomic RNA2 nucleotide sequence identities of currently existing *Cucurbit chlorotic yellows virus* (CCYV) isolates

		Nucleotide sequence identity (%)					
Accession No. of Nucleotide[z]		1	2	3	4	5	6
JN126045 (Taiwan: Erlun, Muskmelon)	1						
JN126046 (Taiwan: Yilan, Melon)	2	99.9					
AB523789 (Japan: Kumamoto, Melon)	3	99.9	99.9				
JF502222 (Taiwan: Yunlin, Muskmelon)	4	99.9	99.9	99.9			
FJ380119 (LCV, *Crinivirus*)	5	68.3	68.3	68.4	68.3		
FJ492808 (CYSDV, *Crinivirus*)	6	58.2	58.2	58.2	58.2	57.2	
NC_001598 (BYV, *Closterovirus*)	7	24.4	24.4	24.4	24.4	24.3	23.9

[z] Details of CCYV nucleotide sequences are listed in Tables 2 and 3.

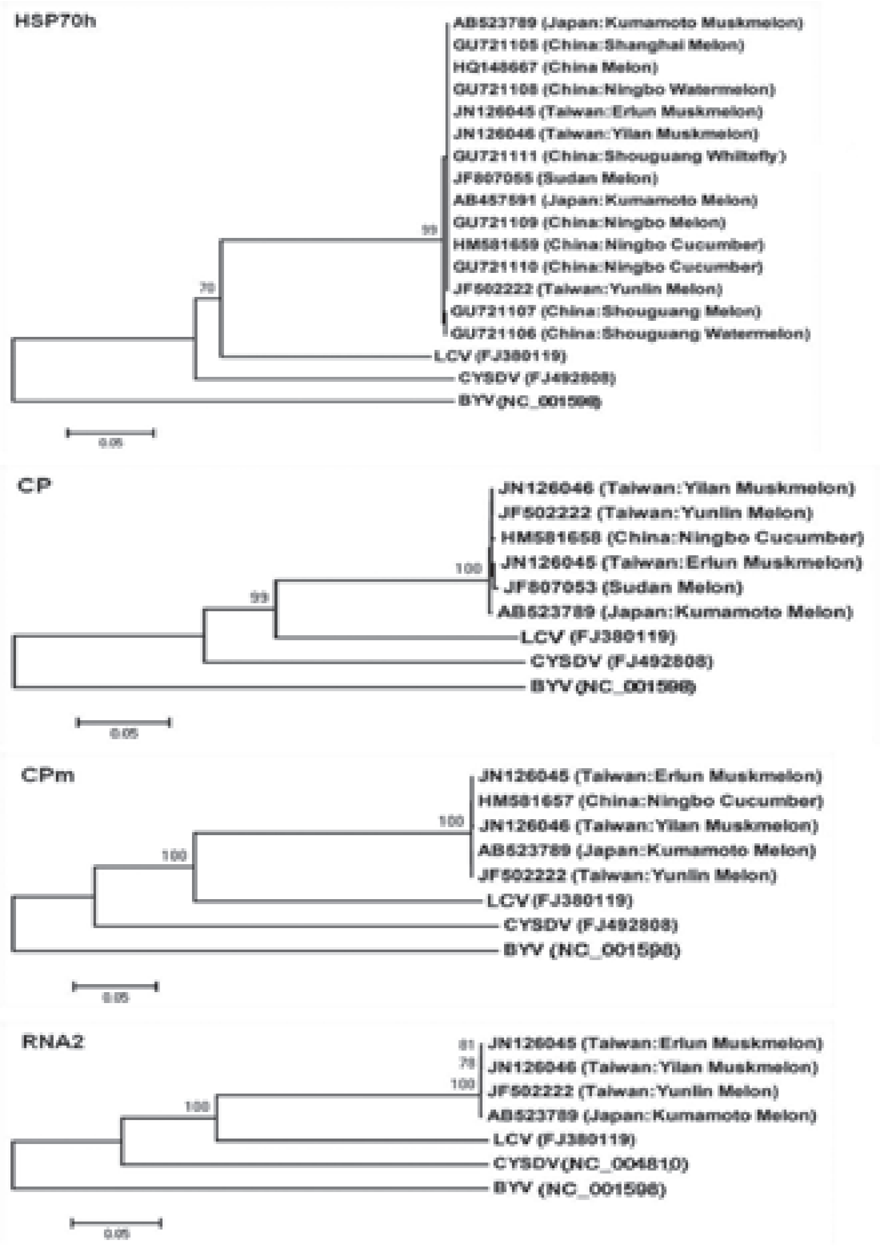
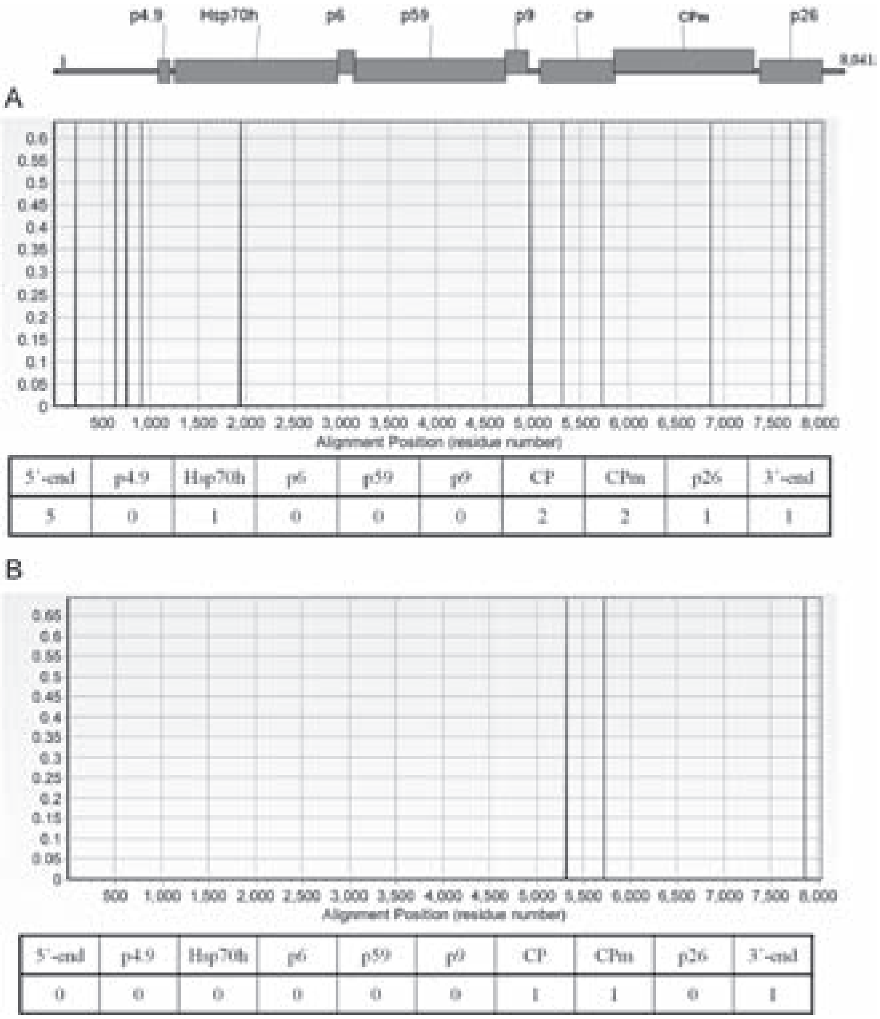


Fig. 2. Phylogenetic dendrograms of respective genes of heat shock protein 70-kDa homologue (*HSP70h*), coat protein (*CP*), minor coat protein (*CPm*) and RNA2 of *Cucurbit chlorotic yellows virus*. Each tree was generated by neighborhood-joining method (MEGA4). Horizontal branch length is proportional to the estimated number of nucleotide substitutions, while boot strap probabilities (shown as percentage) were indicated above or below the internal branches, only values greater than 70% are shown. GenBank accession numbers of the nucleotide sequences used in the phylogenetic analyses are shown and their details are listed in Tables 2 and 3. In addition, CYSDV (NC_004810) is a complete RNA2 sequence of *Cucurbit yellow stunting disorder virus*, Crinivirus, isolated from melon in Spain (Aguilar *et al*. 2003); and BYV (NC_001598) is a closterovirus, set as an outgroup.



