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Molecular Phylogenetic Tracking of Tomato Leaf Curl Virus (ToLCV) Strain in the Philippines

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Abstract

To trace the possible route of the outset of the Tomato Leaf Curl Virus (ToLCV) in the Philippines, the phylogenetic relationships of ToLCV strains in the regions of epidemicity in the Philippines and other countries were investigated. The phylogenetic markers tagged as C1RAPG for ToLCV was specifically investigated to target the replication-associated protein of ToLCV was generated. Phylogenetic analysis indicated that the TolCV strains in the Philippines segregate into two groups: Members of group 1 that include Ilocos Norte, Southern Luzon, and from the islands of Visayas and Mindanao are related to China, Malaysia, and Italy. While group 2 formed a monophyletic cluster with strain isolates from South Korea, Africa, Israel, and the USA implicating that the samples involved in this study are related to the Tomato Yellow Leaf Curl Virus (TYLCV) ancestor of ToLCV from the strains of Israel.

Keywords: Tomato Leaf Curl Virus (ToLCV); Phylogenetic Tracking; Philippine Strain; Tomato Yellow Leaf Curl Virus (TYLCV)

Introduction

Tomato (Lycopersicum esculentum Mill) is one of the most popular and most widely grown fruit vegetable in the world. In the Philippines, the local tomato farming sector has been one of the major contributors to agriculture next to the onion. Tomato ranks fourth among the major vegetables and root crops in the total volume of production which has reached 91.26 thousand MT, 4.1 percent higher than the previous year's output amounting to 87.68 thousand MT [1]. Despite steady development and progressive growth of the local tomato farming sector, production yield and survival of the crop are still being periled by the detrimental effects of several disease-causing entities. Apart from known bacterial and fungal entities that can cause severe damage to tomatoes, several plant viruses also affect the crop. Being ranked second following fungi [2], viruses are known to be one of the most economically important plant pathogens as they consistently induce significant economic loss by limiting plant produce quality and quantity estimated to up to more than a billion dollars per year worldwide.

Amidst the tomato viruses that are endemic in the country, tomato leaf curl virus (ToLCV) and tomato mosaic virus (ToMV) are being construed as the most important, destructive, devastating and contagious viral disease of all [3]. In general, these viruses have been implicated to cause 60% up to almost 100% yield loss in tomato production globally. Also, its mode of infectivity exhibits a host non- specificity denoting that a wide array of crops like cotton, coffee beans, peppers, and tobaccos became susceptible to the injurious effects of the diseases creating a greater negative economic impact on agriculture.

ToLCV is a geminivirus belonging to the family Geminiviridae under the genus Begomovirus. It is first seen in 1959 infecting tomatoes grown in the Jordan Valley, Israel. It has a characteristic morphology described as having twinned quasi-isometric incompletely geminate icosahedra encapsulating a single 2787 nucleotides (total MW 980,000) covalently closed genomic circular ssDNA [4]. Its viral genome is capable of encoding for six major proteins derived from open-reading frames (ORFs) on the viral and complementary viral strands. Introduction and spread of the infection from plant to plant have been attributed to the feeding behavior of its vector, Silverleaf whiteflies or Bemisia tabaci which transmits the virus in a non-propagative and persistent manner. Biotype B is usually the form of B. tabaci involved [5] which spreads with high frequency [6]. Affected plants exhibit outward symptoms several weeks after viral inoculation which include severe stunting, a marked reduction in leaf size, upward cupping and chlorosis of the leaf margins, mottling, flower abscission and significant yield reduction [7]. In a study conducted by [8] Sharma et al in 2011, they confirmed novel strains of ToLCv in the Philippines as indicated by molecular and phylogenetic analyses of three new begomoviral and one beta satellite isolates obtained from tomato plants exhibiting leaf curl symptoms in Laguna the Philippines. Upon analysis, the sequence formed a separate monophyletic cluster with the established strains from different countries hence, a novel strain of leaf curl virus was confirmed which is pertained to as Tomato Leaf Curl Philippine virus (ToLC-Philv).

