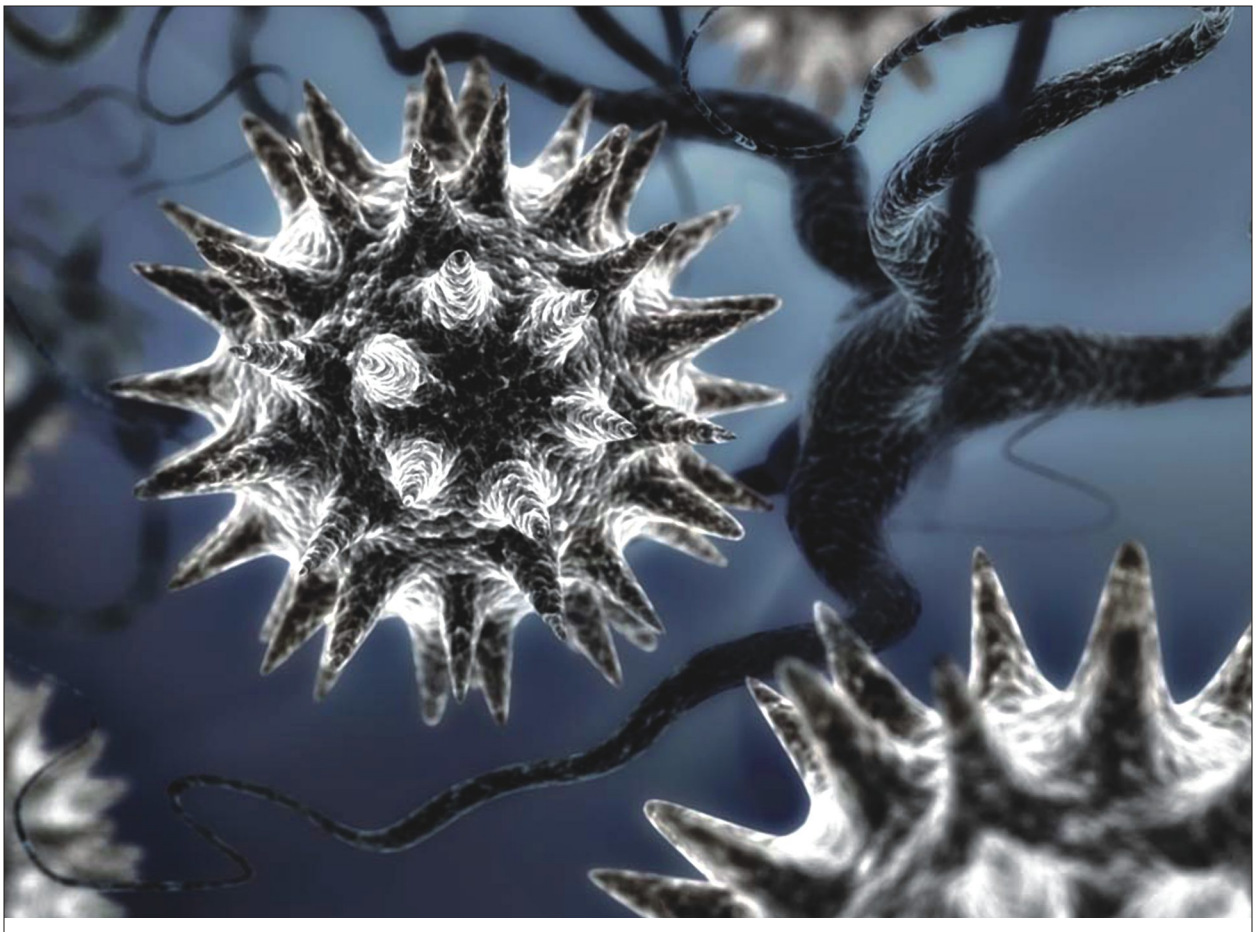

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Research Article
Genetic Diversity, Natural Host Range and Molecular Pathogenesis of Begomovirus-associated Betasatellites in Egypt

