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Research Article

Development of Molecular based Method for Specific Detection of *Begomovirus* Species Causing Tomato Leaf Curl in the Philippines

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ABSTRACT

Tomato is one of the most important vegetable crops due to its economic and nutritional value but due to a disease caused by a *Begomovirus*, tomato production declined over the years. *Begomoviruses* causing tomato yellow leaf curl or tomato leaf curl affect tomato production in many tropical and subtropical areas of the world. Limited detection on these plant viruses made them so difficult to detect and control. The study aims to develop molecular based method for specific detection of ToLCPV and ToLCCeV. This was done by: 1) validating whether the published primers could anneal to a specific *begomovirus* species using multiple sequence alignment, 2) designing a primers for specific detection of ToLCPV or ToLCCeV and optimized by PCR, 3) validating the designed primer for specific detection of ToLCPV and ToLCCeV using field collected samples, 4) To identify RFLP pattern using full length genome DNA in silico. Published primers were able to anneal to ToLCPV strains and ToLCCeV, ToLCMiV species and thus these primers need to be validated in an actual experiment to determine their specificity. Primers ToLCPV19 and ToLCCeV19 were designed to specifically detect ToLCPV and ToLCCeV species respectively and PCR was optimized to a different annealing temperatures: 53°C, 55°C, and 57°C. A ~200 bp amplicons were observed on tomato samples infected with ToLCPV and also tomato samples infected with ToLCCeV when using ToLCPV19 and ToLCCeV19 primers. Also, there was no difference in the band size and the intensity of the bands at different annealing temperatures. Validation of the designed primers were done but to only a few selected tomato samples and thus further validation should be done to determine the specificity of the designed primers. RFLP analysis showed that restriction enzyme EcoRI could distinguish ToLCPV, ToLCCeV, and ToLCMiV as it produced different number of bands per *begomovirus* species.

Keywords: *Begomovirus* species; Optimization; Polymerase chain reaction

INTRODUCTION

Tomato is one of the most important vegetable crops due to its economic and nutritional value. Tomatoes are rich sources of vitamins A, C and folic acid and contain a wide array of beneficial nutrients and antioxidants including alpha lipoic acid, lycopene, choline, folic acid, beta carotene and lutein. In the Philippines, tomato ranks fourth among major vegetables and root crops with a total production volume valued at around 3.9 billion Philippine pesos. Production volume of tomato reaches 280,130 metric tons in 2019 with three major tomato growing regions contributing the most of the tomato production in the Philippines; Northern Mindanao with a production volume of 150,990 metric tons or 56.8% of total production followed by Ilocos region with 8.7% and Zamboanga Peninsula with 6.9%. Ilocos region produces the most tomato for processing and Bukidnon produces the most tomato that is used as fresh [1-5].

Begomoviruses causing tomato yellow leaf curl or tomato leaf curl affect tomato production in many tropical and subtropical areas of the world. The first evidence of the tomato yellow leaf curl disease causing serious damage to tomato crops was recorded in

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1931 in Israel. Since then, the disease spreads and at some point between 1937 and 1952 it has been observed in the Middle East and it was caused by a virus that infects tomato showing leaf curl symptoms but the virus was not identified yet as TYLCV but it was collectively referred to as TYLCV like viruses which are responsible for the TYLCD. The disease is caused by several *begomovirus* species including Tomato Yellow Leaf Curl Virus (TYLCV) or Tomato Leaf Curl Virus (ToLCV) in different countries. *Begomoviruses* belong to Genus Begomovirus of the Family Geminiviridae with genome consisting of either monopartite or bipartite circular single stranded DNA genome. Bipartite *begomoviruses* have DNA-A and DNA-B as their genetic components with a total size of about 5.4 kb, while monopartite *begomoviruses* have DNA-A and lacks DNA-B component. *Begomoviruses* are transmitted by whiteflies (Bemisia tabaci) and infected plants show symptoms such as stunting, upward curling of leaves, chlorosis, and reduction in leaf size.

In the Philippines, tomato leaf curl disease has been first documented in 1968 in Albay, Bicol province. The outbreak of the disease was first recognized 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 1985. Similar disease was observed in the field or in screen houses of tomato growers in Laguna, Batangas, Cavite, Bulacan, and Nueva Ecija where white flies were also prevalent. The disease was observed during 1995 in a few patches, and the virus causing the disease was identified as a Geminivirus using serological assays. The Tomato leaf curl Philippines virus (ToLCPV) has been first identified as the cause of tomato leaf curl in the country. Later, other *begomovirus* species have been identified such as Tomato leaf curl Cebu virus (ToLCCeV). Tomato Leaf Curl Mindanao Virus (ToLCMiV) and Ageratum Yellow Vein Virus (AYVV). Both ToLCPV and ToLCCeV have been detected in Luzon and the Visayas (Cebu province) islands, while AYVV, ToLCCeV, and ToLCMiV in Mindanao Island.

Molecular methods such as Polymerase Chain Reaction (PCR) and Rolling Circle Amplification (RCA) are commonly used to detect *begomoviruses*. Detection by PCR generally involves the use of degenerate primers that amplifies the core coat protein of most *begomoviruses* and another degenerate primers that amplifies the top half of the DNA-A and amplifies the bottom half region of the A genome. In other countries, PCR detection using specific primers such as V2325 and C2714 allows amplification of Tomato yellow leaf curl virus from Israel and VS2308 and CS2698 allows amplification of TYLCV from Italy. Detection by RCA involves amplification of the full length genome of single stranded DNA [5-10].

Begomoviruses causing tomato leaf curl in the Philippines have been detected by PCR using degenerate primers but were not able to distinguish the different *begomovirus* species. Currenty, PCR detection using specific primers ToLCCeVSP-V and ToLCPVSP-V were designed by Tsai et al., 2011 to detect ToLCCeV and ToLCPV respectively. However, the specific primers were able to amplify those 2 viruses in 15 symptomatic tomato samples only and need to be validated using multiple sequence alignment. Therefore, there has been no available method for specific detection of *begomovirus* species particularly the most prevalent, ToLCPV or ToLCCeV in the Philippines. These *begomovirus* species have been identified through nucleotide sequencing of the full length viral genome. Since, they have less than 91% nucleotide sequence identity which is the species demarcation threshold for the classification of *begomovirus* species; they are classified into different virus species based on the International Committee on Taxonomy of Viruses (ICTV) criteria for *begomovirus* species demarcation. Thus, it is possible to develop a molecular based method to specifically detect ToLCCeV and ToLCPV by PCR using species specific primer or by restriction fragment length polymorphism of RCA amplified DNA. Specific detection of these viruses is important in mapping their geographic distribution in the country. It is also important for the development of new tomato varieties particularly in virus resistance screening against ToLCPV and ToLCCeV. Screening for resistance against these viruses is important for the deployment of the resistant varieties to areas where these varieties are effective.

General objective:

To develop molecular-based method for specific detection of ToLCPV and ToLCCeV

Specific objectives:

- To validate whether the published primers could anneal to ToLCPV, and ToLCCeV species using multiple sequence alignment;
- to design primers for specific detection of ToLCPV or ToLCCeV and optimize by PCR;
- to validate the designed primer by PCR for specific detection of ToLCPV and ToLCCeV using selected samples and
- To identify RFLP pattern using full length genome DNA in silico

MATERIALS AND METHODS

Time and place of study

The study was conducted at the Plant Virology and Diagnostic Laboratory (PVDL) at Institute of Weed Science and Plant Pathology (IWEP), College of Agriculture and Food Science, University of the Philippines Los Banos, College, Laguna, Philippines from January 2020 to May 2021.