At present, the Philippines lacks government agencies that would monitor disease incidence in tomatoes. As a result, there are no existing policies, guidelines, programs and projects which are specifically planned and organized to control and eradicate the pathogens affecting the tomatoes. This gap accentuates the importance of awareness of the occurrence, extent, and impact of the ToLCV-induced outbreaks. Therefore, it is important to develop comprehensive surveillance to assess the extent of the incidence of the viruses, to identify the factors that contribute to disease transmission and to come up with a system that records the status of disease occurrence locally.

Methodology

Collection of samples

Samples collected, both symptomatic and asymptomatic were all suspected infected with TolCV. A total of ninety-four (94) samples which comprised 50 tomato leaves, 13 whiteflies, 21 soil and 10 water samples used were purposively obtained from different organic farms **(Table 1)**. Seeds acquired from a regular agricultural supplier were also tested.

Table 1: List of sources/farms with the type of samples and number of samples collected and tested.

Source/Farm	Leaf	Whitefly	Soil	Water	Seeds (Procured from supplier)
CLSU Farm	16	3	8	2	1 pack
Munoz Farm	18	6	3	1	
Sta. Rosa Farm	6	3	4	2	
Benguet Farm 1	3	1	3	1	
Benguet Farm 2	3	0	2	2	
La Union Farm	4	0	2	2	
TOTAL	50	13	21	10	

Nucleic Acid Extraction and Quantification of Nucleic Acid

This study made use of the following samples: leaf tissue, whiteflies, soil, seeds, water samples used in irrigating the plants. For leaf tissue, whiteflies and seeds, nucleic acid extraction was done by maceration with alkaline lysis by 0.5M NaOH added with 100mM Tris-Cl. Water precipitates which were pelleted after subjecting the water samples at full speed centrifugation for 5 min in 1.5 microcentrifuge tubes and were also crudely extracted by alkaline lysis extraction. DNA extracts from soil were purified using standard protocols using a commercial kit; Macherey-Nagel's NucleoSpin® DNA extraction kit per manufacturer's instructions. For kit-extracted eluates, nucleic acids were quantified using IMPLEN P330 Nanophotometer to quantify and determine the concentration (ng/µL) and purity at A260/A280 of the extracts. The samples that met the required DNA concentration (about >50ng/uL) were used in the study. Products were loaded in a 1.5% agarose gel stained with INTRON GelSafe dye, electrophoresed at 100V for at least 45 minutes and viewed under UV light and were scored to obtain results.

The C1RAP Gene/ C1 or Replication Association Protein gene for Phylogenetic analyses

C1RAP gene of ToLCV encodes the Replication-associated protein or better known as REP. It is located on the negative complementary strand of the viral genome consisting of 1088 nucleotides. It plays an important role in the replication and transcriptional regulation of viral ssDNA. The protein that the gene encoded is the sole viral protein required for viral replication [9]. The phylogenetic markers tagged as C1RAPG for ToLCV was specifically designed to target the genes that encode the replication-associated protein of TOLCV was generated using published sequences freely accessible at the National Center for Biotechnology Information (NCBI). Generated oligonucleotide sequences were aligned using the Basic Local Alignment Search Tool (BLAST) accessible at NCBI to verify the specificity of the primers **(Table 2)**.

Various phylogenetic and detection studies demonstrate success in utilizing the genes selected for the study. In this work, the genes were selected as candidate genetic markers for screening of ToLCV infection among the prospective samples. The target sequences remained conserved in position and size [10] hence rendering it ideal for molecular phylogenetic and diagnostic investigations.

The previously described DNA markers were optimized using crude extracts from pooled leaf samples collected from tomato plants manifesting conspicuous symptoms of leaf curl disease. The samples were collected during the summer of 2016 from the tomato growing plots of an organic farm in Nueva Ecija, Philippines and in April 2017 from tomato farms in Benguet and La Union. The primer pairs were successfully optimized via BioRad T100 Thermal Cycler with its corresponding RT-PCR thermal and cycling profile. PCR products of positive representative samples with discrete bands in gels were submitted to 1st Base DNA Sequencing Services in Singapore for direct DNA sequencing to verify the PCR results. The raw data sequence retrieved was assembled on Codon Code Aligner online software. The assembled sequence was then used as a template for designing new sets of primers to be used for detection/routine field testing. Subsequently, it was aligned with the various published strains of ToLCV from NCBI using CLUSTALW available at MEGA 7.0. Further, a dendrogram or phylogenetic tree was constructed utilizing the Maximum Likelihood method with 1000x bootstrap replicates to determine the phylogenetic relationship among the positive field isolate in the study and selected strains from different countries. Meanwhile, a short version of the target gene was also designed

for PCR detection for merely for detection of the presence of the virus.