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Abstract

Background: Whitefly Transmitted Begomoviruses (WTB) cause worldwide economic losses. The WTB have either a monopartite or bipartite genome. The WTB in Egypt have monopartite genome. Recently betasatellite DNAs were found associated with WTB in Egypt. The bipartite squash leaf curl virus was recently introduced into Egypt (SLCEGV). **Materials and Methods:** The incidence of WTB, associated betasatellites and the incidence of SLCEGV was examined in different crops in Egypt for over 4 years. Rolling Circle Amplification (RCA), Polymerase Chain Reaction (PCR) and RCA-random fragment length polymorphism (RCA-RFLP), DNA sequencing and phylogeny analysis were used to study the nature of WTB and associated betasatellites in Egypt. **Results:** Disease symptoms ranged from leaf curling, rugosity, vein yellowing, vein enlargements, mosaic, leaf enations and yellowing. The number of identified WTB was limited to few monopartite WTB and one bipartite WTB. The PCR of RCA products indicated the presence of DNA-B components in several tested samples other than SLCEGV. The RCA-RFLP revealed the presence of several digested products ≥5 kbp in several tested samples with presence of SLCEGV in non-host or presence of other unknown bipartite begomoviruses. Betasatellites were associated with both mono and bipartite WTB and proved to be host-promiscuous in distribution. Phylogenetic analysis involving SLCEGV and the monopartite begomoviruses showed possible genomic mixing. **Conclusion:** The SLCEGV has increased the biodiversity in area with limited biodiversity. Evidence suggests genomic mixing between the local WTB and SLCEGV and possible acquisition of new betasatellites by SLCEGV that may modify viral virulence and fitness.

Key words: Whitefly transmitted begomoviruses, betasatellite DNA, biodiversity of begomoviruses, RCA, IC-PCR

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Geminiviruses are members of the large family Geminiviridae with circular, single-stranded DNA (ssDNA) genomes packaged within geminate particles. Geminiviruses are divided into seven genera including *Becurtovirus*, *Begomovirus*, *Eragrovirus*, *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Turncurtovirus* based on genome organization, nucleotide sequence similarities and biological properties[1].

Begomoviruses replicate through a double-stranded DNA intermediate employing a rolling-circle mechanism[2]. 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[3,4]. Both components are necessary for successful systemic infection. The DNA-A harbors information for replication, transcription and encapsidation, while DNA-B provides movement functions[5]. On the other hand, monopartite begomoviruses carry the necessary genetic information on one DNA molecule.

Bipartite begomoviruses occur in the Eastern and Western hemispheres, resembling Asia and the American continents. Monopartite viruses are found only in the Eastern hemisphere, resembling Europe, Africa, Asia and Australia[6,7]. Though, a monopartite begomovirus was discovered in the new world recently[8].

Whitefly transmitted begomoviruses are among the most widespread and damaging of plant viruses in the world[8]. Begomoviruses cause heavy economic losses in dicotyledonous crops and wild plants in subtropical and tropical areas including arid, irrigated agro-ecosystems in the Delta and along the Nile river in Egypt[9,10]. The symptoms are typically consisted of leaf curling, mosaic, vein yellowing and leaf yellowing often accompanies by stunting of plants[4].

Recently, several novel whitefly transmitted disease complexes comprising a monopartite begomovirus and circular satellites consisted of single-stranded DNAs of approximately 1350 nucleotides and named betasatellites have been recognized as novel and previously unknown plant viral pathogens[11]. They share a highly conserved structure known as satellite conserved region with a single gene, βC1, encoding the βC1 protein. This protein is pathogenecity determinant and suppressor of post transcriptional gene silencing, PTGS[4,12]. Betasatellite molecules are found together with certain monopartite begomoviruses. They share almost no sequence homology with their helper begomovirus other than the presence of a potential stem-loop structure containing the ubiquitous nonanucleotide sequence TAATATTAC that represents for geminiviruses the origin of virus replication, movement in plants and transmission between plants, through insect transmission[12]. They can also substitute for the movement function of DNA-B in some bipartite begomoviruses to permit systemic infection[13].

The demarcation threshold for distinguishing begomovirus species from isolates was set at 89% (taxonomic threshold) for full-length genomic (DNA-A component) sequences[14]. This corresponds to 78% nucleotide sequence identity for full length genomic sequence of betasatellite DNA[12].

