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Molecular Characterization of a *Sweet Potato Leaf Curl Virus* Isolate from Egypt and Its Phylogenetic Relationship with Other Members of the *Begomovirus*

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Authors' contributions

This work was carried out in collaboration between all authors. Author AMAS isolated the virus understudy and associated in the molecular and genetic studies done on this virus. Author AMAS wrote the manuscript, performed the phylogenic study on this virus and collected the necessary literature. Author MMR associated in the molecular and cloning studies on the virus understudy. Author SMES associated in some of the molecular study, collecting the literature on the virus understudy, and also in maintaining the virus isolate in the green house. Author MAES associated in the whitefly transmission experiment for isolating and maintaining the virus isolate in the green house. All authors read and approved the final manuscript.

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ABSTRACT

The present study was undertaken to isolate and molecularly and phylogenetically characterize a *Sweet potato leaf curl virus* isolate infecting sweet potato plants in Giza governorate in Egypt. The virus was isolated from infected plants showing leaf curl symptoms using non-viruliferous *Bemisia tabaci* insects. Total extracted DNA was amplified with polymerase chain reaction (PCR) using the degenerate AV/AC core primers and produced 580 bp of the DNA-A core coat protein in agarose gel. PCR conditions of 6 mM $MgCl_2$, combinatorial enhancer solution, and a 53°C/40s annealing temperature facilitated coat protein amplification. Recovered virus amplicon was ligated into pGEM T-Easy vector and cloned into *Eschericia coli*, strain DH5α. Purified plasmid DNA was submitted to GenBank and given an accession number FJ455517. Sequence comparisons with 13 sweepoviruses indicated that the SPLCV-Giza isolate has the highest nucleotide sequence identity (97%) with the SPLCV-US (KC253238). According to the current taxonomic criteria for *Begomovirus* classification, the Giza isolate of SPLCV in Egypt would be considered as a variant of the SPLCV-US. Phylogenic study using amino acid substitution showed the clustering of the 13 studied sweepoviruses in one major clade and one minor clade. The SPLCV-Giza isolate clustered with SPLCV-US in a monophyletic branch within the large clade circumventing nine sweepoviruses, viz. SPLCV isolates from: Korea, Brazil, Japan, USA, China, India, and *Ipomoea yellow vein virus* (IYVV) from Italy and Spain. The second minor clade involved the clustering of SPLCV isolates from Uganda and Spain in a monophyletic branch apart from the rest of the studied sweepoviruses. Phylogenic analysis of SPLCV-Egypt with five other mono and bipartite begomomoviruses showed its distinctive clustering nature from these viruses. In conclusion, the present study confirms the presence of an isolate SPLCV in Egypt as a variant of the SPLCV-US. The study also illustrates the high worldwide diversity of SPLCV isolates and signifies the economic importance of this newly introduced virus into Egypt.

Keywords: Sweepoviruses; sweet potato leaf curl virus; sweet potato; begomovieses; Bemisia tabaci; Egypt; novel species.

1. INTRODUCTION

Sweet potato (*Ipomoea batatas* (L.) Lam.), *Convolvulaceae*, is grown in all tropical and subtropical areas of the world [1]. Sweet potato is considered as the seventh most important food crop worldwide after wheat, rice, maize, potato, barley, and cassava. Most of the productivity of sweet potato is in eastern Asia with 80% productivity in China [2]. Over 30 viruses infecting sweet potato assigned to nine families, including *Bromoviridae, Bunyaviridae*, *Caulimoviridae*, *Closteroviridae*, *Comoviridae*, *Flexiviridae*, *Geminiviridae*, *Luteoviridae* , and *Potyviridae*, have been identified [3]. Half of these viruses were described as DNA viruses belonging to *Geminiviridae* and *Caulimoviridae.* Most of these viruses cause no disease symptoms upon infection.