Table 2: Phylogenetic and detection DNA markers designed specifically for Philippine strain with their corresponding Annealing Temperatures and Amplicon sizes.

Target Gene	Primer Design		Annealing Temperature	Amplicon Size (bp)
C1RAPG for phylogenetics	Forward Primer (5'-3') ATGGCACCACCCAGAAAATT	Reverse Primer (5'-3') CGCCTGCGTATTGGTCTCCTC	58˚C	1088
C1RAPG for detection	Forward Primer (5'-3') TCAGGGAGCTAAATCAAGC	Reverse Primer (5'-3') AAATCTTTTGGAGCTAACTCC	60˚C	200

Results and Discussion

PCR Detection of C1RAPG for Tomato Leaf Curl Virus by PCR

Mostly, suspected symptomatic plants were collected from the selected organic farms including and a total of ninety-four (94) samples comprised of 50 tomato leaves, 13 whiteflies, 21 soil, and 10 water samples were purposively obtained from the selected organic farms **(Table 1)**. Sources of samples include the CLSU organic farm, Bantug in the Science City of Muñoz, Sta Rosa Farm in Nueva Ecija, Farm 1 and 2 in Benguet and La Union Farm. Seeds used were also tested to verify the source of infection. These seeds were procured commercially from a known regular supplier. Leaf samples and or whole plants were selected either suspected infected or generally with symptoms of ToLCV. These samples underwent crude DNA extraction using alkaline lysis extraction. Samples for detection purposes were processed for PCR. Samples for detection purposes were processed for PCR. DNA markers were designed using the generated sequence from the C1RAP gene only with a shorter target region taken from the gene. PCR positive products have an amplicon size of 200bp.

To evaluate the viability of the designed primer sets, PCR was carried out to detect ToLCV targeting the C1RAPG. Collected samples were processed for crude alkaline lysis extraction and assayed initially using PCR amplification of DNA. Representative PCR products are shown in **Figure 1**. The summary of the assessment of all samples can be referred to in **Table 3**. From the fifty (50) leaf samples, 33 were observed to be positive i.e. 66% of the entire leaf samples collected. Commercial seeds and the soil were also tested and revealed that they essentially can be a potential outset of transmission.

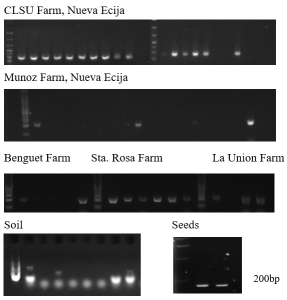

Figure 1: Representative PCR products amplified using C1RAPG DNA markers for detection with an amplicon size of 200-bp. Shown are samples taken from leaf samples from CLSU Farm, Munoz Farm, Benguet, Sta. Rosa and La Union. Seeds were also revealed to be positive with the ToLCV

Table 3: PCR Assessment of Samples Collected for ToLCV

Source/Farm	Leaf	No. of positive	Whitefly	No. of positive	Soil	No. of positive	Water	No. of positive	Seeds (Procured from supplier)	No. of positive
CLSU, Nueva Ecija	16	16 (100%)	3	3 (100%)	8	3 (38%)	2	0	1 pack	Representative seeds (100%)
Bantug	18	5 (28%)	6	6 (100%)	3	2 (75%)	1	0		
Sta. Rosa Farm	6	6 (100%)	3	3 (100%)	4	3 (75%)	2	0		
Benguet Farm 1	3	2	1	1 (100%)	3	1 (33%)	1	0		
Benguet Farm 2	3	2	0	0	2	0	2	0		

La Union Farm	4	2	0	0	2	0	2	0		
TOTAL	50	33	13		21		10			
Percentage		66%		100%		43%		0%		100%

Optimization of PCR amplification for C1RAPG for Phylogenetic Analysis

Singleplex PCR for all samples from identified farms was accomplished using the phylogenetic markers. Upon amplification of C1RAPG gene, an amplicon target of 1088-bp was achieved in the gel after electrophoresis **(Figure 2)**. Representative samples collected from Central Luzon and Northern Luzon for C1RAPG gene-positive were submitted for sequencing to verify the gel result. BLAST (Basic Local Alignment Search Tool) from NCBI nucleotide analysis of the positive sample confirmed 96-97% identity similar to fifteen (15) Tomato Leaf Curl Philippine virus strains published at NCBI **(Figure 3)**.

