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Molecular Detection of Whitefly-Transmissible Geminiviruses (Family *Geminiviridae*, Genus *Begomovirus*) in the Philippines

Dennis B. Bela-ong[1*] and **Narceo B. Bajet**[2, 3]

[1]Genetics and Molecular Biology Division, Institute of Biological Sciences
University of the Philippines Los Baños, College, Laguna
[2]United States Department of Agriculture, Agricultural Research Service
Konnowac Pass Road, Wapato, WA, USA
[3]Washington State University Prosser, Irrigated Agricultural Research and
Extension Center, Hamilton Hall, N Bunn Rd., Prosser, WA, USA

Whitefly-transmissible geminiviruses (family *Geminiviridae*, genus *Begomovirus*) were detected by the polymerase chain reaction (PCR) in total nucleic acid preparations of tomato and squash leaf samples from different areas in the country. *Begomovirus* DNA fragments were detected by PCR using 3 sets of degenerate primers that amplify different regions of the genomic A DNA component of begomoviruses. The core coat protein primer pair gave the diagnostic ~560 bp amplicons; the top half primers produced ~1.6 kb PCR products; and the bottom half primers yielded ~1.2 kb amplicons. Not all samples produced amplicons of the expected sizes in PCR. Some samples produced bands in all 3 primer sets, some in only 2 of the 3 sets of primers, while some produced PCR fragments in only 1 of the 3 primer pairs, which suggests variation in the virus DNA sequence in terms of the presence or absence of primer annealing sites. Southern blot analysis using as probe the PCR product amplified from a top half clone of the genomic A DNA component of the Philippine isolate of *Tomato leaf curl geminivirus* (ToLCV-Ph), confirmed the *Begomovirus* identity of the top half primer pair-generated amplicons. Results confirmed that begomoviruses are responsible for some tomato and squash leaf curl diseases and the prevalence of these diseases threatens sustainable production of tomato and squash in the country.

Key Words: whitefly-transmissible geminiviruses, *Begomovirus*, polymerase chain reaction (PCR), Southern blot analysis, leaf curl disease, tomato, squash, E-value

INTRODUCTION

Whitefly-transmissible geminiviruses (family *Geminiviridae*, genus *Begomovirus*) are serious pathogens of a large number of important crop plants in tropical and subtropical areas worldwide. Many of the diseases that they cause are among the world's most economically significant diseases, resulting in yield losses ranging from negligible to 100% and amounting to more than US$7 billion in value of food and export crops (Thresh et al. 1997; Engel et al. 1998; Briddon et al. 2001). Economically important diseases caused by begomoviruses include leaf curl and mosaic diseases of cassava, cotton, tomato, pepper, tobacco, bean, and cucurbits. Begomoviruses infect dicots and are vectored by the whitefly *Bemisia tabaci* (Gennadius).

*Corresponding author: dbelaong@yahoo.com
dennis.bela_ong16@nus.edu.sg
Dennis.Bela-Ong@stud.unibas.ch

In the Philippines, leaf curl disease of tomato is one of the most prevalent, particularly during March to May (Truong 1981; Soriano et al. 1988) and can drastically reduce yield. Retuerma et al. (1971) and Benigno (1976) reported studies on the transmission by *B. tabaci* of the causal agent of tomato leaf curl disease, which was named *Philippine tomato leaf curl virus* (PTLCV). Based on host range, Retuerma et al. (1971) concluded that PTLCV was not identical with either *Tomato yellow leaf curl virus* (TYLCV)-Israel reported by Cohen and Nitzany (1966) or *Tobacco leaf curl virus* (TLCV).

Dolores and Bajet (1995) provided conclusive evidence that a geminivirus, *Tomato leaf curl virus* (ToLCV) causes tomato leaf curl disease in the Philippines. The virus reacted with anti-TYLCV monoclonal antibodies and hybridized with TYLCV nucleic acid probes. The study partially characterized the local *Tomato leaf curl virus* (ToLCV). However, there had not been many studies conducted to ascertain the full identity of the local geminiviruses and their relationship with other reported geminiviruses. In 2002, Kon et al. showed that the Philippine isolate of *Tomato leaf curl virus* (ToLCV-Ph) is a monopartite *Begomovirus*, and the single circular DNA molecule consisted of 2755 nucleotides. Nucleotide sequence comparisons of ToLCV-Ph genomic DNA with those of other begomoviruses revealed significant but relatively low sequence identity, signifying that it is a distinct geminivirus (Kon et al. 2002).

On the other hand, leaf curl disease of squash was a major constraint in squash production in some farms in Sta. Cruz, Laguna. Affected plants are stunted and their leaves exhibit severe deformations such as reduction in size and upward curling, vein enlargement, discoloration of interveinal areas, and enations. The disease agent was transmitted in a semi-persistent manner by *B. tabaci* (Benigno 1978). Squash plants exhibiting these symptoms had been observed in a number of locations wherever tomato leaf curl disease and whiteflies occur. The disease is the most destructive of all virus diseases that afflict squash, in which the plant seldom yields marketable fruits (Dolores & Valdez 1988). The disease had been attributed to *Begomovirus* infection because of its association with whiteflies and tomato leaf curl disease.

Kon et al. (2003) demonstrated that the causative agent of squash leaf curl disease in the Philippines is a bipartite *Begomovirus*. The A and B genomic DNA components consisted of 2739 and 2705 nucleotides, respectively. DNA-A sequence comparison with those of other geminiviruses showed that the squash leaf curl virus isolate from the Philippines had low sequence similarity to *Squash leaf curl virus* (SLCV) and *Squash mild leaf curl virus* (SMLCV), but was closely related to that of *Squash leaf curl China virus* (SLCCNV). Hence, the Philippine squash virus is considered an isolate of SLCCNV, and named *Squash leaf curl China virus*-Philippines (SLCCNV-Ph) (Kon et al. 2003).

Tomato leaf curl disease was reported a serious problem in Albay in 1968, which reduced yields up to 80% (Retuerma et al. 1971). The importance of the disease was recognized when outbreak occurred at the breeder's field and in the greenhouses of the Institute of Plant Breeding, University of the Philippines at Los Baños (IPB, UPLB) in March to May 1985. The disease caused significant reduction in yield and vigor of tomato plants (IPB 1986). There were also outbreaks in the greenhouses of the Department of Plant Pathology, UPLB. Similar abnormalities were observed in the field or in screenhouses of tomato growers in Laguna, Batangas, Cavite, Bulacan, and Nueva Ecija where whiteflies were also prevalent.