Phylogeographic relationships have also indicated possible movement of betasatellites across plant species, being promiscuous and their congruent evolution with the helper begomovirus in the region[4,15-17]. Betasatellites have been found in association with begomoviruses in the old world, namely in Asia and the Nile Basin of Africa[11,18]. Betatellites contribute to the production of symptoms and enhanced helper virus accumulation in certain hosts[3,11]. One class of betasatellites has been found uniquely associated with begomoviruses from the Nile Basin ranging between 660-1350 nucleotides[11,18]. Among these betasatellite isolates, a further unique feature has been noted that has not been observed for Asian betasatellites. Each has a characteristic signature sequence that is shared in common with the helper virus and may in fact function in replication specificity[18].

An additional satellite-like component, DNA 1 currently known as alphastellites[19] can multiply autonomously. Alphasatellite components are associated with the majority of begomovirus. It is a single stranded DNA molecule of ca. 1370 nucleotides and consists of a single ORF. The alphasatellite molecule requires the helper begomovirus for movement in plants[19,20]. Two alphasatellite have been associated with hollyhock and cotton plants in Egypt[19].

Previously beta and alphasatellites were not known for association with bipartite begomoviruses in the new world. Now, accumulative evidence have shown the association of beta and alphsatellites with bipartite begomoviruses of the old and new world[3,21-24].

Most of approaches being used nowadays for studying epidemiology and diversity of begomoviruses rely entirely on molecular methods. These include Polymerase Chain Reaction (PCR) with the right degenerate or specific primers to demonstrate the presence of begomoviruse[4]. The PCR or PCR-derivative methods may be coupled with serology as in immunocapture PCR (IC-PCR) and IC-loop mediated isothermal amplification as powerful techniques for diagnosis of begomoviruses[9,25,26]. Rolling Circle Amplification (RCA) followed by Restriction Fragment Length Polymorphism

(RFLP) has proven as a useful tool for the detection of many begomoviruses[27,28]. Additional PCR amplification using the RCA product as template may be applied to enrich for or pre-amplify viral circular DNA-A[29].

In 2005, the bipartite begomovirus, Squash Leaf Curl Virus (SLCV) was detected in Egypt[9,10]. It was probably introduced from neighboring Middle Eastern countries through movement of viruliferous whitefly or infected plant material[30,31]. Prior to its spread to Egypt and its neighbors, SLCV was prevalent mainly in Central and Northern America[32]. The introduction of the bipartite SLCV into new areas is thought to increase virus diversity through potential exchange of genome components[33].

This investigation was undertaken to examine the diversity of begomoviruses in fiber and vegetable crops in Egypt and elucidate the role played by weed and ornamental plants as possible alternative hosts for begomoviruses. Further this study is concerned with examining the effect posed by the introduction of the bipartite SLCV on local monopartite begomoviruses encapsidating betasatellites in many samples collected from various geographic locations in Egypt during the last few years. This information will help elucidate possible emergence of new species of begomoviruses in Egypt.

MATERIALS AND METHODS

Viruses sources and associated symptoms: Isolates of viruses undertaken in this study were collected from different governorates in Egypt. Samples were from several different plant families and included fiber, medicinal, vegetable and ornamental crops. Infected plants had large infestations with *Bemisia tabaci* whitefly. One sample had infestation with *Trialeurodes ricini* whitefly. Collected samples had symptoms of begomovirus infection including vein yellowing, vein thickening, leaf curling, rugosity and stunting. Samples were preserved in 100% glycerol and stored at -20°C. The previously identified begomoviruses, okra leaf curl Egypt virus (OLCEGV), hollyhock leaf crumple Egypt virus (HLCrEGV), tomato yellow leaf curl Egypt virus (TYLCEGV) and squash leaf curl Egypt virus (SLCEGV) were kept as live cultures in their respective hosts, in insect proof cages under green house conditions and used as positive controls.

DNA extraction and Rolling Circle Amplification (RCA) and cloning: Total DNA was extracted from preserved leaves by first washing samples with sterilized distilled water to remove glycerol residues and then drying between tissue papers. Extractions were prepared according to the CTAB-based method of Haible *et al.*[27]. The RNase-treated DNA samples were used as the source of virus templates for RCA analysis. The RCA of total DNA genomes using Phi29 DNA polymerase (Templiphi™, Amersham Biosciences) was performed according to Shepherd *et al.*[34]. The genomic concatemers generated during Phi 29 DNA polymerase amplification were used as templates for polymerase chain reaction of DNA-A (core coat protein), DNA-B, DNA-β satellites and DNA 1 satellites.