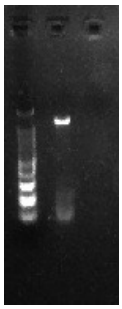

Figure 2: Representative PCR result of C1RAPG DNA markers.

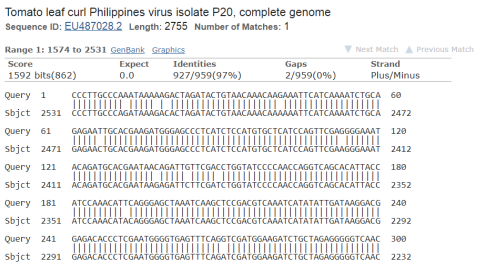

Figure 3: Result of BLAST between Positive Field Isolate and ToLVPhil viral isolate P20. BLAST hits comparison was shown between the Query and Top 1 subject sequence.

Multiple sequence alignment was performed via CLUSTALW in MEGA 7.0 with various strains of ToLCV from The Philippines and other countries **(Table 4)**. The resultant alignment was utilized for phylogenetic tree analysis which was then performed to determine the relationship of the field isolates among several ToLCV sequences obtained from NCBI. After 1000 times bootstrap replicates using the Maximum Likelihood method, phylogenetic results revealed that the current strain of ToLCV from Central and Northern Luzon involved in this study is distantly related to other identified Philippines strains **(Figure 4)**.

The field isolates formed a monophyletic cluster with isolates strains from South Korea, Africa, Israel, and the USA implicating that the samples involved in this study are related to the Tomato Yellow Leaf Curl Virus (TYLCV) ancestor of ToLCV from the strains from Israel. TYLCV was reported firstly in Israel region in 1920 which appear in epidemic form in 1960- [11]. Another group that includes Ilocos Norte, Southern Luzon, and from the islands of Visayas and Mindanao is related to China, Malaysia, and Italy.

Table 4: Reference sequences of different ToLCV Strains from the Philippines and Other Countries used in Multiple Sequence Alignment Analysis with their Accession Number and Strain Description.

Accession Number	Strain	Origin
Philippine Strains		
	From this Study	Central Luzon
	From this Study	Benguet-Northern Luzon
EU487042	P134	Cebu
KU946996	SBN2-Ilocos Norte	Ilocos Norte
KX063715	LB4-Laguna	Laguna
EU487046	P162	Mindanao
EU487028	P20	Pangasinan
Strains from Other Countries		
AY044137	ToLCSDV-Gez	Central Sudan, Africa
NC_003896	Tomato leaf curl virus-[AU]	Australia
AM236785	GX-2	Guangxi, China
GU951759	TYLCSV-Sar-[IT:Sic:04]	Italy, Sicily, Europe
AB110217	Ng	Israel
AF327436	MT1-5	Klang, Malaysia
GU322424	Tomato yellow leaf curl virus-Wailuku	Hawaii, USA

TLCV belongs to the genus Begomovirus. Begomoviruses have icosahedral single-stranded DNA (ssDNA) genomes and are transmitted by the sweet potato whitefly (Bemisia tabaci) in a persistent-circulative manner [12]. The TYLCV "type-strain" itself is reported to be an ancestor of the begomovirus ToLCV [13].

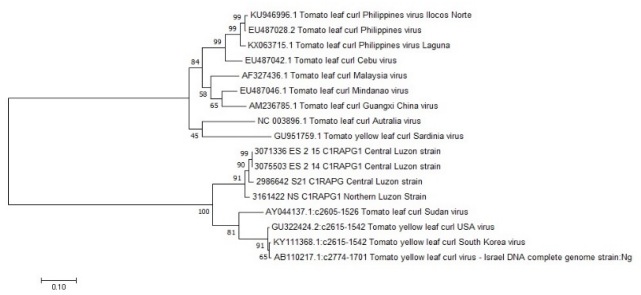

Figure 4: Phylogenetic Tree Analysis among the Field Isolate and Various Strains of ToLCv from the Philippines and other countries using MEGA 7 (Molecular Evolutionary Genetic Analysis version 7.14).