Reports of diseases attributed to begomoviruses have escalated in recent years. Some previously identified geminiviruses were found to infect new crops. In Pakistan, Mansoor et al. (2000) reported the prevalence of *Cotton leaf curl virus* in radish. *Tomato leaf curl virus* was found to cause yellow leaf disease in cantaloupe and wax gourd (Samretwanich et al. 2000b) and muskmelon (Samretwanich et al. 2000c) in Thailand. Begomoviruses have also spread to regions where they were previously unknown. For instance, *Tomato yellow leaf curl virus*-Israel had been identified in the Canary Islands (Font et al. 2000) and in The Bahamas (Sinisterra et al. 2000). Literature abounds with first reports of the occurrence of these diseases and new begomoviruses identified (Banks et al. 1999; Idris et al. 1999; Momol et al. 1999; Peterschmitt et al. 1999; Brown & Idris 2000; Idris & Brown 2000; Pappu et al. 2000; Pietersen et al. 2000; Raj et al. 2000; Roye et al. 2000; Mandal et al. 2001; Rodriguez et al. 2001; Samretwanich et al. 2000a).

Tomato and squash with varying types of leaf curl and mosaic symptoms collected from some tomato growing areas of Luzon were found positive for putative begomoviruses in nucleic acid hybridization using the Philippine tomato leaf curl DNA probe (Dolores 2000). At present, tomato leaf curl disease is becoming prevalent, affecting tomato production in major tomato production areas in the Philippines.

Effective management of these diseases entails early detection and breeding for resistance. Development of resistant cultivars necessitates a sound knowledge of the virus, its variants, and their distribution. This requires rapid and accurate techniques for virus detection and subsequent identification to facilitate studies of virus

epidemiology and genetic diversity. This information would be important in the design of more efficient crop protection strategies. This is particularly significant for begomoviruses, which exhibit unusual heterogeneity of nucleotide sequences among isolates from different countries (Navot et al. 1991).

While symptom-based detection remains helpful in initial disease identification, it is not reliable because disease symptoms due to viruses resemble those caused by other pathogens or by environmental factors. Symptoms also vary depending on the host genotype or the presence of mixed virus populations. The tools of molecular biology and biotechnology/genetic engineering offer novel and more precise ways for analyzing organisms because of their specificity and sensitivity, as well as an approach to obtaining non-conventional resistance for the control of virus diseases (Hull & Davies 1992).

This study aims to detect and identify local whitefly-transmissible geminiviruses using molecular techniques. The specific objectives are: (1) to amplify DNA fragments of local begomoviruses and (2) to confirm by Southern hybridization analysis the *Begomovirus* identity of the amplicons generated using top half primers, which amplify the top half of the A DNA genome component of most begomoviruses, containing regions that are among the highly conserved nucleotide sequences in the *Begomovirus* genome.

MATERIALS AND METHODS

Sources of Virus Collections

Several leaf curl samples of tomato and squash were collected from selected locations in the country. Each of the collected samples was considered as a separate collection and appropriately labeled (Table 1). Four tomato collections from the IPB- UPLB showing varying degrees of leaf curling, and 4 from the Bureau of Plant Industry (BPI), Los Banos showing different symptoms were obtained and correspondingly labeled (IPB-A, IPB-B, IPB-C, IPB-D, BPI-A, BPI-B, BPI-C and BPI-D). The 3 squash collections from BPI exhibiting varying degrees of leaf curling, enation, and vein thickening, as well as the 2 squash samples that showed varying degrees of leaf curling from Central Luzon State University (CLSU) were also correspondingly labeled (BPI-A, BPI-B, BPI-C, CLSU-A, and CLSU-B). Each sample showed 1 or more of the following symptoms typical of geminivirus infection: leaf curl, little leaf, crinkling and rugosity of leaves, yellowing, and stunting, and enation in some squash samples (Figure 1). In cases in which the author personally collected the samples, prevalence of whiteflies were observed. Some leaf samples harbored insect larvae when collected. The identity of the larvae was confirmed as *B. tabaci* by Marita D. Salinas, CLSU.

Table 1. Tomato and squash samples collected from different locations in the Philippines

SAMPLES	SYMPTOMS[a]	PLACE OF COLLECTION	COLLECTOR
Tomato			
PPTH	LC, St	Greenhouse, Department of Plant Pathology, UPLB	D. B. Bela-ong
IPB-A	LC	Greenhouse, Institute of Plant Breeding, UPLB	D. B. Bela-ong
IPB-B	LC	Experimental plot 1, IPB, UPLB	D. B. Bela-ong
IPB-C	LC	Experimental plot 2, IPB, UPLB	D. B. Bela-ong
IPB-D	LC	Experimental plot 3, IPB, UPLB	D. B. Bela-ong
HORTI	LC	Greenhouse, Department of Horticulture, UPLB	D. B. Bela-ong
BPI-A	LC, MMo	Experimental plots, Bureau of Plant Industry, Los Baños	D. B. Bela-ong
BPI-B	LC, Mo	Experimental plots, Bureau of Plant Industry, Los Baños	D. B. Bela-ong
BPI-C	LC, Ll	Experimental plots, Bureau of Plant Industry, Los Baños	D. B. Bela-ong
BPI-D	LC, SMo	Experimental plots, Bureau of Plant Industry, Los Baños	D. B. Bela-ong
NCPC	LC, Mo	Experimental plots, National Crop Protection Center, UPLB	D. B. Bela-ong
CAV	LC	Backyard garden, Bacoor, Cavite	D. B. Bela-ong
CLSU	LC	Experimental field, Central Luzon State University, Nueva Ecija	V. Viernes, Jr.
BAT	LC	Lipa, Batangas (field)	D. B. Bela-ong
ILO	LC	Backyard garden, Barotac Nuevo, Iloilo	E. B. Braga

continuation on page 90 . . . . .

Table 1 continued . . . .