Geminiviruses are members of the large family *Geminiviridae* with circular, single-stranded DNA (ssDNA) genomes packaged within geminate particles. Based on genome organization, nucleotide sequence similarities, and biological properties, the *Geminiviridae* circumvents seven genera including *Becurtovirus*, *Begomovirus*, *Eragrovirus*, *Mastrevirus*, *Curtovirus*, *Topocuvirus*, and *Turncurtovirus* [4].

Begomoviruses have either a monopartite (DNA A component) or bipartite genome (DNA A and DNA B components) of about equal size of 2.6-2.8 kb for each component [5]. Begomovirues are among the most widespread and damaging of plant viruses in the world. Begomoviruses are transmitted by the whiteflies; mostly by *Bemisia tabaci* [6,7].

Several begomoviruses including *Sweet potato leaf curl virus* (SPLCV) were isolated from infected *Ipomoea* species from all continents of the world [3]. Begomoviruses infecting the *Ipomoea* have monopartite genome; resembling the OldWorld begomoviruses. Phylogenically they are, however, distinct and clustered apart from both the monopartite and bipartite begomoviruses and are called sweepoviruses [8,9]. Several investigators have pointed out to the high diversity of SPLCV due to mixed infection with virus isolates or strains to the *Ipomoea* leading to genetic recombination and the induction of new SPLCV strains [10,11,12]. SPLCV was first isolated from Taiwan [13] and

Japan [14]. SPLCV was detected in the USA [15], Spain [16], Peru [17], Italy [18], Kenya [19], China [20], India [21], Uganda [9], Korea [22], and Argentina [1]. SPLCV caused considerable reduction in quality and tuber yield [3,23,24].

Symptoms induced by SPLCV include inward leaf curling, vein swelling, darkening of tuber skin, and grooving of tubers in some sweet potato cultivars [3,1]. Upon aging, however, most symptoms disappear from leaves. Host range study revealed that SPLCV infection is restricted to plants in the genus *Ipomoea* within the family *Convolvulaceae*. In total, 38 of 45 *Ipomoea* species tested were susceptible to SPLCV infection [25].

As for all sweepoviruses, SPLCV is transmitted persistently by the whitefly *B. tabaci* [15] and through vegetative propagation of sweet potato, using storage roots, shoot tips and stem cuttings [26]. These propagation methods, in turns, lead to accumulation of viruses over subsequent propagations. Recently Kim et al. [26] reported SPLCV in Korea as the first seed-transmitted begomovirus.

In Egypt, severe incidence of whitefly on sweet potato plants coupled with leaf curling symptoms was observed in several locations in Giza governorate in Egypt in 2012. Using touchdown polymerase chain reaction (PCR) and the begomovirus degenerate primers SPG1/ SPG2 described by Li et al. [27] a 912 bp amplicon was amplified indicating the presence of a begomovirus in the symptomatic sweet potato leaves [28]. The purpose of this study is to identify the nature of the isolated geminivirus from sweet potato plants on the molecular level and illustrates its phylogenic relationship with other isolates of sweepoviruses and begomoviruses present worldwide.

2. MATERIALS AND METHODS

2.1 Virus Isolation and Whitefly Transmission

The virus isolate understudy was previously isolated from sweet potato plants showing leaf curl symptoms from the Experimental Station, Faculty of Agriculture, Cairo University, Giza governorate, Egypt [28]. This virus isolate was maintained in an insect proof greenhouse on sweet potato plants and renewed through serial transfer using none-viruliferous *B. tabaci* insects.