Sample collection

Tomato leaf samples were collected from farmers fields located in three municipalities specifically Paoay, Batac and Dingras in Ilocos Norte. In each tomato farm, at least 10 leaf samples from infected tomato plants were collected. After collection, samples were stored in icebox and processed within 24 hr after collection or samples were stored in a refrigerator prior to processing at the PVDL. Furthermore, DNA samples from infected tomato plants collected from Tal-ot, Cagay, and Sudlon in Cebu from the Visayas; Digos in Davao; and Malaybalay in Bukidnon from Mindanao were provided by Dr. Maria Pascual. At least five DNA samples from each location were used for the analysis.

DNA Extraction

Extraction of DNA was conducted following the Dellaporta method. Leaves were punched in a 1.5 ml tube and dip in liquid nitrogen until it freezes for about 5-10 min. Tissues were ground until it becomes brittle. About 500 µL of 500 mM Dellaporta buffer was added to the tube and ground. This is followed by addition of 60 µL 20% SDS then vortexed for 2 min (50 counts). Tubes were incubated at 65°C for 10 min using thermo shaker. Incubated tubes were filled by about 160 µL of 5 M Potassium acetate and then tubes were vortexed for 2 min. Tubes were spun down at 14,000 rpm for 10 min and 450 µL of supernatant was collected and placed unto a new tube followed by addition of 225 µL of 90% isopropanol (ice cold) then vortex quickly. Tubes were spun down again at 14,000 rpm for 10 min and supernatant was discarded. An addition of 500 µL 70% EtOH was poured to the tube and spin again at 14,000 rpm for 5 min. Supernatant was removed, and pellets were air dried for 3 hrs followed by suspension with 50 µL 500 mM TE buffer [11-20].

Alignment of published primers with ToLCPV, ToLCCeV, and ToLCMiV species

Alignment of published primers with ToLCPV, ToLCCeV, and ToLCMiV species primers from published primers were aligned with different strains of ToLCPV sequences, different species of ToLCCeV and ToLCMiV using ClustalW in MEGA X software (Table 1).

Table 1: Published primers designed for detection of Philippine *begomoviruses* infecting tomato.

Degenerate primer	Sequence (5'-3')	Product size	Gene	Reference
PAL1v 1978	GCATCTGCAGGCCCAC ATYGTCTTYCCNGT	1.6 kbp	ALII	Rojas, et al.
PARIc715	GATTTCTGCAGTTDATR TTYTCRTCCATCCA	1.6 kb	ALII	Rojas, et al.
ToLCPV primer				
ToLCPVSP_V	AYCAYACAGAGAACGC TTTAC	831 bp	IR	Tsai, et al.
LB2433_V	ATGAGCACATGGAGAT GAGG	1454 bp	C4	Sta.Cruz, et al.
ToLCCeV primer				
ToLCCeVSP_V	TCATATTGGACCTTGAC AGCT	902 bp	IR	Tsai, et al.
BK2560_V	CGAAATTGTCTGGGAG GTGC	1617 bp	IR	Sta.Cruz, et al.
ToLCMiV primer				
ToLCMiVSP_V	CGCATCGAAGGTACGT CGTC	790 bp	IR	Tsai, et al.

Primer design

The procedure was based on the properties to consider in order designing a good and effective primer. Sequences of ToLCPV-LB5 isolate (KU946995.1) and ToLCCeV-BK isolate were used in designing the primers. Sequences were aligned using MEGA X software. The following were considered in designing the primers: the length of the bases (18-24), having 40%-60% G/C content, should start and end with 1-2 G/C pairs, melting temperature should be within the range of 50°C-60°C, primer pairs should have Tm within 5°C of each other; primers should not have complementary regions. Oligoanalyzer software was used to check the validity of the designed primers (Integrated DNA Technologies Oligoanalyzer, n.d.). The designed primers are expected to amplify the V1 gene of ToLCPV-LB5 and the other set is expected to amplify the V2 gene of ToLCCeV-BK.

Optimization of PCR amplification

The procedure was based on the protocol used by Lorenz. DNA samples were amplified using the designed primers. In a 15 µL per reaction mix, the polymerase chain reaction mixture contains 7.5 µL of 1 x of GreenTaq (green direct load reaction buffer), 0.6 µL of each 10 mM of forward and reverse primers, 1 µL of 100 ng/uL DNA template, and nuclease free water to a final volume of 15 µL. The amplification was carried out using thermo fisher thermo cycler. Sterile distilled water was added first before adding the other reagents. Computation was done to determine the exact volume of water to be added to the PCR

Mix. Optimization of PCR conditions was conducted for the designed primers. The key condition optimized was the annealing temperatures: 53°C, 55°C, and 57°C; and template concentrations: 100 mg, 50 mg, 5 mg, and 1 mg. Initial denaturation was set to 95°C for 10 min followed by denaturation of 95°C for 1 min which melts all dsDNA into ssDNA, annealing temperature was set to 5°C lower than the melting temperature of the primer pair (50°C-55°C) for 1 min to promote the binding to the template, and extension with the temperature increase to 72°C for 1 min in order for the hybridized primer to be extended. The cycle was repeated thirty times to allow production of multiple copies of the target DNA.

Detection of ToLCPV and ToLCCeV by PCR

All DNA samples from different locations were subjected to spectrophotometry to determine the quantity of DNA which is the basis in determining what DNA samples should be used. For each location, three DNA samples with high DNA quantity were used for the detection of ToLCPV and ToLCCeV. ToLCPV-LB5 primer was used to detect DNA samples from Paoay, Ilocos Norte and Los Banos and ToLCCeV-BK primer was used to detect DNA samples from Cebu and Bukidnon. PCR was conducted in a 15 μL reaction volume containing 5.3 μL of sterile d H2O, 7.5 uL of GreenTaq, 0.6 μL of reverse and forward primers and 1 μL of the DNA template.

Restriction fragment length polymorphism

RFLP pattern using RCA Amplification was done using five different restriction enzymes. About 7 μl of the RCA-products were digested with 5 units of the enzymes RsaI, HaeIII, MspI, HinfI, and AluI. Restriction fragments were separated by gel electrophoresis in a 2% agarose gel stained with and analyzed with an ImageMaster VDS (Pharmacia Biotech) using the software Image Master 1D Elite v 3.01.

RESULTS AND DISCUSSION

Primer sequence alignment with Philippine *begomovirus* sequences

Sequence alignment of the available primers for *begomovirus* detection was conducted to determine if these primers can be used for specific detection of *begomovirus* species such as ToLCPV, ToLCCeV, ToLCMiV infecting tomato in the Philippines. The primers used in the alignment are 1) degenerate primer PAL1v 1978 designed for whitefly transmitted Geminiviruses to anneal within the AL11 Open Reading Frame (ORF), which codes for a replication-associated protein; 2) ToLCPVSP_V designed from the intragenic region of ToLCPV; 3) LB2433_V designed from the C4 gene of ToLCPV; 4) ToLCCeVSP_V designed from the IR of ToLCCeV; 5) BK2560_V designed also from the IR of ToLCCeV; 6) ToLCMiVSSP_V designed from the IR of ToLCMiV. The number of mismatches and percent mismatch were observed in order to evaluate whether these published primer could be used to specifically detect different species of *Begomovirus* Sequence of each primer was aligned with ToLCPV species consisting of ToLCPV-A (LB4), ToLCPV-A (L3), ToLCPV-B, ToLCPV-B (P7), ToLCPV-C (LB5) and ToLCPV-C (Lag); ToLCCeV (BK2 and P2-1); and ToLCMiV species were used to align with each designed primer using MEGA X software through multiple sequence alignment(Table 2 and Figure 1).