SAMPLES	SYMPTOMS[a]	PLACE OF COLLECTION	COLLECTOR
Tomato			
LND	LC	Sugod, Sultan Naga Dimaporo, Lanao del Norte	I. Dimaporo
CDO	LC	Backyard garden, Gusa, Cagayan de Oro City, Misamis Oriental	I. Dimaporo
SAR	LC	Saranggani (field)	M.G. Dacuma
Squash			
BPI-A	LC, En, Vt	Experimental plots, Bureau of Plant Industry, Los Baños	D. B. Bela-ong
BPI-B	LC, En, Vt	Experimental plots, Bureau of Plant Industry, Los Baños	D. B. Bela-ong
BPI-C	LC, En, Vt	Experimental plots, Bureau of Plant Industry, Los Baños	D. B. Bela-ong
MARK	LC, En	San Pablo, Laguna (bought from Batong Malake public market, Los Banos)	D. B. Bela-ong
CAV	LC, En	Backyard garden, Bacoor, Cavite	D. B. Bela-ong
CLSU-A	LC	Experimental field, Central Luzon State University, Nueva Ecija	V. Viernes, Jr./ A. Manipon
CLSU-B		Experimental field, Central Luzon State University, Nueva Ecija	V. Viernes, Jr./ A. Manipon
PAN	LC, Mo	Pangasinan	N. B. Bajet
ILO	LC	Barotac Nuevo, Iloilo (backyard garden)	E. B. Braga
MAK	LC	Makato, Aklan (field)	R.I.D. Ballena
NUM	LC	Numancia, Aklan (field)	R.I.D. Ballena
SKUD	LC	Tacurong, Sultan Kudarat (field)	R.I.D. Ballena

[a] LC = leaf curling; St = stunting; Mmo = mild mosaic; Mo = mosaic; Ll = little leaf; Smo = severe, mosaic; En = enation; Vt = vein thickening

Amplification of *Begomovirus* DNA Fragments Using Degenerate Primers

Total nucleic acid was extracted from 1 g of leaf tissue taken from symptomatic and asymptomatic plants essentially as described by Gawel and Jarret (1991). Three sets of *Begomovirus*-specific degenerate primers (Table 2) were used in PCR. All sets of primers were provided by Dr. Christine Rey, University of the Witwatersrand, Johannesburg, South Africa.

The first set of primers (Av494 and Ac1048) had been designed to amplify the core coat protein of most begomoviruses and produces a ~560 bp fragment upon PCR (Wyatt & Brown 1996). The second set (PALIv1979 and PARIc715) had been designed to amplify the top half of the A DNA genome component of most whitefly-transmissible geminiviruses (WTGs) and yields a ~1.6 Kb product following PCR (Rojas et al. 1993). The third primer set (PARI722 and PALIc1960) produces a ~1.2 Kb band upon PCR and had been designed to amplify the bottom half region of the A genome component of most WTGs (Rojas et al. 1993). The sequences of the 3 sets of oligonucleotide primers are shown in Table 2. The relative binding positions of the primers and expected products are shown in Figure 2.

PCR was conducted with Biometra UNO-Themoblock (Gottingen, Germany) DNA thermal cycler. Reaction mixtures (25 µL) contained 1 µL of the DNA extract (~1 µg DNA) as template, 1.5 mM $MgCl_2$, 1x reaction buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 0.2 mM dNTPs, 30 pmol of each primer, and 2.5 U Taq DNA polymerase. The control consisted of all the reaction components minus the template DNA or using DNA from healthy plants as template. After a "hot start" procedure and the reaction mixtures overlaid with mineral oil, the cycling conditions were continued following the cycling protocols of Paximadis et al. (1997). The amplified products, along with 1 kb DNA ladder, were resolved in 2% agarose gel in Tris-borate EDTA (TBE), pH 8.0 buffer with 0.25 ug/mL ethidium bromide. Gel electrophoresis was carried out at 70 V until tracking dye has reached the bottom of the gel. The DNA bands were viewed and photographed using a gel documentation system (BioRad, Hercules, CA, USA).

Southern Analysis of PCR Products

Amplicons generated using the top half primers and fractionated by electrophoresis in 2% agarose gel were transferred to nylon membranes (Boehringer Mannheim cat.

Figure 1. Healthy and symptomatic tomato and squash. (A) A tomato plant showing symptoms of the leaf curl disease and (B) a tomato plant that is apparently free of leaf curl symptoms. (C) Leaf samples taken from squash plants exhibiting symptoms of the leaf curl disease and (D) squash leaves that are apparently free of leaf curl symptoms. (E) the underside of a leaf curl infected squash plant exhibiting enation (arrows) symptoms and (F) the underside of the leaf of an apparently healthy squash plant

Table 2. Oligonucleotide primers used for the amplification of *Begomovirus* DNA fragments

PRIMER[1]	SEQUENCES (5' 3')	DEGENERACY[2]
Av494[a]	GCCYATRTAYAGRAAGCCMAG	32
Ac1048[a]	GGRTTDGARGCATGHGTACATG	36
PALIv1979[b]	GCATCTGCAGGCCCACATYGTCTTYCCNGT	16
PARIc715[b]	GATTTCTGCAGTTDATRTTYTCRTCCATCCA	24
PARIv722[b]	ATATCTGCAGGGNAARATHTGGATGGA	24
PALIc1960[b]	TGGACTGCAGACNGGNAARACNATGTGGGC	128

[1]Nucleotides at degenerate positions are represented by a single letter of the IUPAC ambiguity code: D=A,G,T; H=A,C,T; K=G,T; M=A,C; N=A,C,G,T; R=A,G; W=A,T; Y=C,T.

[2]Degeneracy is the product of the numbers that designate the number of bases or nucleotides that may occur at each position in that primer.

[a]Nomenclature of primer is coded as follows: AV=viral sense primer (anneals to complementary sense strand of the genomic A DNA component and gives viral sense sequence); AC=complementary sense primer (anneals to viral sense strand and produces complementary sense sequence); number= nucleotide number for bean golden mosaic geminivirus from Puerto Rico used as the prototype CP core region (Wyatt & Brown, 1996).