Restriction Fragment Length Polymorphism (RFLP): The RCA products (~300 ng DNA) were digested by the restriction enzymes *Ssp*I, *Nco*1, *BamH*1, *Cla*1, *Sac*1 and *EcoR*1 (New England Biolabs, Frankfurt, Germany), in 10 µL final volume for 2 h at 37°C, following the manufacturer's protocol, separated in 1% agarose gels in TBE and stained with 0.5 µg mL^{-1} ethidium bromide.

PCR, cloning, DNA sequencing and analysis: The PCR reactions were either conducted on DNA directly extracted from tissue samples or amplified RCA products. The PCR was conducted with the Perkin-Elmer (Norwalk, CT) DNA thermal cycler. Primers AV core (5' GCCHATRTAYAGRAAGCCMAGRAT 3') and AC core (5' GGRTTDGARGCATGHGTACANGCC 3') were designed to amplify 580 bp of the DNA-A core coat protein. Primers BV1855 (5' AC(A/G)CAA(A/G)TG(A/G)TC(A/T/G)AT(C/T) TTCAT 3')[35] and BC2571 (5' GGTAATATTATA(A/C/T)CGGATGG 3')[36] were designed to amplify 665 bp of DNA-B. Primes β01 (5' GGTACCACTACGCTACGCAGCAGCC 3') and β02 (5' GGTACCTACCCTCCCAGGGGTACAC 3')[37] were designed to amplify the full length, 1350 bp of DNA-betasatellites. Primers DNA 1F (5' ctgcagACAACGTCAGCTATCAG 3') and DNA 1R (5' ctgcagATCCTCCACGAGTGTAG 3') were designed to amplify 1300 bp of DNA-alphasatellites.

The reaction mixture contained 1-2.5 µL of DNA template (used directly or diluted 1/10 with water), 2.5 mM $MgCl_2$, 5X GoTaq DNA polymerase reaction buffer (Cat No. M8301, Promega Madison, WI, USA), 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.

The DNA amplification parameters were 30 cycles of denaturation at 95°C for 1 min, primer annealing for 1 min at 58°C for DNA-A, DNA-β, DNA 1 primers and 50°C for DNA-B primers. Primer extension was at 72°C for 1 min per cycle with a final extension cycle of 7 min at 72°C. The DNA amplicons were evaluated by electrophoresis using a 1% agarose gel

prepared in TAE buffer and stained with 0.5 μg mL^{-1} 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.*[38]. Plasmid from selected colonies was purified by miniprep then sequenced using automated, capillary DNA sequencing.

The DNA sequences were compared with all available begomovirus and satellite reference sequences using BLASTn available at the NCBI website. Nucleotide sequence identity and phylogenetic relationships was measured using NCBI/blastn, www.ncbi.nlm.nih.gov and MEGA6 programs. DNA sequences were submitted to GenBank, accession references are shown in Table 1.

Immunocapture PCR: The PCR tubes were coated with a mix of begomovirus specific antisera prepared from begomoviruses including African cassava mosaic virus and Solanum yellow leaf curl virus (R.W. Briddon, NIBGE, Faisalabad, Pakistan), bean dwarf mosaic Egypt virus, cotton leaf curl mosaic Egypt virus, CLCMEGV[39], HLCrEGV[40], okra leaf curl Egypt virus, OLCEGV, squash leaf curl Egypt virus, SLCEGV[9], tomato greenhouse whitefly transmitted Egypt virus, TGWFEGV[41], tomato yellow leaf curl Egypt virus, TYLCEGV and an unidentified begomovirus isolated from potato in WA, USA (P.E. Thomas, Prosser, WA, USA). The IC-PCR was conducted as described by Abdel-Salam[42]. Three virus controls of non-begomoviruses were included in the IC-PCR. These included plant samples infected with tobacco mosaic virus (*Tobamovirus*), faba bean necrotic yellows virus (*Nanovirus*) and a putative badnavirus causing sweet potato leaf curl disease.

RESULTS AND DISCUSSION

Symptomatology: Natural infection with whitefly-transmitted begomoviruses of fiber, vegetable, medicinal and ornamental crops were observed in many plants. Virus symptoms which ranged from leaf curling, rugosity, vein yellowing, vein enlargements, mosaic, leaf enations and yellowing (Fig. 1, Table 1) are typical for those caused by beogmovirus infections[4]. The high diversity of symptom manifestation represents a potential high diversity of begomoviruses present in Egypt including monopartite, bipartite and potentially beta satellite associations.