Table 2: Number of mismatches and percent mismatch in relation with the total base pair length of each published primers when aligned with different Begomoviruses.

Begomoviruses	**Published primers**					
	PALv1979	**ToLCPVSP_V**	**LB2433_V**	**ToLCCeVSP_V**	**BK2560_V**	**ToLCMiVSSP_V**
ToLCPV-A (L3)	9 (30%)	0 (100%)	2 (10%)	7 (33.33%)	4 (20%)	11 (55%)
ToLCPV-B (LB4)	9 (30%)	0 (100%)	0 (100%)	8 (38.10%)	4 (20%)	11 (55%)
ToLCPV-B	8 (26.67%)	0 (100%)	0 (100%)	8 (38.10%)	4 (20%)	11 (55%)
ToLCPV-B (P7)	8 (26.67%)	0 (100%)	0 (100%)	7 (33.33%)	4 (20%)	11 (55%)
ToLCPV-B (SBN)	8 (26.67%)	0 (100%)	0 (100%)	8 (38.10%)	4 (20%)	11 (55%)
ToLCPV-C (LB5)	9 (30%)	0 (100%)	2 (10%)	7 (33.33%)	10 (50%)	11 (55%)
ToLCPV-C (Lag	7 (23.33%)	0 (100%)	2 (10%)	7 (33.33%)	6 (30%)	11 (55%)
ToLCCeV-P2-1	10 (33.33%)	4 (19.05%)	0 (100%)	0 (100%)	3 (15%)	11 (55%)
ToLCCeV-BK	10 (33.33%)	4 (19.05%)	0 (100%)	0 (100%)	0 (100%)	11 (55%)
ToLCMiV	10 (33.33%)	5 (23.81%)	1 (5%)	8 (38.10%)	5 (25%)	0 (100%)

Species/Abbrv	
1. PAL1v1978	--GCATCTGCAGGCCCACATYGTCTTYCCCGT--
2. ToLCPV-A_(L3)	CCCAGAGATCTAGCCCACATCGTCTTCCCCGTTC
3. ToLCPV-B_(LB4)	CCCAGAGATCTAGCCCACATCGTCTTCCCCGTCC
4. ToLCPV-B_(P7)	CCCAGTGATCTAGCCCACATCGTCTTCCCCGTTC
5. ToLCPV-B	CCCATAGACCTAGCCCACATCGTCTTCCCCGTCC
6. ToLCPV-B_(SBN)	CCCAAAGACCTAGCCCACATCGTCTTCCCTGTCC
7. ToLCPV-C_(LB5)	CCCAGAGATCTAGCCCACATCGTCTTCCCCGTTC
8. ToLCPV-C(Lag)	CCCAGAGTTCTAGCCCACATCGTCTTCCCCGTTC
9. ToLCCeV_(P2-1)	CCCAGAGATCTAGCCCACATCGTCTTTCCTGTTC
10. ToLCCeV_(BK2)	CCCAGAGATCTAGCCCACATCGTCTTTCCTGTTC
11. ToLCMiV	CCCAGTGACCTAGCCCACATCGTCTTCCCTGTCC

Figure 1: Multiple sequence alignment of degenerate PAL1v 1978 primer against sequences of Philippine *begomoviruses* infecting tomato: (1) PAL1v 1978 primer; (2) ToLCPV-A (L3); (3) ToLCPV-B (LB4); (4)ToLCPV-B (P7); (5)ToLCPV-B; (6) ToLCPV-B (SBN); (7)ToLCPV-C (LB5); (8) ToLCPV-C (Lag); (9) ToLCCeV-P2-1; (10) ToLCCeV-BK2; (11) ToLCMiV using Clustal in Mega X software.

Sequence of PAL1v 1978 primer did not match all ToLCPV strains A, B and C, as well as ToLCCeV and ToLCMiV species. Out of the total 30 bp length of the primer, it has seven mismatched base pairs or 23.3% mismatched when aligned with ToLCPV-Lag (C); nine mismatched base pairs (30% mismatched) with ToLCPV-B (LB4), ToLCPV-A (L3) and ToLCPV-C (LB5); eight mismatched base pairs (26.67% mismatched) with ToLCPV-B and ToLCPV-B (P7) that are all located at the 5' end of the sequence (Figure 1).

When the primer was aligned with ToLCCeV-BK2, and ToLCCeV-P2-1, it resulted in ten mismatched base pairs or 33.33% mismatched in which five are located at the 5'end of the sequence and two the 3' end of the sequence. The same result was observed when the primer was aligned with ToLCMiV.

Based on the alignment, PAL1v 1978 anneals and therefore may detect the ToLCPV, ToLCCeV, and ToLCMiV species since the primer mismatches mostly occur at the 5' end and not the 3' end of the sequences for ToLCPV, ToLCCeV and ToLCMiV. Specificity of the primer as whether it could attach to the target sequence or not depends on the location of the match or mismatch. Mismatches located in the 3′ end region (defined as the last 5 nucleotides of the 3′ end region) of a primer have significantly larger effects on priming efficiency than more 5′ located mismatches since 3′ end mismatches can disrupt the nearby polymerase active site. Furthermore, mismatches between primers and templates are known to affect both the stability of the primer template duplex and the efficiency with which the polymerase extends the primer and potentially leading to biased results or even PCR failure. Every mismatch, irrespective of its location within the primer sequence, will result in a decreased thermal stability of the primer-template duplex, thus potentially affecting PCR specificity (Figure 2).

Species/Abbrv	
1. ToLCPVSP_V	--AYCAYACAGAGAACGCTTTAC--
2. ToLCPV-A_(L3)	TAACCATACAGAGAACGCTTTACTG
3. ToLCPV-B_(LB4)	TAACCATACAGAGAACGCTTTACTG
4. ToLCPV-B_(P7)	TAACCATACAGAGAACGCTTTACTG
5. ToLCPV-B	TAACCATACAGAGAACGCTTTACTG
6. ToLCPV-B_(SBN)	TAACCATACAGAGAACGCTTTACTG
7. ToLCPV-C_(LB5)	TAACCATACAGAGAACGCTTTACTG
8. ToLCPV-C(Lag)	TAACCATACAGAGAACGCTTTACTG
9. ToLCCeV_(P2-1)	TAACCACACAGAAAATGCTTTGTTG
10. ToLCCeV_(BK2)	TAACCACACAGAAAATGCCTTGTTG
11. ToLCMiV	CAATCATACTGAGAATGCCTTGTTA

Species/Abbrv	
1. LB2433_V	--ATGAGCACATGGAGATGAGG--
2. ToLCPV-A_(L3)	GGATAAGCACGTGGAGATGAGGGC
3. ToLCPV-B_(LB4)	GGATGAGCACATGGAGATGAGGGC
4. ToLCPV-B_(P7)	GGATGAGCACATGGAGATGAGGGC
5. ToLCPV-B	GGATGAGCACATGGAGATGAGGGC
6. ToLCPV-B_(SBN)	GGATGAGCACATGGAGATGAGGGC
7. ToLCPV-C_(LB5)	GGATAAGCACGTGGAGATGAGGGC
8. ToLCPV-C(Lag)	GGATAAGCACGTGGAGATGAGGGC
9. ToLCCeV_(P2-1)	GGATGAGCACATGGAGATGAGGGC
10. ToLCCeV_(BK2)	GGATGAGCACATGGAGATGAGGGC
11. ToLCMiV	GTACGAGCACGTGGAGATGAGGGC

Figure 2: Multiple sequence alignment of ToLCPVSP_P and LB2433_V primers with sequences of Philippine *begomoviruses* infecting tomato: (1) ToLCPVSP_V and LB2433_V primer; (2) ToLCPV-A (L3); (3) ToLCPV-B (LB4); (4) ToLCPV-B (P7); (5) ToLCPV-B; (6) ToLCPV-B (SBN); (7) ToLCPV-C (LB5); (8) ToLCPV-C (Lag); (9) ToLCCeV-P2-1; (10) ToLCCeV-BK2; (11) ToLCMiV using ClustalW in Mega X software.