[b]Nomenclature of primer is coded as follows: P=primer; ARI-open reading frame (ORF) for ARI; ALI=ORF for ALI; V=viral sense primer (hybridizes to complementary sense strand of the replicative form and produces viral sense sequence); C=complementary sense primer (hybridizes to viral sense strand and produces complementary sense sequence); number= nucleotide number for bean golden mosaic geminivirus from Guatemala for the 3' nucleotide primer (Rojas et al., 1993).

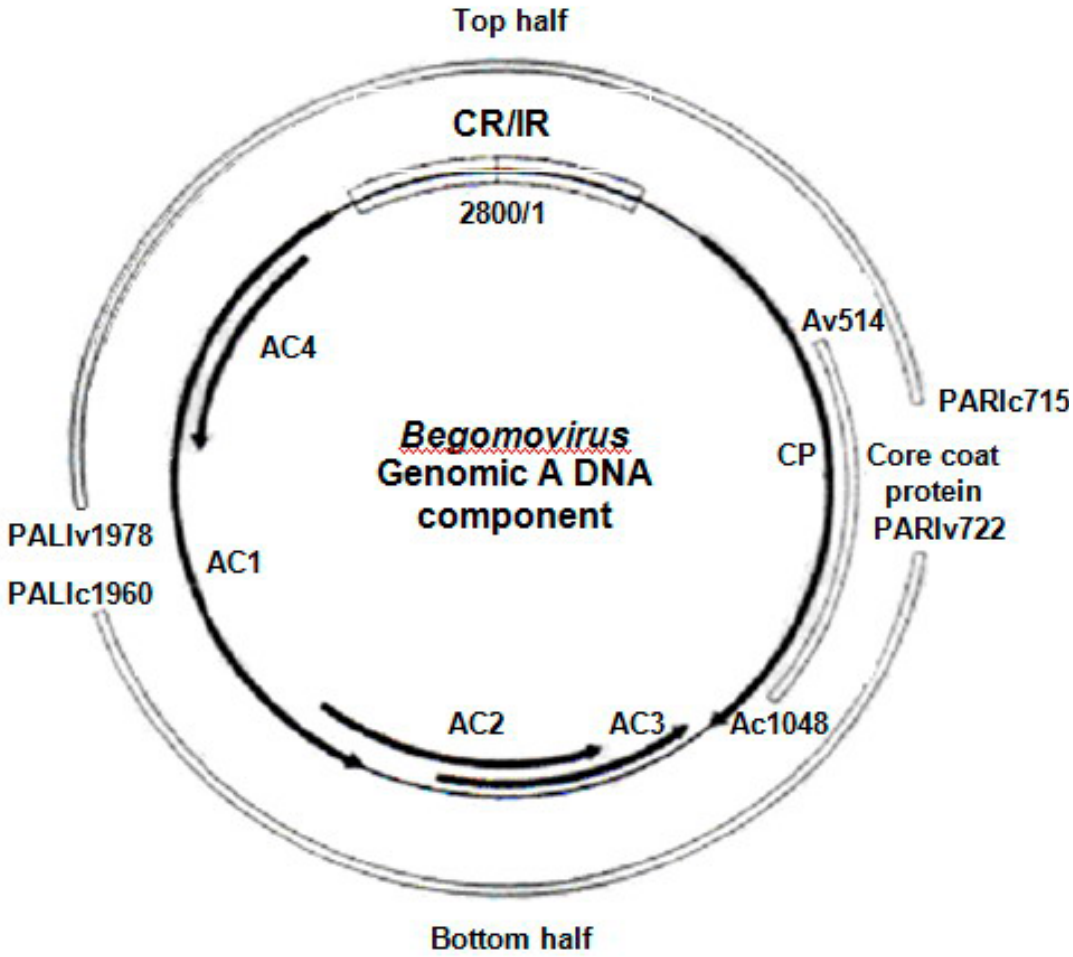

Figure 2. General *Begomovirus* A genomic DNA component map showing the relative annealing positions of the degenerate primers (Av514 and Ac1048, core coat protein primers; PALIv1978 and PARIc715, top half primers; PALIc1960 and PARIv722, bottom half primers) and corresponding amplicons and amplicon sizes

1417240) by the capillary method and baked-dry for 2h at 80°C as described by the Amersham Pharmacia Biotech EC nucleic acid labeling and detection protocol booklet (1999). The probe was labeled PCR product obtained using the top half primers and as template a clone of the top half region (~1.6 Kb) of genomic A DNA component of *Tomato leaf curl virus*-Philippine isolate (ToLCV-Ph), which contains partial rep gene, intergenic region and partial CP gene, inserted in the *Pst* I site of plasmid pBluescript II KS (+/-). The clone was kindly provided by Dr. Sylvia K. Green of the Asian Vegetable Research and Development Center (AVRDC), Taiwan, Republic of China and was propagated in *Escherichia coli* DH5α following the procedure of Sambrook et al. (1989) with some modifications. To generate the probe for Southern blot hybridization, 2 µL of the purified plasmid DNA was used as template and the top half primers in PCR. PCR components and cycling conditions were the same as in previous PCRs using the same primer pairs. The probe was labeled by random priming with digoxigenin (DIG)-UTP using the DIG DNA Labeling and Detection Kit of Boehringer Mannheim. Hybrids were detected by enzyme immunoassay using the chromogenic substrate nitroblue tetrazolium (NBT)/X-phosphate for alkaline phosphatase. Prehybridization was done in pre-warmed (68ºC) standard hybridization buffer (5X SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, and 1% blocking reagent) for 30 min in a hybridization oven (Techne, Inc., Princeton, NJ). The denatured DIG-labeled probe was added to pre-warmed standard hybridization buffer and mixed well. Hybridization was carried out at high stringency at 68ºC for 12 h. Washing of blots was carried out as follows: twice in 2X SSC, 0.1% SDS at room temperature for 5 min each; twice in 0.1X SSC, 0.1% SDS at 68ºC for 15 min each. After binding of an anti-DIG-alkaline phosphatase conjugate to the hybridized DIG-labeled probe, hybridization was detected by a purple color reaction initiated at alkaline pH by the addition of 5-bromo, 4-chloro, 3-indolyl phosphate and nitroblue tetrazolium salt. When the desired band intensities were achieved, the reaction was stopped by washing the membrane with distilled water for 5 min. Results were documented by photography.