Confirmation of ToLCV using seed samples

To verify that the tomato seeds used in the experimental study are a potential source of infection, they were subjected to PCR assay. Results demonstrated that the target region of the C1RAP gene was also amplified through PCR and LAMP proving that the commercial seeds used in the study were also contaminated with the virus **(Figure 5)** [14-17].

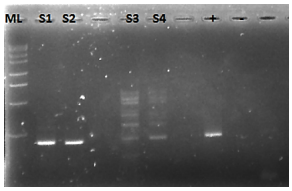

Figure 5: Gel electrophoretic results using C1RAPG DNA markers for the detection of ToLCV in representative seeds used in the experimental study. M- Marker, N- No Template Control S1 and S2 are PCR products

Summary

A total of ninety-four (94) samples which comprised 50 tomato leaves, 13 whiteflies, 21 soil, and 10 water samples were purposively obtained from different tomato farms with cases of ToLCV. Commercial seeds used in the experimental study were also tested [18]. The collection of samples comprising of both symptomatic and asymptomatic was processed and conducted an initial assessment using the PCR technique. PCR amplification of C1RAPG marker was successfully optimized initially for phylogenetic analysis and subsequently applied in the detection of ToLCv in a leaf, whiteflies, soil and seed samples by which C1RAP gene was verified utilizing direct DNA sequencing. Accordingly, designing primer sets for PCR, for this gene were routinely tested on various samples initially from selected farms in Central and Northern Luzon [19].

The phylogenetic results revealed that there are 2 groups formed: Members of group 1 that include Ilocos Norte, Southern Luzon, and from the islands of Visayas and Mindanao are related to China, Malaysia, and Italy [20]. While group 2 formed a monophyletic cluster with strain isolates from South Korea, Africa, Israel, and the USA implicating that the samples involved in this study are related to the Tomato Yellow Leaf Curl Virus (TYLCV) ancestor of ToLCV from the strains of Israel. This implicates that the latter group is related to the Tomato Yellow Leaf Curl Virus (TYLCV) ancestor of ToLCV from the strains of Israel [21]. ToLCV was initially recognized in the Jordan Valley, Israel, in the 1930s, but it was not until the early 1960s that TYLCV was identified. Subsequently, the virus has spread persistent into the Mediterranean basin and most tropical and sub-tropical regions of the world and is recognized as one of the world's most devastating pathogens of tomato. Tomato leaf curl virus is a serious threat complex of virus strains. This complex of viruses in Pakistan, India, and Australia region is known as ToLCV (tomato leaf curl virus) and in Israel and Europe, the region called as TYLCV (tomato yellow leaf curl virus [22].

ToLCV is transmitted by the insect vector Bemisia tabaci in a persistent-circulative non-propagative manner (viruses that manage to pass through the gut into the hemolymph and then to the salivary glands; circulative viruses include both those that disseminate but do not replicate in the body of the insect). Tomato leaf curl disease is not transmitted in handling but it is harbored in infected host plants, some of which may be hosts that do not show symptoms [23-26]. The whitefly is a serious pest in tomatoes and other vegetable crops.

Conclusion

We have tested the possibility of the presence of the virus in the soil and seeds and they proved positive and can, therefore, suggest that they can be probable causes of infection.
Tomato yellow leaf curl virus (TYLCV) is also a well-known tomato-infecting begomovirus and transmitted by Bemisia tabaci. Seed transmission was also possible and also detected in seeds and their seedlings of TYLCV-infected tomato plants that were infected by both viruliferous whitefly-mediated transmission and agro-inoculation. Taken together, ToLCV or TYLCV can also be transmitted not only via the insect vector but the seeds and the soil as a source of infection. Restrictions may require to subject screening the imported seeds through molecular assays to prevent the spread of the virus from other countries that can be damaging to agriculture.

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