ToLCPVSP_V primer has a 100% match when aligned with ToLCPV-A (LB4), ToLCPV-A (L3), ToLCPV-B, ToLCPV-B (P7), ToLCPV-C (LB5), and ToLCPV-C (Lag). Out of 21 bp length of the primer, it has four mismatched base pairs or 19.05% mismatched with ToLCCeV-P2-1 including two mismatched base pairs with ToLCCeV-BK2 at the 3' end of the sequence. Five mismatched base pairs (23.81% mismatched) with ToLCMiV includes three mismatched base pairs at the 3' end of the sequences (Figure 2). Based on the number of mismatches at the 3' end of the sequence, this primer can anneal to ToLCCeV and ToLCMiV species due to less number of mismatches at the 3' end of their sequences (Figure 2).

LB2433_V primer has a length of 20 bps and has a 100% match when aligned with ToLCPV-A (LB4), ToLCPV-B, ToLCPV-B (P7) but has two mismatch base pairs or 10% mismatched with ToLCPV-A (L3), ToLCPV-C (LB5), and ToLCPV-C (Lag). It has a 100% match with ToLCCeV-BK2, and ToLCCeV-P2-1 at the 5' end of their sequence. One mismatched base pair (5% mismatched) with ToLCMiV species is at the 5' end of the sequences. Based on the number and location of the mismatched base pairs, the primers can anneal to all ToLCPV strains, and both ToLCCeV and ToLCMiV species (Figure 3).

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Species/Abbrv             *  ***** ****  * *  
1. ToLCCeVSP_V       --TCATATTGGACCTTGACAGCT--
2. ToLCCeV_(P2-1)    AATCATATTGGACCTTGACAGCTAT
3. ToLCCeV_(BK2)     AATCATATTGGACCTTGACAGCTAT
4. ToLCMiV           TTCTGTGTTGGACTTTGATGGGTAC
5. ToLCPV-A_(L3)     TTTTGTGTTGGACTTTGATGGGTAT
6. ToLCPV-B_(LB4)    TTTTGTGCTGGACTTTGATGGGTAC
7. ToLCPV-B_(P7)     TTTTGTGTTGGACTTTGATGGGTAC
8. ToLCPV-B          TTTTGTGCTGGACTTTGATGGGTAC
9. ToLCPV-B_(SBN)    TTCTGTGCTGGACTTTGATGGGTAC
10. ToLCPV-C_(LB5)   TTTTGTGTTGGACTTTGATGGGTAC
11. ToLCPV-C(Lag)    TTTTGTGTTGGACTTTGATGGGTAC

Species/Abbrv             **       ** *  *   
1. BK2560_V          --CGAAATTGTCTGGGAGGTGC--
2. ToLCCeV_(P2-1)    TTTGGAATTTTCTGGGAGGTGCCA
3. ToLCCeV_(BK2)     TTCGAAATTGTCTGGGAGGTGCCA
4. ToLCMiV           TTTGGAATTTTCTCGGTGGTGCCA
5. ToLCPV-A_(L3)     TTTTAAATGGTCTTGGAGGAGCCA
6. ToLCPV-B_(LB4)    TTTGGAATTTTCTGGGTGGTGCCA
7. ToLCPV-B_(P7)     TTTGGAATTTTCTGGGTGGTGCCA
8. ToLCPV-B          TTTGGAATTTTCTGGGTGGTGCCA
9. ToLCPV-B_(SBN)    TTTGGAATTTTCTGGGTGGTGCCA
10. ToLCPV-C_(LB5)   TTCTAAAACGACGGGGTGTAGGCA
11. ToLCPV-C(Lag)    TTTTAAAACGTTTGGGTGGTGCCA
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Figure 3: Multiple sequence alignment of ToLCCeV primers with sequences of Philippine begomoviruses infecting tomato: (1) ToLCCeVSP_V and BK2560 primer; (2) ToLCCeV-P2-1; (3) ToLCCeV-BK2; (4)ToLCMiV; (5) ToLCPV-A (L3); (6) ToLCPV-B (LB4); (7)ToLCPV-B (P7); (8) ToLCPV-B; (9) ToLCPV-B (SBN); (10)ToLCPV-C (LB5); (11) ToLCPV-C (Lag) using ClustalW in Mega X software.

Out of 21 total bp length of ToLCCeVSP_V, it has a 100% match when aligned with ToLCCeV-BK2 and ToLCCeV-P2-1 but the primer failed to match with ToLCMiV having eight mismatched base pairs or 38.1% mismatched including four mismatches at the 5' end and three mismatches at the 3' end of the sequence. It has eight mismatch base pairs (38.1% mismatched) with ToLCPV-A (LB4) and ToLCPV-B including four mismatches at the 5' end and 3 mismatches at the 3' end of the sequences.; seven mismatched base pairs (33.3% mismatched) with ToLCPV-A (L3), ToLCPV-B (P7), and ToLCPV-C (LB5) including three mismatches at the 5' end and three mismatches at the 3' end (Figure 3). Based on the number of mismatches and the location of mismatches, ToLCCeVSP_V can predicably detect both ToLCPV and ToLCMiV. The same with ToLCPVSP_V primer, this primer was used for cloning begomovirus species and not for specific detection. The result of Tsai, et al. contradicts the result from the alignment as ToLCCeV species were only detected using ToLCCeVSP_V primer which targets the IR of the virus. It implies that there are several factors involved for a primer to be specific and not only based on the number of mismatches.

BK2560_V primer, having a length of 20 bps, has a 100% match when aligned with ToLCCeV-BK2 but failed to match with ToLCCeV-P2-1 having three mismatched base pairs or 15% mismatched including two mismatches at the 5' end. The primer has five mismatched base pairs (25% mismatched) including two mismatches at the 5' end with ToLCMiV. It has four mismatched base pairs (20% mismatched) with ToLCPV-A (LB4), ToLCPV-A (L3), ToLCPV-B, and ToLCPV-B (P7), and ToLCPV-B (SBN) including two mismatches at the 5' ends of the sequences. ToLCPV-C (LB5) has the most mismatched base pairs having ten mismatched base pairs (50%), three mismatches at the 5' end of the sequence and four mismatches at the 3' end of the sequence. It was followed by ToLCPV- C (Lag) having six mismatched base pairs (30% mismatched) including four mismatches at the 5' end of the sequence. The same with LB2433_V primer, this primer could also detect ToLCPV and ToLCMiV species based on the number of mismatch base pairs at the 3' end of their sequences (Figure 4).

```
Species/Abbrv           * ** ****    *  *    
1. ToLCMiVSSP_V      --CGCATCGAAGGTACGTCGCC--
2. ToLCMiV           CCCGCATCGAAGGTACGTCGCCGT
3. ToLCCeV_(P2-1)    AATGTATAGAAGCCGTGATGTTCC
4. ToLCCeV_(BK2)     AATGTATAGAAGCCGTGATGTTCC
5. ToLCPV-A_(L3)     AATGTATAGAAGCCGTGATGTTCC
6. ToLCPV-B_(LB4)    AATGTATAGAAGCCGTGATGTTCC
7. ToLCPV-B_(P7)     AATGTATAGAAGCCGTGATGTTCC
8. ToLCPV-B          AATGTATAGAAGCCGTGATGTTCC
9. ToLCPV-B_(SBN)    AATGTATAGAAGCCGTGATGTTCC
10. ToLCPV-C_(LB5)   AATGTATAGAAGCCGTGATGTTCC
11. ToLCPV-C(Lag)    AATGTATAGAAGCCGTGATGTTCC
```

Figure 4: Multiple sequence alignment of ToLCMiV primer with sequences of Philippine *begomoviruses* infecting tomato: (1) ToLCMiVSSP_V primer; (2) ToLCMiV; (3) ToLCCeV-P2-1; (4) ToLCCeV-BK2; (5)ToLCPV-A (L3); (6) ToLCPV-B (LB4); (7) ToLCPV-B (P7); (8)ToLCPV-B; (9) ToLCPV-B (SBN); (10) ToLCPV-C (LB5); (11) ToLCPV-C (Lag) using ClustalW in Mega X software.