RESULTS AND DISCUSSIONS

Amplification of *Begomovirus* DNA fragments from tomato and squash

Figures 3 and 4 show PCR amplification products resolved in agarose gels. Only 17 of the 30 samples produced amplicons of the diagnostic sizes in PCR (Table 3). Of these, 12 were tomato (out of 18 tomato samples) and 5 were squash samples (out of 12 squash samples).

Using the core coat protein primers, 13 samples (8/12 tomato samples and 5/5 squash samples) produced ~560 bp bands (Figure 3, lanes 6-9, 11-13, 18-19, 21, 23, and 25). IPB-C tomato sample also produced the ~560 bp amplicon (Bela-ong 2003). The top half primer pair amplified ~1.6 Kb *Begomovirus* DNA fragments (Figure 4) from 10 samples

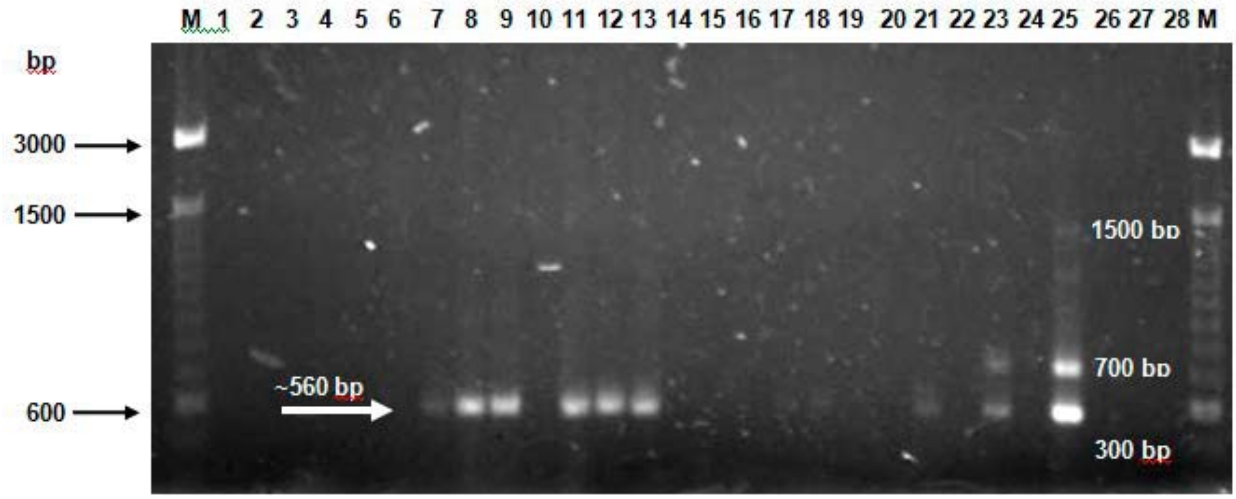

Figure 3. Polymerase chain reaction (PCR) amplification of ~560 bp DNA fragments from leaf total nucleic acid extracts of healthy or symptomatic tomato or squash using the core coat protein primers. M- 100 bp DNA ladder; Lane 1- No DNA (water control); 2-Healthy tomato; 3-Healty squash; 4- CDO Tomato; 5- LND Tomato; 6- NCPC Tomato (very faint); 7-CAV-Tomato; 8- BPI-A-Squash; 9- BPI-B-Squash; 10- BPI-C-Squash (very faint); 11- BPI-A Tomato; 12- BPI-B Tomato; 13- BPI-C Tomato; 14- BPI-D Tomato; 15- IPB-A Tomato; 16- IPB-B Tomato; 17- MARK Squash; 18- CLSU-A Squash (very faint); 19- CLSU-B Squash (very faint); 20- CLSU Tomato; 21-HORTI-Tomato; 22- Blank lane; 23- PPTH-Tomato; 24- Blank lanes; 25- PPTH Tomato; 26- Healthy tomato; 27- Healthy squash; 28- Distilled water only

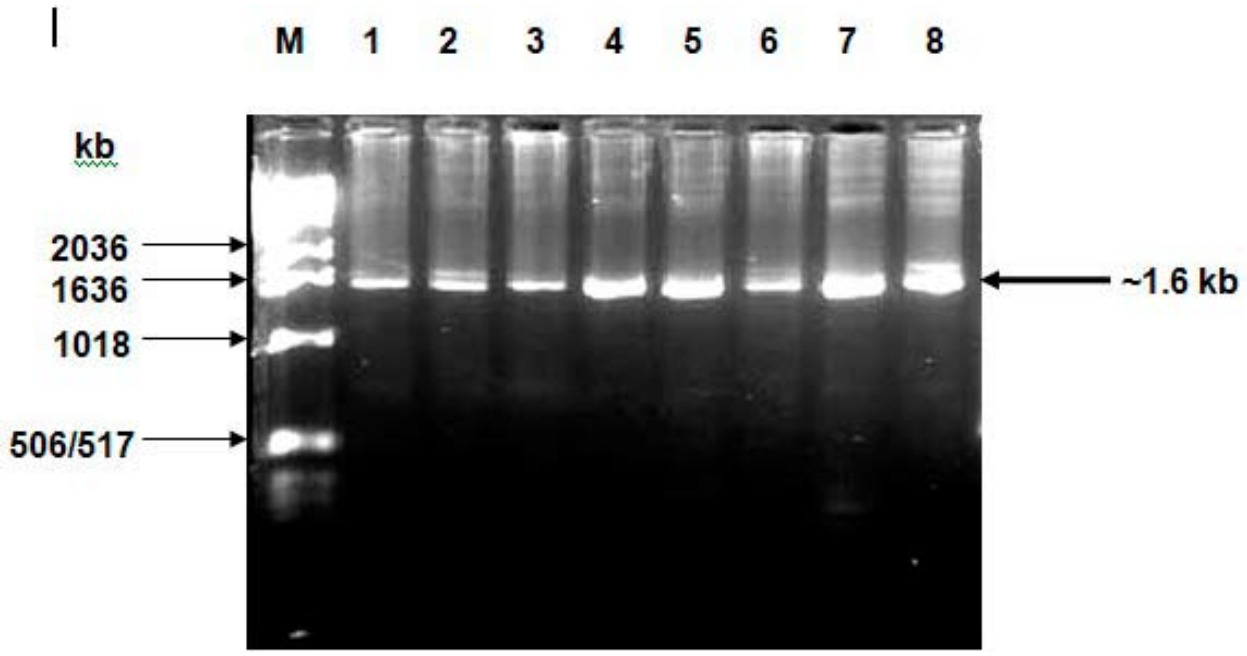

Figure 4. Polymerase chain reaction amplification of the diagnostic ~1.6 kb DNA fragments from leaf total nucleic acid extracts of tomato samples with leaf curl symptoms using the top half primers. M- 1 Kb DNA ladder; Lane 1- BPI-B; 2- BPI-C; 3- BPI-D; 4- PPTH; 5- IPB-A; 6- IPB-B; 7- IPB-D; 8- HORTI