5'-end	p4.9	Hsp70h	p6	p59	p9	CP	CPm	p26	3'-end
5	0	1	0	0	0	2	2	1	1

5'-end	p4.9	Hsp70h	p6	p59	p9	CP	CPm	p26	3'-end
0	0	0	0	0	0	1	1	0	1

Fig. 3. The entropy (Hx) plot generated with BioEdit v 7.053 showing the nucleotide sequence variability among two Taiwan isolates (JN12604, JN126046) and a Japan isolate (AB523789) of *Cucurbit chlorotic yellows virus* RNA2 (A), and the difference between two Taiwan isolates (B). On the top, the diagram represents the genomic organization of CCYV RNA2; and the numbers of different nucleotides are shown at the bottom of each diagram in corresponding to the names of respective ORFs.

alignment of the entire sequence of RNA2 with 8041 nt, only 12 nt were different as recognized by BioEdit and the major sequence divergences were located in the 5' and 3' end non-translated regions shown by entropy plots (Fig. 3). The sequence variability between the two indigenous Taiwan isolates was localized in 3 divergent nucleotides located respectively in regions of *CP*, *CPm*, and 3'-end. The remarkable homogeneity and extremely close cluster in phylogenetic trees (Fig. 2) of all CCYV nucleotide sequences tested indicate that all the currently existing CCYV isolates are likely derive from the same origin which was found in Kumamoto Prefecture, Kyushu, Japan.

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台灣瓜類退綠黃化病毒 RNA2 之全長核苷酸序列及其與現有株系的比較[1]

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摘 要

林羿廷、廖吉彥、蔡錦慧、鄧汀欽。2012。台灣瓜類退綠黃化病毒 RNA2 之全長核苷酸序列及其與現有株系的比較。台灣農業研究 61:132–143。

瓜類退綠黃化病毒 (*Cucurbit chlorotic yellows virus*, CCYV) 雲林縣二崙鄉分離株 (CCYV-Erlun) 與宜蘭縣壯圍鄉分離株 (CCYV-Yilan)，以反轉錄聚合酶鏈式反應 (RT-PCR) 及 Rapid amplification of 5’ complementary DNA ends (5’ RACE) 等方法將其 RNA2 完全解序，各得全長為 8041 bp 之核苷酸序列；除 5’- 和 3’- 未轉譯端外，包括 *p4.9* 基因 (132 nt)、*HSP70h* 基因 (1671 nt)、*p6* 基因 (165 nt)、*p59* 基因 (1554 nt)、*p9* 基因 (240 nt)、*CP* 基因 (753 nt)、*CPm* 基因 (1425 nt) 及 *p26* 基因 (642 nt)。利用其中 CP 基因序列設計引子對進行 RT-PCR，可提高 CCYV 檢測的敏感性與準確性。藉由 RNA2 核苷酸序列比對及親緣分析，結果顯示於台灣發現之CCYV-Erlun (JN126045) 及CCYV-Yilan (JN126046) 都與日本、台灣、中國及蘇丹之 CCYV 相關核苷酸序列相同度達 99–100%。熵圖 (Entropy plot) 顯示兩個台灣分離株與日本原始分離株的 RNA2 核苷酸差異只有 12 nt (在總數 8041 nt 中)，且主要都發生在 5’- 及 3’- 端未轉譯區。本研究發現目前全球各地發生之 CCYV 分離株彼此間基因同質性極高，親緣關係非常近，因此推論這些病毒都是同一來源。

關鍵詞：瓜類退綠黃化病毒、反轉錄聚合酶鏈式反應、親緣分析、熵圖。

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1. 行政院農業委員會農業試驗所研究報告第 2679 號。接受日期：101 年 5 月 2 日。
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