ToLCMiVSSP_V primer, having a length of 20 bp, has a 100%matched with ToLCMiV. The primer has 11 mismatch bases or 55% mismatched including five mismatches at the 5' end and five mismatches at the 3' end of the sequences when aligned with ToLCCeV-BK2, and ToLCCeV-P2-1. The primer also has 11 mismatch base pairs (55%) including five mismatches at the 5' end and five mismatches at the 3' end of the sequences when aligned with ToLCPV-A (LB4), ToLCPV-A (L3), ToLCPV-B, ToLCPV- B (P7), ToLCPV-C (LB5), and ToLCPV-C (Lag). This implies that ToLCMiVSSP_V primer could only detect ToLCMiV species and could not detect ToLCPV and ToLCCeV species due to the number of mismatched base pairs greater than five at the 3' end of their sequences.

PAL1v 1978 degenerate primer, when used to detect *Begomovirus* species, produces amplicon with a product size of 1600 bp. ToLCPVSP_V has a product size of 831 bp when used to amplify IR of ToLCPV. LB2433_V has a product size of 1454 bp when used to amplify the C4 gene of ToLCPV. ToLCCeVSP_V has a product size of 902 bp when used to amplify IR of ToLCCeV species. BK2560_V has a product size of 1617 bp when used to amplify IR of ToLCCeV. ToLCMiVSP_V has a product size of 790 bp when used to amplify IR of ToLCMiV species. Primers designed at the Intergenic Region (IR) were intended to capture all the variability among the isolates. Although these primers were intended for cloning ToLCPV and ToLCCeV species and not for specific detection, these primers

should be further validated using field-collected samples in order to determine their specificity.

Primers designed for specific detection of *begomovirus* species

Since the leaf curl disease in the Philippines is caused by *begomovirus* complex consisting of different virus species, there is a need to design a primer to specifically detect ToLCPV, ToLCCeV and ToLCMiV species. Primers for specific detection of ToLCPV, ToLCCeV or ToLCMiV were designed using Primer3 software. They were designed from the V2 gene of the virus based on the product generated by the Primer3 and their characteristics were checked using an Oligoanalyzer tool. The primers generated have the following features: 1) ToLCPV19 having a length of 22 bps (Forward) and 24 bps (Reverse), 45.45% GC for the forward and 41.67% GC for the reverse, melting temperature of 58.81 C (Forward), and 59.73% (Reverse). Hairpin structure value of -0.1 kcal.mole^{-1} and -1.47 kcal.mole^{-1}; 2) ToLCCeV19 with a length of 20 bps for the forward and 24 bps for the reverse. GC content of 55% (Forward) and 41.67% (Reverse), a melting temp of 59.82 C (Forward) and 59.73 C (Reverse). Hairpin structure of 0.47 kcal.mole^{-1} and a value-1.57 kcal.mole^{-1} for the self-dimer (Table 3).

Table 3: Forward and reverse primers for ToLCPV-LB5 and ToLCCeV-BK isolates.

Primer name	Sequence (5' - 3')	Length	% GC	Melting temp	Gene	Hairpin ΔG (kcal.mole-1)	Self-dimer ΔG (kcal.mole-1)
ToLCPV19	Forward:				V2	-0.1	-1.47
	5'- AAATGTGGG ATCCTCTGGT AC A-3'	22 bp	45.45%	58.81°C			
	Reverse:						
	5'- CTTCGGCATA ATTCTTACTT CG CA-3'	24 bp	41.67%	59.73°C			
ToLCCeV19	Forward:				V2	0.47	-1.57
	5' – GCGAACATG TGGGATCCT CT - 3'	20 bp	55%	59.82°C			
	Reverse:						
	5'- CTTCGGCATA ATTCTTACTT CG CA-3'	24 bp	41.67%	59.73°C			

The primer sequence determines several things that include the length of the product, its melting temperature and lastly the yield (Table 4). Forward primer ToLCPV19 (5'-AAATGTGGGATCCTCTGGTACA-3') has a length of 22 bp while its reverse primer (5'-CTTCGGCATAATTCTTACTTCGCA-3') has a length of 24 bp. Both specificity and temperature and time of annealing are at least partly dependent on primer length. Primers should be at least 18 nucleotides in length to minimize the chances of encountering problems with a secondary hybridization site on the vector or insert.

Primers with long runs of a single base should generally be avoided. It is especially important to avoid four or more G's or C's in a row. In terms of the GC content of the desired primer, primers should have a desired GC content between 40 and 60 percent. Our primer fits the criteria having a GC% of 45.45 for the forward primer and a GC% of 41.67 for the reverse primer. Percent GC is an important characteristic of DNA and provides information about the strength of annealing. Although the GC content of some primers would be less than 50%, it may be necessary to extend the primer sequence beyond 20 bases to keep the melting temperature above the recommended lower limit of 50°C. GC content, melting temperature and annealing temperature are strictly dependent on one another.

In terms of melting temperature, the forward primer has a Tm of 58.81 C while its reverse primer has a Tm of 59.73°C. The optimal melting temperatures for primers in the range 52°C-58°C generally produce better results than primers with lower melting temperatures. On the other hand, primers with melting temperatures above 65°C cause secondary annealing. A good working approximation of this value can be calculated using the formula of Wallace, et al., Tm=2(A+T)+4(G+C). The primer pair has a product size of approximately 147 bps. This primer shows good characteristics based on the length, value of melting temperature, GC content and a good value of hairpin structure (-0.1) and self-dimer (-1.47). A poorly designed primer can result in little or no product due to non-specific

amplification and/or primer-dimer formation, which could be enough to suppress product formation (Figure 5).

The forward primer of ToLCCeV19 (5'–GCGAACATGTGGGATCCTCT-3') has a length of 20 bp while its reverse primer (5'-CTTCGGCATAATTCTTACTTCGCA-3') has a length of 24 bp. This also makes a good primer based on the length as it is within 18-24 bps. In terms of GC content, the forward primer has a GC% of 55 and its reverse primer has a GC% of 41.67. Melting temperature of the forward primer is 59.82°C while the reverse primer has a melting temperature of 59.73°C. The primer pair has a product size of approximately 184 bps. This primer also showed good characteristics based on the length of bps, value of melting temperature, GC content and a good hairpin structure value of 0.47 and a good self-dimer value of -1.57. Primers are also prone to form dimers and false priming; thus, they should not contain any complimentary (palindromes) within themselves which form hairpins. It results in an unproductive priming event that decreases the signal obtained. Both self-dimers and hairpins Delta G value of ToLCPV19 and ToLCCeV19 primers are below the threshold level (-9.0 kcal/mole) which indicates that no actual secondary structure will form at all. In terms of Delta G value of any self-dimers, hairpins and heterodimers, weaker or more positive value than -9.0 kcal/mole is considered desirable (IDT, n.d.).