Table 3. Amplification of *Begomovirus* DNA fragments from different leaf curl samples using 3 pairs of degenerate primers by the polymerase chain reaction (PCR)

SAMPLES/ TEMPLATES	PRIMER PAIRS		
	Core Coat Protein Primer (Av494 and Ac1048)	Top Half Primer (PAL1v1979 and PAR1c715)	Bottom Half Primer (PAR1v722 and PAL1c1960)
Tomato			
BPI-A	+	+	+
BPI-B	+	+	+
BPI-C	+	+	+
BPI-D	-	+	+
CAV	+	-	+
HORTI	+	+	-
IPB-A	-	+	-
IPB-B	-	+	-
IPB-C	+	-	+
IPB-D	-	+	-
PPTH	+	+	+
NCPC	+	+	-
Squash			
BPI-A	+	-	+
BPI-B	+	-	+
BPI-C	+	-	-
CLSU-A	+	-	+
CLSU-B	+	-	+
Total	13/17	10/17	11/17

(10/10 tomato samples), including tomato samples BPI-A and NCPC (data not shown). Bottom half primers generated ~1.2 Kb amplicons (data not shown) in 11 samples (7/12 tomato samples and 4/5 squash samples) using the bottom half primers (Bela-ong 2003).

The rest of the tomato samples (BAT, ILO, LND, CDO, and SAR) and squash samples (CAV, PAN, ILO, MAK, NUM, and SKUD) did not produce any PCR product with any of the 3 sets of degenerate primers, indicating the absence of WTGs in these samples. Yet the leaf curl symptoms typical of geminivirus infection cannot be discounted. WTGs may be present in the samples but were not detected by PCR due to contaminants in the total nucleic acids used as templates. A number of these samples were partially decomposed and total nucleic acid preparations from them have brownish color, most likely due to contamination with phenolic compounds. Polyphenols impart brown color to DNA preparations and interfere with restriction enzymes, ligases, and polymerases (Mannerlof & Tenning 1997) and that these bind to nucleic acids (Maliyakal 1992). While DNA can be extracted from dried tissues (Rojas et al. 1993), a major limiting factor in DNA suitable for molecular analyses is the presence of interfering plant secondary metabolites, especially polyphenols that get oxidized during prolonged periods between sample collections and processing. An approach that circumvents the need to ship fresh plant samples long distances and the usual requirement for extraction of total nucleic acids involves the direct absorption of a viral template from clarified extracts to polypropylene microcentrifuge tubes (Wyatt & Brown 1996). Another strategy is to print the cut edge of the plant's stem onto a nylon membrane in several spots and directly apply the printed material in the PCR reaction mixture (Nagata et al. 2004). These approaches were not employed in this study because the samples were directly sent to us from their respective places of collection.

It is also possible that begomoviruses are present in the samples but the primers may not be specifically targeted to hitherto uncharacterized *Begomovirus* DNA sequences, a typical situation with the emergence of WTGs as important plant pathogens in different locations around the globe.

Some samples produced PCR products with only 2 of the primers; there were samples that produced bands with all 3 degenerate primers, while some samples produced PCR DNA bands with only 1 primer pair (Table 3). That some samples could produce amplicons using a pair of primers but not with other primer pairs, could be due to the absence of sequences of sufficient complementarity in the genome of some of the viruses in the samples with which the primers would anneal, indicating to some extent variability among the viruses present in the samples tested. This could be confirmed by sequencing the genome of the viruses or the full-genome length PCR product.

In the design of the CP primer Av494, not all base differences in the alignment of the coat protein gene nucleotide sequences of geminiviruses were accounted for. Also, 5 alternate bases were omitted because of their rare occurrence (Wyatt & Brown 1996). The omission of the respective mismatched base might have hindered amplification of DNA fragments from some of the samples. In instances when the CP primers would not work, the bottom and/or top half primers may be used, which could reveal false negative results. A repetition of PCR using another set of primers could reveal a positive reaction. Only sequencing of the amplicons can reveal mismatches at certain positions in the primer, which could account for non-amplification in 1 set of primer but testing positive in another primer.

While the core coat protein primers had the most number of amplifications (Table 3), the number of samples that produced amplicons did not differ significantly with that obtained using the top and bottom half primers. Also, the number of samples tested may not be sufficient to say with certainty that the likelihood of detecting begomoviruses by PCR, at least in the Philippines, is higher with the use of the core coat protein primers than with the top half and bottom half primers.

No amplicon was produced in both healthy tomato and squash samples, as well as in the reaction in which distilled water was substituted for the template. These indicate that the primers were specific for the DNA of the viruses being detected. Also, the specificity of these 21-mer to 30-mer primers for amplifying *Begomovirus* A component sequences, was evaluated/verified/confirmed *in silico* by BLASTing 16 to 20 degenerate primer sequence combinations to GenBank using the Search for Short Nearly Exact Match of the nucleotide BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990) module of NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov/blast). At least 16 positive matces were obtained for each of the primers and 20 alignments with the lowest expectation values (E- values) from the BLAST search (Bela-ong 2003). The E-values are displayed in the report containing the results of BLAST analysis that compared the primer sequences with entries in the databank. E-values show the number of chance hits in database search. The match is significant the closer the value is to zero (http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=handbook.glossary.1237). E-values give information regarding the likelihood that a given sequence alignment is significant. The E-value of an alignment indicates the number of alignments with a score greater than or equal to the score of the observed

alignment in a search against a random database. Based on the search results, different combinations in the degenerate positions of each of the degenerate primers matched known sequences of *Begomovirus* A genome component, and are indeed targeted to amplify these sequences. In most cases, matches with *Begomovirus* sequences produced the most significant alignments i.e., such alignments have the lowest E-values. An E-value of 5 or 10 is high and indicates that the alignment probably has occurred by chance, and that the query sequence has been aligned to an unrelated sequence in the database (Gibas & Jambeck 2001). The E-values of the positive matches obtained in this study fell within the E-value cutoff of 0.1 or 0.05, hence, these matches were obtained from alignments with closely related sequences in the database, in this case, *Begomovirus* sequences. It is thus expected that the PCR fragments that could be amplified using these primers would be *Begomovirus* DNA fragments and such amplification will indicate the presence of *Begomoviruses* in the samples tested.