2.2 DNA Extraction

Total DNA was extracted using the silica based methods of Echevarría-Machado et al. [29]. For each tested sample, 0.1 g of tissue was put in a 2 ml Eppendorf tube containing about 100-200 mg polyvinyl pyrrolidone. Tubes were dipped in a container containing liquid nitrogen. The frozen tissue was pulverized using a small sterilized plastic rod, or a small glass pipette with sealed tip. The powder was suspended in 0.8 ml of extraction buffer (10 mM Tris-HCl, 50 mM EDTA, 500 mM sodium chloride, 10 m*M* ß-mercaptoethanol, pH 7.0). 100 µl of 20% SDS were then added and the mixture was incubated at 65°C for 10 min. 250 µl of 10 M potassium acetate were added to the mixture to precipitate the SDS and the mixture was incubated on ice for 20 min, and then spun at 16,000 g for 20 min at 4°C. The supernatant was transferred to a new tube and 300 µl of well mixed silica were added to the solution, mixed manually for 3-5 min, then tube was spun at 16,000 g/1 min/4°C. The pellet was washed twice with 70% ethanol, dried, re suspended in 50 µl of distilled water, then incubated at 55°C/5 min. The tube was spun at 16,000 g for two min and the supernatant was transferred to a new 500 µl Eppendorf tube and stored at –17°C. Samples of other begomovirus DNAs including the monopartite *Tomato yellow leaf curl viru*s (TYLCV) and the bipartite *Squash leaf curl virus* (SqLCV) were used as positive controls.

2.3 PCR, Cloning, Sequencing and Phylogenetic Study

PCR was conducted with the Techne TC-312: DNA thermal cycler, England. Primers AV Core (5' GCCHATRTAYAGRAAGCCMAGRAT 3') and AC Core (5' GGRTTDGARGCATGHGTACANGCC 3') (Brown and Idris, unpublished) were designed to amplify 580 bp of the DNA-A core coat protein of begomoviruses. The reaction mixture contained 2.0 µl of DNA template (used directly or diluted 1/10 with water), 5 mM $MgCl_2$, 1X GoTaq DNA polymerase reaction buffer (Cat No. M8301, Promega Madison, WI, USA), 1X combinatorial enhancer solution [30], 0.2 mM dNTPs, 10 picomol of each primer and 1.25 U of Taq polymerase. The reaction mixture was completed to 25 µl with water. DNA amplification parameters were as follow: one cycle of initial denaturation at 95°C for 1 min, 35 cycles each of denaturation at 94°C for 20 s, primer annealing

Table 1. Sweepoviruses and begomovirues used in comparative sequence analyses with their corresponding countries, abbreviations, and GenBank accession numbers

	Begomovirus	Country	Abbreviation	Accession numbers
	Sweet potato leaf curl virus	**Egypt**	**SPLCV-Giza**	**FJ455517**
Sweepoviruses	Sweet potato leaf curl virus	USA	SPLCV-US	KC253238
	Sweet potato golden vein associated virus	Brazil	SPGVaV-Br	FJ969829
	Sweet potato leaf curl Korean virus	South Korea	SPLCV-K	FJ560719
	Ipomoea yellow virus	Italy: Sicily	IYVV-IT	AJ586885
	Sweet potato leaf curl Uganda virus	Uganda	SPLCUV	FR751068
	Sweet potato leaf curl Bengal virus	India	SPLCBenV	FN432356
	Sweet potato lead curl virus	China	SPLCCNV	EU253456
	Sweet potato leaf curl Spain virus	Spain	SPLCV-Sp	EF456741
	Sweet potato leaf curl virus	USA	SPLCV-USA	AF104036
	Ipomoea yellow vein virus	Spain	IYVN-Sp	EU839577
	Sweet potato leaf curl virus	Brazil	SPLCV-Br	FJ969835
	Sweet potato leaf curl virus	Japan	SPLCV-Jp	AB433786
	Cotton leaf curl Gezira virus	Egypt	CLCuV-Giza	FJ030874
	Hollyhock leaf crumple virus	Egypt	HLCrV-Giza	FJ030876
	Okra leaf curl virus	Egypt	OLCV-Giza	FJ030878
	Tomato yellow leaf curl virus	Egypt	TYLCV-Giza	FJ030876
	Squash leaf curl virus	Egypt	SqLCV-Cairo	DQ285019

at 53°C for 40 s, and elongation at 72°C for 30 s. The final elongation was done at 72°C for 7 min. DNA amplicons were evaluated by electrophoresis using a 1% agarose gel prepared in TAE buffer and stained with 0.5 µg/ml ethidium bromide. Bands of interest were excised, purified, cloned into pGEM T-Easy vector (Promega). The ligation mixtures were used to transform *Eschericia coli*, strain DH5α, using the procedure of Sambrook et al. [31]. Three plasmids from selected colonies were purified by miniprep then sequenced in both directions using automated, capillary DNA sequencing and 'dye-terminator sequencing'. DNA sequence for the SPLCV-Giza isolate was submitted to the GenBank to obtain an accession number.