Table 4: Number of mismatches and percent mismatch in relation with the total base pair length of each designed primers when aligned with different *Begomoviruses*.

Begomoviruses	**Designed primers**	
	ToLCPV19	**ToLCCeV19**
ToLCPV-A (L3)	0 (100%)	8 (40%)
ToLCPV-B (LB4)	0 (100%)	8 (40%)
ToLCPV-B	1 (4.54%)	9 (45%)
ToLCPV-B (P7)	1 (4.54%)	10 (50%)
ToLCPV-B (SBN)	1 (4.54%)	9 (45%)
ToLCPV-C (LB5)	0 (100%)	8 (40%)
ToLCPV-C (Lag	0 (100%)	8 (40%)
ToLCCeV-P2-1	5 (22.73%)	0 (100%)
ToLCCeV-BK	5 (22.73%)	0 (100%)
ToLCMiV	6 (27.28%)	5 (23.81%)

Figure 5: Multiple sequence alignment of ToLCPV19 primer sequence with sequences of Philippine *begomoviruses* infecting tomato: (1) ToLCPVSP_V and LB2433_V primer; (2) ToLCPV-A (L3); (3) ToLCPV-B (LB4); (4) ToLCPV-B (P7); (5) ToLCPV-B; (6)ToLCPV-B (SBN); (7) ToLCPV-C (LB5); (8) ToLCPV-C (Lag); (9)ToLCCeV-P2-1; (10) ToLCCeV-BK2; (11) ToLCMiV using ClustalW in Mega X software.

ToLCPV19 primer was designed to amplify ToLCPV species and has a length of 22 base pairs. The primer matches 100% with both strain A of ToLCPV (ToLCPV-LB4 and ToLCPV-L3) and strain C of ToLCPV (ToLCPV-LB5 and ToLCPV-Lag). The designed primer has one mismatched base pair or 4.54% mismatched located in the 5' end of their sequences in both strain B of ToLCPV (ToLCPV, ToLCPV-SBN, and ToLCPV- P7). It also has five mismatched base pairs (22.73% mismatched) which include one mismatch at the 5' end and four mismatches at the 3' end when aligned with ToLCCeV- BK2 and ToLCCeV-P2-1 (Figure 5). It has six mismatched base pairs (27.28% mismatched) with ToLCMiV which includes one mismatch at the 5' end and four mismatches at the 3' end of the sequence. Mismatches are distributed at the 5' end and 3'ends that could result in a detection not only of ToLCPV species but also ToLCCeV and ToLCMiV species. Several factors could affect the specificity of primers. Aside from number of mismatch base pairs, type of mismatches such as purine-purine (A-A, A-G, G-A and G-G) and C-C mismatches had the most detrimental effect, followed by the remaining pyrimidine-pyrimidine mismatches (T-T, C-T, and T-C) and the relatively minor effects of purine-pyrimidine (A-C, C-A, G-T, and T-G) mismatches. ToLCPV19

primer has only one purine-purine mismatches (A-A) at the 3' end of the sequence when aligned with ToLCCeV-BK2 and ToLCCeV-P2-1 and one purine-pyrimidine mismatch (G-T) also at the 3' end of the sequence. Hence, the mismatches have a significant effect on the specificity of the primer which makes the primer not specific enough to only detect ToLCPV species (Figure 6).

Species/Abbrv						*			*	*	*	*	*	*	*				*					
1. ToLCCeV19	-	-	G	C	G	A	A	C	A	T	G	T	G	G	G	A	T	C	C	T	C	T	-	-
2. ToLCCeV_(P2-1)	T	G	G	C	G	A	A	C	A	T	G	T	G	G	G	A	T	C	C	T	C	T	A	G
3. ToLCCeV_(BK2)	T	G	G	C	G	A	A	C	A	T	G	T	G	G	G	A	T	C	C	T	C	T	A	G
4. ToLCMiV	T	C	C	C	A	A	A	C	A	T	G	T	G	G	G	A	T	C	C	G	T	T	A	G
5. ToLCPV-A_(L3)	C	T	T	T	A	A	A	A	A	T	G	T	G	G	G	C	C	T	C	G	T	C	G	G
6. ToLCPV-B_(LB4)	C	T	T	T	A	A	A	A	A	T	G	T	G	G	G	C	T	C	C	G	T	C	G	G
7. ToLCPV-B_(P7)	T	A	C	T	A	A	G	A	A	T	G	T	G	G	G	C	T	C	C	G	T	C	G	G
8. ToLCPV-B	A	A	T	T	A	A	G	A	A	T	G	T	G	G	G	C	T	C	C	G	T	C	G	G
9. ToLCPV-B_(SBN)	A	A	T	T	A	A	G	A	A	T	G	T	G	G	G	C	T	C	C	G	T	C	G	G
10. ToLCPV-C_(LB5)	C	T	T	T	A	A	A	A	A	T	G	T	G	G	G	C	T	C	C	G	T	C	G	G
11. ToLCPV-C(Lag)	C	T	T	T	A	A	A	A	A	T	G	T	G	G	G	C	T	C	C	G	T	C	G	G

Figure 6: Multiple sequence alignment of ToLCCeV19 primer sequence with sequences of Philippine *begomoviruses* infecting tomato: (1)oLCCeVSP_V and BK2560 primer; (2)ToLCCeV-P2-1; (3) ToLCCeV-BK2; (4)ToLCMiV; (5)ToLCPV-A (L3); (6)ToLCPV-B (LB4); (7)ToLCPV-B (P7); (8)ToLCPV-B; (9)ToLCPV-B(SBN); (10)ToLCPV-C (LB5); (11) ToLCPV-C (Lag) using ClustalW in Mega X software.

ToLCCeV19 primer, having a length of 20 base pairs, has a 100% matched with both ToLCCeV-BK2 and ToLCCeV-P2-1. The designed primer has four mismatched base pairs or 20% mismatched including two mismatches at the 5' end and two mismatches at the 3' end when aligned with ToLCMiV (Figure 6). It has eight mismatched base pairs or 40% mismatched with ToLCPV-B (LB4), ToLCPV-A (L3), ToLCPV-C (LB5), and ToLCPV-C (Lag) which includes 3 mismatches at the 3' end. It has nine mismatched base pairs (45% mismatched) with ToLCPV-B and ToLCPV-B (SBN) which includes 3 mismatches at the 3' end. When aligned with ToLCPV-B (P7), it resulted to ten mismatches (50% mistmached) which include five mismatches at the 3' end. The designed primer could anneal to ToLCPV strains LB4, L3, LB5 and Lag and ToLCMiV species as the number of mismatches in the 3' end of the sequences does not exceed five or mismatches percentage does not exceed to more than 49% but could not anneal to ToLCPV-L3, and P7.

Optimization of Polymerase Chain Reaction (PCR)

Optimization of PCR was done by changing only the annealing temperature (53°C, 55°C, and 57°C). Specificity of the designed primer was tested by using PCR assay with genomic DNAs from a tomato sample with ToLCPV and ToLCCeV collected by Dr. Pascual. DNA templates were diluted to 50 mg/uL. In a 15 uL PCR reaction, 7.5 uL of 20 mM GoTaq green master mix was used, 100 mM each of diluted forward and reverse primers, 1 uL of 10 ng/uL DNA template and 5.3 uL of DEPC water. Samples were subjected to gradient PCR thermo cycler with the following conditions: 95°C initial denaturation for 10 mins followed by denaturation of 95°C for 1 min which melts all dsDNA into ssDNA, annealing temperatures of 53°C, 55°C, and 57°C for 1 min to promote the binding to the template and to know what annealing temperature is more efficient for the binding, and extension with the temperature increase to 72°C for 1 min in order for the hybridized primer to be extended. Cycle was repeated for thirty times. PCR products were subjected to gel electrophoresis to view the amplified target sequence (Figures 7,8).