The 3 pairs of primers were designed from the conserved regions of the genomic A DNA component of most Begomoviruses. Of these regions, the coat protein gene sequences from which the coat protein primers were designed are the most highly conserved within the *Geminiviridae* family (Harrison & Robinson 1988). The CP primers were designed to hybridize to 2 highly conserved sequences that flank the capsid protein gene, the most highly conserved gene of Begomoviruses. By design, the CP primers specifically target the middle or 'core' region of the coat protein gene of WTGs, and have been used to amplify *Begomovirus* coat protein gene fragments from plants infected with biogeographically diverse WTG isolates irrespective of the host plant. (Wyatt & Brown 1996). The CP primers did not amplify virus genes from plants infected with geminiviruses of different genera, thus, the primer pair is specific for assaying WTGs. Brown et al. (1994) as cited by Wyatt and Brown (1996) have demonstrated the versatility and broad spectrum capability of the CP primers in more than 80 isolates of WTGs.

The top half primer pair (PAR1c715 and PAL1v1978) was designed to amplify the top half region of the genomic A DNA component of Begomoviruses (Rojas et al. 1993), amplifying part of the rep gene, the cp gene, and the common region (CR) (Roye et al. 2000). PAR1c715 anneals to the viral sense strand of AR1 open reading frame while PAL1v1978 hybridizes to the complementary sense strand of the replicative form of AL1 sequence that encodes the conserved amino acid sequence TGKTMWA, a putative NTP-binding site present in proteins associated with viral replication (Gorbalenya & Koonin 1989; Hanson et al. 1991). These regions are among the highly conserved nucleotide sequences in the *Begomovirus* genome. PAL1v1978 had been used with another primer and had been able to produce 1.1 kb amplicons from geminivirus-infected samples from the Western Hemisphere and 1.4 kb PCR products from samples obtained from Africa (Rojas et al. 1993), consistent with observations that Eastern Hemisphere geminiviruses possess larger genomic A DNA components than those from the Western Hemisphere (Stanley & Gay 1983; Navot et al. 1991 as cited by Rojas et al. 1993). These results have demonstrated the ability of PCR to discriminate among geminiviruses coming from different geographical regions based on the sizes of their genomic A DNA components. The PAR1c715 and PAL1v1978 pair had been used to amplify ~1.6 kb genomic A DNA fragments from tomatoes infected with geminiviruses from India and Taiwan (Rojas et al. 1993). Roye et al. (1997) used the same primer pair to amplify 1.3 kb fragments from geminiviruses associated with weed species from Jamaica. In this study, the use of the same primer pair resulted in the amplification of ~1.6 kb genomic A DNA fragments. This indicates that the top half segments of the genomic A DNA component of the Begomoviruses in this study have similar sizes with those from India and Taiwan but not with that of Begomoviruses from Jamaica.

On the other hand, the bottom half primers (PAR1v722 and PAL1c1960) were designed to amplify the bottom half region of the A component of the genome of Begomoviruses. These were used to detect *Begomovirus* infection in some tobacco leaf curl samples in southern Africa (Paximadis & Rey 1997) and in South India (Paximadis et al. 2001).

Of the samples that produced PCR fragments of the expected sizes for the core coat protein primer pair, additional bands were observed with the PPTH tomato sample. In addition to the diagnostic ~560 bp fragment, fragments of the sizes ~1500 bp, ~700 bp and ~300 bp were also observed (Figure 3, Lanes 23 & 25; Bela-ong 2003). The bands with sizes greater than ~560 bp could be amplified *Begomovirus* DNA fragments, which could have insertions within the region flanked by the annealing sites of the primers used. On the other hand, the band whose size is less than ~560 bp could be amplified *Begomovirus* DNA fragments that could have deletions within the region flanked by the annealing sites of the primers used. Such insertions and/or deletions could have been due to non-homologous recombination that may have resulted in the addition or deletion of DNA fragments, hence the larger (for those with insertions) or smaller (for those with deletions) the size of the additional bands compared with the bands of the expected size. Similar observations had not been noted in any of the literatures so far reviewed. Analysis of these additional bands and the diagnostic fragment through nucleotide sequencing would confirm this hypothesis.

The core coat protein primers used to produce the diagnostic and additional fragments were degenerate (degeneracies of 32 and 36 for the forward and reverse primers, respectively). The template for PCR was the total nucleic acid extract that also possibly contains tomato DNA and DNA of other organisms and viruses that may have been present in the leaf tissue. The possibility of priming and amplifying cognate but undesired DNA sequences from other sources was ruled out by BLAST (Altschul et al. 1990) search for short nearly exact matches of the coat protein primer pair. No significant match was obtained with any of the possible sequences that could be present in the total nucleic acid extract (data not shown). Also, the control reaction with nucleic acid from healthy samples did not produce amplicons, hence non-specific amplification with tomato DNA did not occur.

In addition, the stringency of the annealing temperature used for the core coat protein primer pair was high, i.e. 55ºC annealing temperature and 50 mM KCl concentration, thus avoiding the possibility of mispriming. The fact that these additional bands are produced only using PPTH sample total DNA rules out the possibility of non-specific amplification from tomato DNA, and that these additional bands may be geminivirus sequences that are unique to geminiviruses present in the PPTH sample.