DNA sequences were compared with some available sweepovirus and begomovius reference sequences (see Table 1) using NCBI/blastn, www.ncbi.nlm.nih.gov. Phylogenetic relationships were measured using MEGA6 programs.

3. RESULTS AND DISCUSSION

3.1 Field Symptoms

SPLCV-infected sweet potato plants showed inward leaf curling and vein swelling (Fig. 1). Such symptoms were typical to those described by Lotrakul et al. [15] for the SPLCV-US strain infecting *I. batatas* plants.

3.2 PCR

Primary attempt to amplify the core coat protein gene of the Egyptian isolate of SPLCV using the AV/AC core-PCR protocol described for SqLCV amplification [32] failed. Similarly PCR products for the SPLCV-US could not be visualized by ethidium bromide staining [15]. Such problems in SPLCV PCR amplification could be attributed to low concentration of DNA template, presence of DNA inhibitors, or poor primer annealing with the DNA template. It is known that the replication origins of the various begomovirus and nanovirus genome have GC rich regions that have the potential to form secondary structures that hamper PCR amplification. Therefore, a modification of the AV/AC core-PCR protocol entailed the use of lower annealing temperature using 53°C instead of 60°C, increasing the $MgCl_2$ concentration to 5 mM instead of 2.5 mM, and the addition of CES to the PCR reaction mixture. Increasing the Mg^{++} up to 6 mM is known to enhance amplification. Low annealing temperature is in favor of DNA-primer binding. The CES is composed of 4 M betaine, 16 mM DTT, 16% DMSO, and 83 µg/ml bovine serum albumin. Such compounds are known for decreasing DNA secondary structure, stabilize Taq polymerase enzymes, and neutralize inhibitory contaminants that may be present in the DNA; leading to enhancing the yield and/or the specificity of PCRs particularly for the amplification of highly GC-rich sequences up to

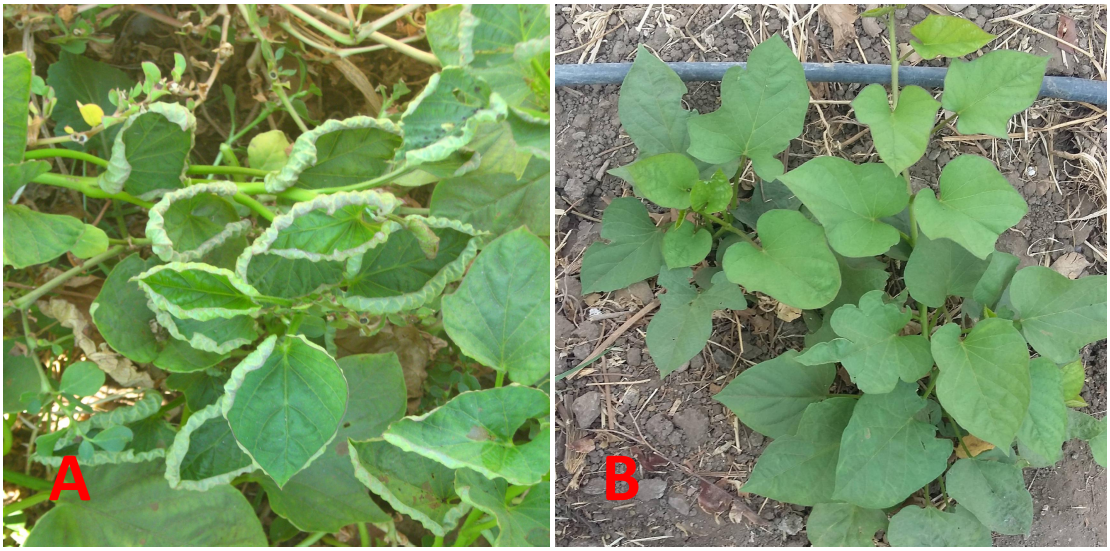

Fig. 1. Field symptoms of SPLCV-infected sweet potato plants (A); B, healthy control

75% [30]. The previous modification in PCR protocol led to successful amplification of the core coat proteins of the Giza isolates of SPLCV, TYLCV, and SqLCV (Fig. 2).