Figure 7: Target sequence amplified using ToLCPV19 Primer Lane 1: 1 kb Ladder, Lane 2: ToLCCeV (T47) 53 C, Lane 3: T47 55 C, Lane 4: T47 57 C, Lane 5: oLCPV (T221) 53 C, Lane 6: T221 55 C, Lane 7:T221 57 C, Lane 8: Healthy, Lane 9: Negative (-) Control, Lane 10: Positive (+) Control, Lane 11: Positive (+) Control.

Amplicons of ~200 bps were detected at Lanes 2, 3, and 4 which are samples of ToLCPV (T4) ran with 53°C, 55°C, and 57°C annealing temperatures and using ToLCPV19 primer. There was no difference in the size of the amplicons and the intensity of the bands at different annealing temperatures. Amplicons of ~200 bps were detected at Lanes 5,6 and 7 amplified at annealing temperatures of 53°C, 55 °C, and 57°C with no difference in terms of presence or absence of amplicons. Amplification was detected on ToLCPV (T4) and ToLCCeV (T221) when ToLCPV19 primer was used at all three annealing temperatures. No amplicons were detected in healthy (Lane 8) and negative control (Lane 9) while amplicons were detected in both positive controls (Lane 10 and 11) which should amplify only Tomato Leaf Curl Philippines Virus. However, amplicons were also detected on T47 sample which is infected with ToLCCeV (Figure 7). Although no amplicons were detected on healthy sample and negative control, ToLCPV19 primer was not specific enough to detect ToLCPV species which is possible as previous studies showed that tomato samples infected with tomato leaf curl disease here in the Philippines were caused by multiple infections of different ToLCV species which includes both ToLCPV and ToLCCeV.

Figure 8: Target sequence amplified using ToLCCeV19 Primer; Lane 1:1 kb Ladder, Lane 2: T221 53 C, Lane 3: T221 55C, Lane 4: T221 57 C, Lane 5: T47 53 C, Lane 6: T47 55 C, Lane 7: T47 57 C, Lane 8: Healthy, Lane 9: Negative (-) Control, Lane 10: Positive (+) control.

Figure 8 shows that amplicons that are ~200 bps were detected at Lanes 2, 3, and 4 which are samples of ToLCPV(T4) ran using ToLCCeV19 primer at annealing temperatures of 53°C, 55°C, and 57°C, respectively. There is no difference in the amplicon size and intensity at different annealing temperatures. No amplicon was detected at Lanes 5, 6, and 7 having annealing temperatures of 53 °C, 55 °C, and 57 °C with no difference in terms of presence or absence of amplicons. Amplification was detected on ToLCPV (T4) and ToLCCeV (T221) when ToLCCeV19 primer was used at three different annealing temperatures. No amplicon was detected at healthy (Lane 8) and negative control (Lane 9) while it was expectedly detected in positive control (Lane 10).

PCR validation for specific detection using field collected samples

Validation was done to determine whether ToLCPV19 primer can specifically detect ToLCPV species and whether ToLCCeV19 primer can only detect ToLCCeV species. In order to test the specificity of the two designed primers, each primer was used to detect two ToLCPV samples (T66 and T80) and two ToLCCeV samples (T235 and T306) (Figures 9 and 10).

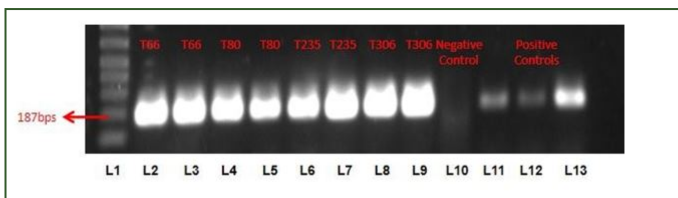

Figure 9: Target sequence amplified using ToLCPV19 primer. Lane 1: 1 kb ladder, Lane 2 and 3: T66, Lane 4 and 5: T80, Lane 6 and 7: T235, Lane 8 and 9: T306, Lane 10: Negative Control, Lane 11-13: Positive Controls, Lane 14: Blank (water).

All samples were subjected to PCR with the exact same conditions and both designed primers were used on all tomato samples. Amplicons were detected using ToLCPV19 primer on infected tomato samples T66 and T80 which are infected with ToLCPV and an expected PCR product of 200 bps was obtained from those samples (Lane 2 to Lane 5) (Figure 9). No amplicon was detected on negative control (Lane 10 healthy) and DEPC water (Lane 14). Amplicons were detected with a PCR product size of 200 bps on positive controls (Lane 11 to Lane 13). The results alone could not determine whether the ToLCPV19 primer is a viable designed primer due to the primer were validated against very few samples only.

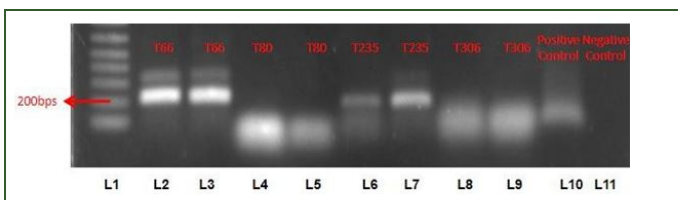

Figure 10: Target sequence amplified using ToLCCeV19 primer. Lane 1: 1 kb ladder, Lane 2 and 3: T66, Lane 4 and 5: T80, Lane 6 and 7: T235, Lane 8 and 9: T306, Lane 10: Positive Control, Lane 11: Negative Control, Lane 12: Blank (water).

Based on Figure 10, amplicons were detected using ToLCCeV19 primer on T66 but not in T80 which are tomato samples from Ilocos and are infected with ToLCPV. Amplicons were detected on T235 but no amplicon was detected on T306 which was infected with ToLCCeV. Band was observed in positive control (Lane10) and no band was observed in negative control (Lane 11) and blank (Lane 12). Tomato sample used in the optimization and validation were infected by a multiple *begomovirus* species based on the Next Generation Sequencing result of Dr. Pascual. Amplicons detected on samples T235 and T306 using ToLCPV19 primer could possibly different *begomovirus* species and not ToLCCeV. The same result for ToLCCeV19 primer as it detected T66 sample which could be a different *begomovirus* species aside from ToLCPV. Validation should be tested further on more tomato samples to evaluate the specificity of these designed primers.

Restriction fragment length polymorphism of ToLCPV, ToLCCeV, and ToLCMiV using selected restriction enzymes

Rolling Circle Amplification (RCA) was performed to amplify the complete genome of DNA a of ToLCPV species using *in situ* restriction digestion NEW ENGLAND BioLabs NEB cutter following the standard procedure. RCA products were digested using different restriction endonucleases (BamHI, EcoRI, HindIII, MspI, and SalI). The fragments were produced and viewed in a virtual gel that was separated on 1% agarose gel, and with a 1 kb+ DNA ladder (Figure 11 and Table 5). Among all the restriction endonucleases, EcoRI was the only enzyme that could distinguish ToLCPV, ToLCCeV, and ToLCMiV species. When cut by EcoR1, ToLCCeV species produce 1 band, three bands on ToLCV strains, and four bands on ToLCMiV species.