The addition of hot start PCR could have also increased the stringency and specificity of the PCR conditions. Hot start PCR prevents DNA synthesis in the first cycle when there is heating up to 95°C and the polymerase enzyme has not yet been added, thus providing a tighter control over the conditions that allow annealing of the template and primer, which can significantly reduce background amplification (Vandenbussche & Gerats 2002).

The results of PCR amplification confirms the presence of Begomoviruses in the samples tested. Amplification in at least 1 primer pair strongly indicates *Begomovirus* presence. Non-amplification from healthy samples rules out background amplification. The 3 primer pairs have been repeatedly used to detect the presence of begomoviruses (Rojas et al. 1993; Wyatt & Brown 1996; Zeidan et al. 1998; Roye et al. 1997; 1999).

Southern Hybridization Analysis

Eight of the 10 amplicons generated using the top half primers were blotted. Two amplicons from tomato samples BPI-A and NCPC were not blotted because these were already degraded. CP and bottom half amplicons were not blotted and probed because the available probe is not expected to hybridize to amplicons derived from other regions of the genomic A DNA component of Begomoviruses.

All the blotted top half amplified fragments gave strong hybridization signals with the probe under high stringency hybridization (68º C without formamide), and both low stringency (2X SSC, 0.1% SDS at room temperature) and high stringency (0.1X SSC, 0.1% SDS at 68º C) washing conditions. This indicates that each of these fragments contains nucleotide sequences complementary to that of the probe (Figure 5). This confirms that the top half amplicons from the different

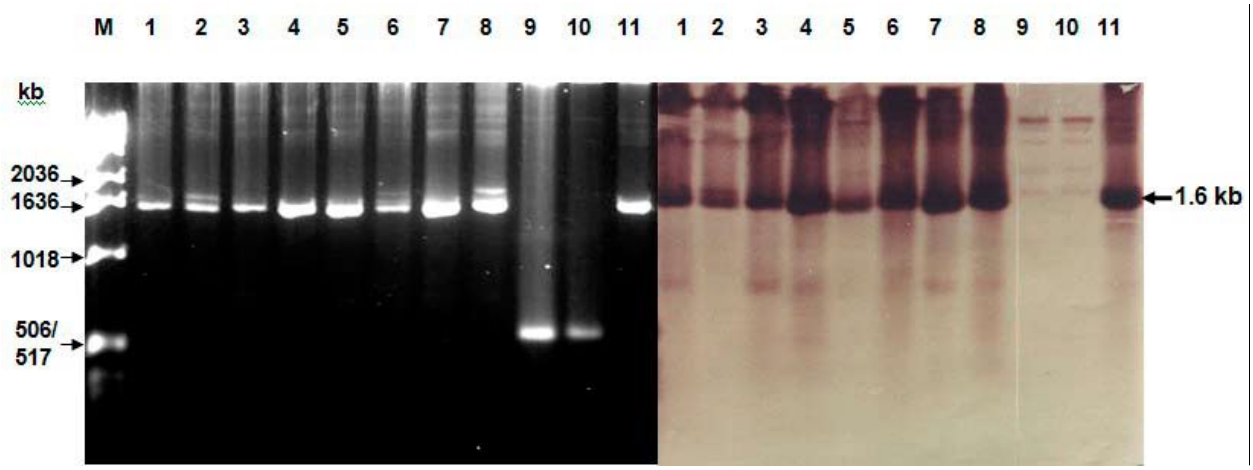

Figure 5. Southern blot hybridization of polymerase chain reaction-amplified ~1.6 kb DNA fragments obtained using the top half primer pair (PAL1v 1979 and PAR1c715) and leaf curl diseased tomato DNA extracts as templates (right photo). The amplicons blotted onto a nylon membrane was probed with digoxigenin (DIG)-labeled PCR product amplified from the top half clone of the Philippine isolate of tomato leaf curl virus (TLCV-Ph). DIG-labeled 1 kb ladder was used to hybridize to the marker DNA and was used together with the TLCV-Ph probe during hybridization. The photograph on the left shows appearance of the amplicons in an agarose gel prior to transfer to the membrane. M- 1 kb DNA ladder; Lane 1- BPI-B; 2- BPI-C; 3- BPI-D; 4- PPTH; 5- IPB-A; 6- IPB-B; 7- IPB-D; 8- HORTI; 9-10- Amplicons obtained using the coat protein primer (negative controls); 11- TLCV-Ph amplicon (positive control)

tomato leaf curl samples have *Begomovirus* origin or are authentic DNA fragment amplified from the respective *Begomovirus* DNA sequence. This also substantiates PCR results that Begomoviruses are present in the plant samples that exhibit symptoms typical of geminivirus infection.

CONCLUSIONS AND RECOMMENDATIONS

Results of PCR and Southern blot analysis prove that Begomoviruses are present in some tomato and squash leaf curl samples from Los Baños, Laguna, Bacoor, Cavite, and Munoz, Nueva Ecija. These suggest that Begomoviruses are involved in the leaf curl diseases sampled in this study. Together with the report of Dolores (2000), this study demonstrated that Begomoviruses are present in many areas of Luzon and confirmed through molecular analysis in this study that they are responsible for some of the tomato and squash leaf curl diseases. The increasing incidence of leaf curl diseases in tomato and squash growing areas in the country could be attributed to the emergence of these viruses, and threatens sustainable production of these 2 crops. This provides the opportunity for the detection and identification of Begomoviruses in larger number of samples from these areas. Any of the 3 primer pairs used in this study may be employed in PCR-based detection. Should 1 of the primer pairs fail to work, any of the 2 other primer pairs may be used to show false negatives. Negative results obtained because of poor quality DNA extracts from leaf samples necessitates the improvement of nucleic acid extraction procedures, particularly for dry leaf samples. A PCR-based assay that employ minimal sample preparation should also be developed if such assay were to become a practical way of virus detection in large number of samples. Detection of Begomoviruses across several locations and in large number of samples would facilitate detailed studies on the distribution of still uncharacterized Begomoviruses and genetic diversity among Begomoviruses in the Philippines. Such information is needed in formulating effective and durable crop protection strategies such as breeding for and deployment of resistant cultivars to Begomoviruses specific to individual production areas, targeting of conserved nucleotide sequences for generating resistant cultivars through genetic engineering, and using variable sequences to design specific diagnostic tools to identify virus isolates in different locations.

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