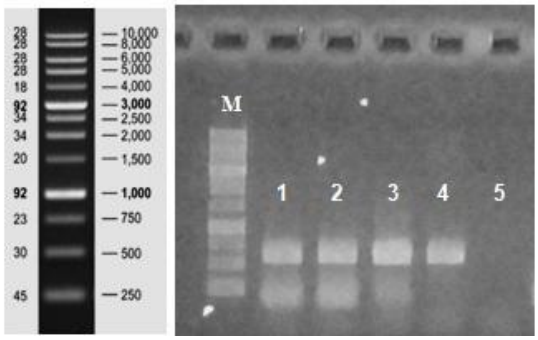

Fig. 2. Agarose gel electrophoresis showing DNA core coat protein amplicons (580 bp) of three amplified begomoviruses. M, 1kb DNA Ladder; 1 and 2, SPLCV-Giza; 3, TYLCV; 4, SqLCV; 5, healthy control

3.3 Sequence Comparison and Phylogenic Studies

Sequence comparison studies using the pairwise sequence analysis of the NCBI/blastn, www.ncbi.nlm.nih.gov, indicated that the local virus isolate had the highest sequence identity (97%) with the SPLCV-US. Therefore, according to the current taxonomic criteria for begomovirus by the ICTV, the SPLCV-Giza isolate from Egypt could be considered as a variant of the SPLCV-US. The lowest sequence identity was with SPLCUV (FR751068); being 87%.

Phylogenic study (Fig. 3) using amino acid substitution showed the clustering of the 13 studied sweepoviruses in one major clade and one minor clade. The Giza isolate of SPLCV from Egypt (FJ455517) clustered with SPLCV-US (KC253238) in a monophyletic branch within the large clade circumventing nine sweepoviruses, viz. SPLCV isolates from: Korea (FJ560719), Brazil (FJ969829, FJ969835), Japan (AB433786), USA (AF104036), China (EU253456), India (FN432356), and *Ipomoea yellow vein virus* (IYVV) from Italy (AJ586885) and Spain (EU839577). SPLCBenV (FN432356) clustered, however, in a separate branch in the large major clade. The minor second clade involved the clustering of SPLCV isolates from Uganda (FR751068) and Spain (EF456741) in a monophyletic branch apart from the rest of the studied sweepoviruses. Such results indicate genetic diversity between these viruses as previously suggested by several investigators [10,11,12] due to mixed infection of a given plant with more than one sweepovirus. The involuntarily distribution of segments of SPLCV-infected sweet potato plants between countries in different geographic locations for reproduction purposes in the presence of an active vector as *B. tabaci* insects has led to the presence of new foreign genetic elements in several countries; leading to new recombinants of SPLCV strains in sweet potato plantations.