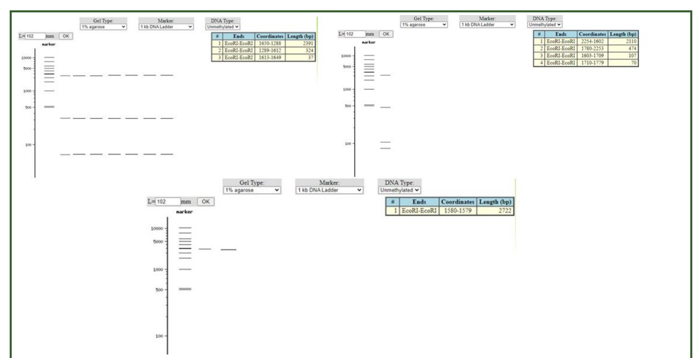

Figure 11: Restriction digestion on ToLCPV, ToLCCeV, and ToLCMiV species using EcorR1 under 1% agarose gel electrophoresis, and 1 kb ladder. (A) Digestion on ToLCPV strain; (B) Digestion on ToLCMiV species; (C) Digestion on ToLCCeV species.

Table 5: Restriction digestion of ToLCPV strains, ToLCCeV, and ToLCMiVspecies using BamHI, EcoRI, HindIII, MspI, SalI.

Number of Bands and Sizes					
Restriction	One band (2700-	Two bands	Two bands	Three	Four bands
Enzymes	2780 bp)	(2670 bp-2690 bp, 40 bp-91 bp)	(1683 bp, 1078 bp)	bands (2419 bp, 324 bp, 37 bps)	(2110 bp, 474 bp, 107 bp, 70 bp)
BamHI	ToLCPV				
	(LB4,LB5,L3,SBN ,Lag ,P7) ToLCCeV-BK2 ToLCCeV-P2-1 ToLCMiV				
EcoRI	ToLCCeV-BK2 ToLCCeV-P2-1			ToLCPV (LB4,LB5, L3,SBN,Lag,P7)	ToLCMiV
HindIII	ToLCMiV	ToLCPV (LB4,LB5,L3,S BN,Lag,P7) ToLCCeV-BK2 ToLCCeV-P2-1			
MspI	ToLCPV (LB4,LB5,L3,SBN ,Lag ,P7) ToLCCeV-BK2		ToLCMiV		
	ToLCCeV-P2-1 ToLCPV (LB4,LB5,L3,SBN ,Lag ,P7) ToLCCeV-BK2 ToLCCeV-P2-1				

DNA of ToLCPV strains were digested using restriction endonucleases (BamHI, EcoRI, HindIII MspI, and SalI) and viewed less than 1% agarose gel with 1 kb ladder *in situ* using NEB cutter software. There was only one amplicon produced when ToLCPV- LB5 was digested using BamHI restriction enzyme having a product size of 2780 bps. When digested with EcoRI, it produces three amplicons having product sizes of 2419 bps, 324 bps, and 37 bps. When digested using HindIII, it produces two amplicons having product sizes of 2689 bps and 91 bps. One amplicon was detected when ToLCPV-LB5 strain was digested using MspI and SalI same with ToLCPV-P7 strain. ToLCPV-P7 was digested with BamHI, EcoRI, and HindIII. There were no unique band patterns observed between ToLCPV-LB5 and ToLCPV-P7 when digested with the three restriction enzymes (Table 5).

Sequences of both ToLCCeV-BK2 and ToLCCeV-P2-1 were digested using restriction endonucleases (BamHI, EcoRI, HindIII MspI, and SalI) and viewed under 1% agarose gel with 1 kb ladder *in situ* using NEB cutter software. There is only one amplicon produced when ToLCCeV-BK2 was digested using BamHI, EcoRI, MspI, and SalI restriction enzymes having a product size of 2722 bps. When digested with HindIII, it produces two amplicons having product sizes of 2677 bps, and 45 bps. Band patterns of ToLCCeV-P2-1 was digested using BamHI, EcoRI, HindIII MspI, and SalI and viewed under 1% agarose gel with 1 kb ladder *in situ* using NEB cutter software. It produces one amplicon with a size of 2722 bps but when ToLCCeV-P2-1 was digested with HindIII, it produces two amplicons with product sizes of 2677 bps, and 45 bps (Table 5). No difference was observed on both ToLCCeV species as they produced the same banding patterns when digested with each restriction enzyme. Sequence of ToLCMiV isolate was digested using restriction endonucleases (BamHI, EcoRI, HindIII MspI, and SalI) and viewed less than 1% agarose gel with 1 kb ladder *in situ* using NEB cutter software. There is only one amplicon produced when ToLCMiV is digested using BamHI, HindIII, and SalI restriction enzymes having a product size of 2761 bps. When digested using EcoRI, it produces four amplicons having product sizes of 2110 bps, 474 bps, 107 bps, and 70 bps. When digested using MspI, it produces two amplicons having product sizes of 1683 bps and 1078 bps (Table 5).

Comparing all species of ToLCV used in the study, EcoRI restriction enzyme produce unique banding patterns when used in all ToLCV isolates: three bands when ToLCPV-LB5 or ToLCPV-P7 is digested using EcoRI, one band when ToLCCeV-BK2 or ToLCCeV-P2-1 is digested using EcoRI , and four bands when ToLCMiV is digested using EcoRI. EcoR1 restriction enzymes may be considered as key enzyme to specifically detect species of ToLCPV, ToLCCeV, and ToLCMiV.

CONCLUSION

This is the only study that tries to develop a molecular based method to specifically detect ToLCPV, and ToLCCeV species.

The study successfully showed the alignment of the published and the self-designed primers with ToLCPV strains and ToLCCeV, and ToLCMiV species.

Published primers were able to anneal to ToLCPV strains and ToLCCeV, ToLCMiV species and thus these primers need to be validated in an actual experiment to determine their specificity. Primers ToLCPV19 and ToLCCeV19 were designed to specifically detect ToLCPV and ToLCCeV species respectively. Designed primer ToLCPV19 were also able to anneal to both ToLCPV strains and ToLCCeV, ToLCMiV species and based on the multiple sequence alignment, the primer was able to detect both tomato samples infected with ToLCPV and ToLCCeV and thus could not distinguish one from the other. Further validation on ToLCPV19 primer should be done on several field collected samples to test whether this primer is specific to ToLCPV species or not. On the other hand, designed primer ToLCCeV19 could anneal only to ToLCCeV species and not to ToLCMiV and ToLCPV strains based on the number of mismatches at the 3' end and percent mismatch. PCR result showed that ToLCCeV19 primer detected one tomato sample infected with ToLCPV but no amplicons was observed on the other tomato sample infected with ToLCPV. The primer also detected tomato sample infected with ToLCCeV but did not detect another tomato sample infected with ToLCCeV. This could signify that ToLCCeV19 detected another *begomovirus* species aside from ToLCPV as the tomato samples used were infected with multiple *begomovirus* species based on the NGS result by Dr. Pascual. Due to COVID pandemic, the experiment was not able to proceed and resulted in the use of limited samples for PCR validation of the primers which hinders the need for further validation to satisfy the main objective of the study. Also, RCA was not actually performed and the study used in silico restriction digestion using NEB cutter software. RFLP analysis is important to determine which restriction enzymes can distinguish one species of ToLCPV to another and can be verified using RCA as RCA alone could not distinguish ToLCPV, ToLCCeV and ToLCMiV species. It is recommended to continue this study when the pandemic is over to further analyze and verify the specificity of the designed primers and that more tomato samples should be used and RCA must be performed.

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