Begomovirus-host range and associated beta satellites: The incidence of begomoviruses and their associated betasatellites in these samples (Table 1) was confirmed with PCR, IC-PCR (using the AV/AC core primers), RCA analysis and/or DNA sequence. The presence of whiteflies (*Bemisia tabaci* Gennadius) associated with the above described symptoms in the affected fields suggest a common denominator for plant infections with begomoviruses[43]. Some crop species have previously been reported as hosts for begomoviruses in Egypt including HLCrEGV[40], CLCMEGV[39] (Phylogenically known as cotton leaf curl Gezira virus CLCuGV-EG/okra), TYLCEGV[44,45], OLCEGV[18], okra yellow vein Egypt virus, OYVEGV and okra enation Egypt virus, OEEGV[11], SLCEGV[9,10].

Results indicate that 26 out of 33 hosts mostly in the fibers, medicinal, weeds and ornamental crops are reported herein as possible hosts for new or previously recorded begomoviruses in Egypt. This raises an alarm for their being functioning as a reservoir for begomoviruses infecting economic crops in Egypt. Many investigators have pointed out to the significance of ornamentals and weeds as alternative hosts for begomoviruses[4,46].

Symptoms detected on bean, squash, cucumber, melon and watermelon (Fig. 1a-c, Table 1) are most likely caused by the newly introduced SLCEGV isolates from squash SLCEGV-SQ (DQ285019) and watermelon SLCEGV-W (KT35935). Both isolates have 92% DNA-A sequence identity; though IC-PCR, based on intensity of bands in gel may suggest that they differ in antigenecity of coat protein epitopes. The SLCEGV-SQ isolate had 98 and 99% DNA-A sequence identity with SLCV-E (M38183) from USA and SLCV (HQ184436) from Israel, respectively. The SLCEGV-W had 91 and 92% sequence identity with the same isolates from USA and Israel, respectively. The SLCV-E is known for its wide host range[32] and has high sequence identities with similar isolates from Egypt and Israel. Perhaps this explains the high virulence of the present SLCV in Egypt. Interestingly, SLCV was reported in Israel in 2002)[30] and in Egypt in 2005[9].

Hibiscus piment = syn. *Malvaviscus arboreus* showed bright yellow mosaic and yellow vein symptoms (Fig. 1d). Begomovirus incidence in this plant was confirmed with PCR and RCA. Closer symptoms were described for this host in Brazil where begomovirus infection was associated with alphasatellite-like nonanucleotide structure[47].

The vein yellowing symptoms observed on *Hibiscus cannabinus* and *Hibiscus sabdariffa* (Fig. 1e, f) may be related to new undiscovered begomovirus(s) in these two hosts. Chatterjee *et al.*[46] isolated a new monopartite begomovirus from *Hibiscus cannabinus* and *H. sabdariffa* with leaves showing yellow veins in India.