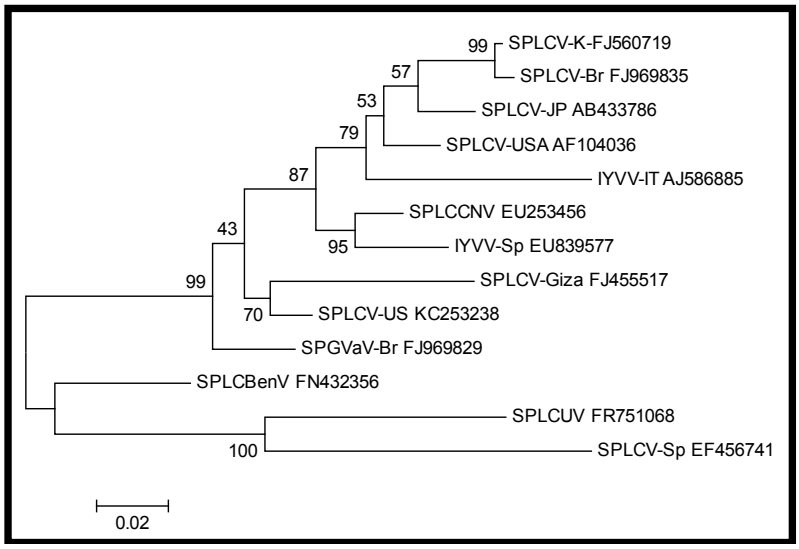

Fig. 3. Phylogenetic tree depicting the relationships between SPLCV-Giza DNA-A isolate from Egypt with sweepoviruses based on amino acid sequences. Tree was constructed using the Maximum Likelihood method based on the JTT matrix-based model (33). The dendrogram was boosted 500 times. The tree was drawn to scale; with branch lengths measured the number of substitutions per site. The analysis involved 13 amino acid sequences. Evolutionary analysis was conducted in MEGA6 (34). Abbreviation of sequences and accession numbers are mentioned in Table 1

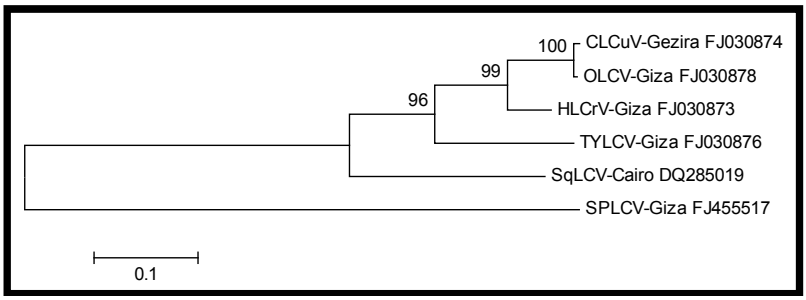

Fig. 4. Phylogenetic tree depicting the relationships between SPLCV-Giza DNA-A isolate from Egypt with other mono and bipartite begomoviruses based on amino acid sequences. Tree was constructed using the Maximum Likelihood method based on the JTT matrix-based model [33]. The dendrogram was boosted 500 times. The tree was drawn to scale; with branch lengths measured the number of substitutions per site. The analysis involved 6 amino acid sequences. Evolutionary analysis was conducted in MEGA6 [34]. Abbreviation of sequences and accession numbers are mentioned in Table 1

Phylogenic analysis (Fig. 4 above) between the SPLCV-Giza isolates (FJ455517) and other begomoviruses from Egypt including monopartite begomoviruses, viz. CLCuV-Giza (FJ030874), HLCrV-Giza (FJ030876), OLCV-Giza (FJ030878), TYLCV-Giza (FJ030876) and the bipartite SqLCV-Cairo (DQ285019) indicated the distinctive nature of SPLCV-Giza as a sweepovirus where the latter clustered separately from all the mono and bipartite begomovirses as previously found by several investigators [8,9].

4. CONCLUSION

The present study clarifies for the first time the nature of the begomovirus infecting sweet potato in Egypt that was previously detected [28]. It indicates the nature of SPLCV-Giza isolate as a variant of SPLCV-US strain infecting sweet

potato in Egypt. The study also showed the wide distribution of the sweepoviruses worldwide and the high diversity between these viruses. Several factors are involved in the worldwide spread out of SPLCV. These include the multiple methods of transmission of SPLCV through stem cuttings, storage roots, and true seeds. Secondly, the presence of the *B. tabaci* whitefly vector known to play critical role in SPLCV transmission. With the absence of resistant sweet potato cultivars to SPLCV, it is expected to have high built up of SPLCV in Egypt in the forthcoming future.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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