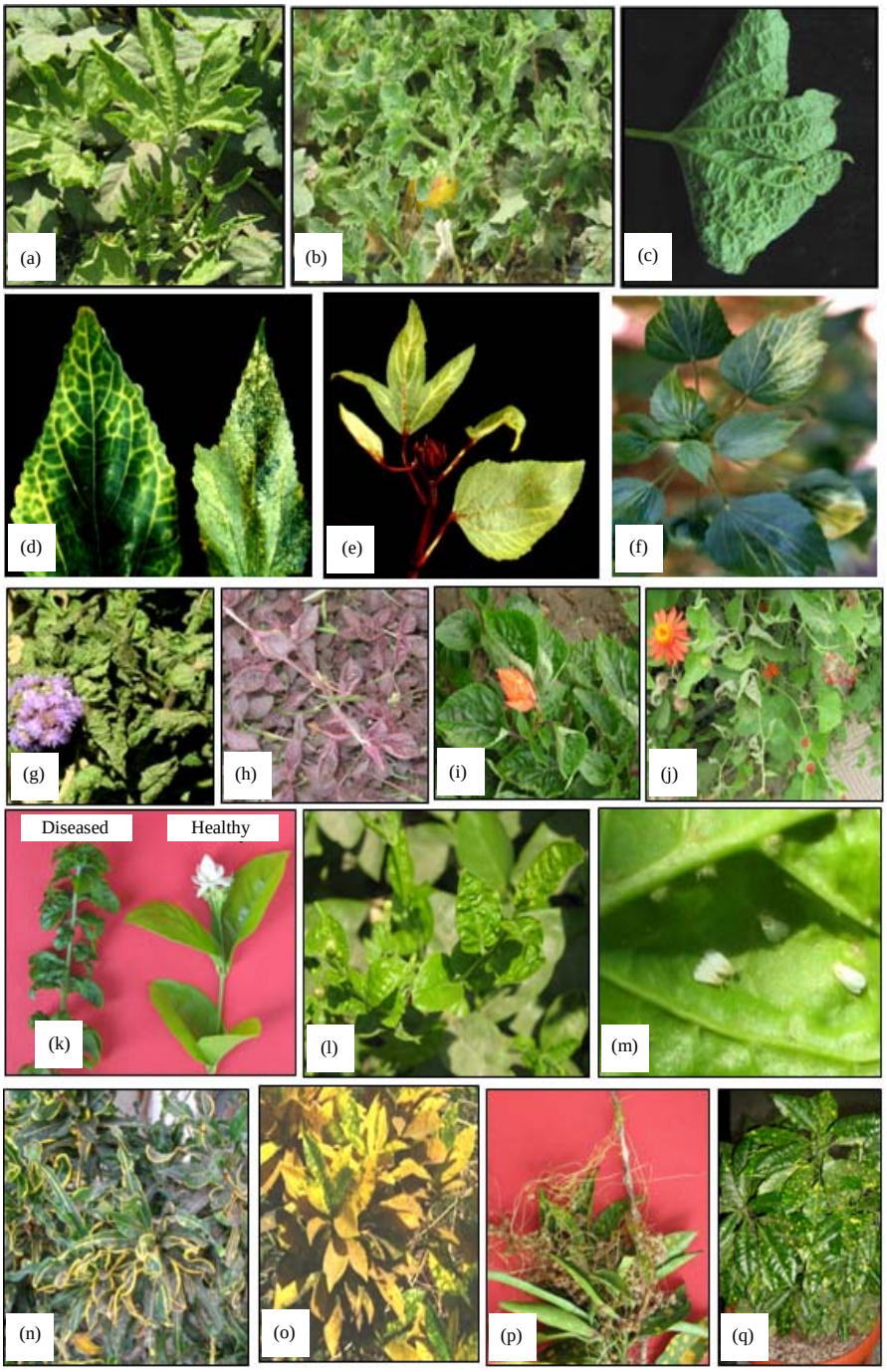

Fig. 1(a-q): Symptoms caused by begomoviruses detected in this study, (a) SLCEGV/squash, (b) *Cucumis melo* leaf curl disease/melon, (c) *Cucumis sativus* leaf curl disease/cucmber, (d) *Hibiscus piment* yellow vein chlorosis disease/*Hibiscus piment*, (e) *Hibiscus sabdariffa* yellow vein disease/*Hibiscus sabdariffa*, (f) *Hibiscus cannabinus* yellow vein disease/*Hibiscus cannabinus*, (g) SLCEGV/*Ageratum conyzoides*, (h) *Iresine herbstii* leaf curl disease/*Iresine herbstii*, (i), *Salvia splendens* leaf curl disease/*Salvia splendens*, (j) *Senecio macroglossus* leaf curl disease/*Senecio macroglossus*, (k) *Jasminum sambac* rugosity disease/*Jasminum sambac*, (l) Rugosity symptoms mentioned in (k), (m) *Trialeurodes ricini* whiteflies on *Jasminum sambac*, (n) *Codiaeum variegatum* malformation disease/*Codiaeum variegatum*, (o) *Codiaeum variegatum* cv goldfinger yellowing disease/*Codiaeum variegatum* cv goldfinger, (p) *Codiaeum variegatum* cv goldfinger parasitized by dodder and (q) Healthy *Codiaeum variegatum* cv goldfinger

Table 1: Begomovirus-infected hosts tested positive for PCR, IC-PCR and/or RCA analysis

Host plant/family	Virus or virus-like disease*	Associated begomo-virus/ GenBank accession No.	Symptoms	Locations	PCR	IC-PCR	RCA	Associated beta saellites/ GenBank accession No.
Fiber and medicinal crops								
Malvaceae								
Gossypium barbadense (Cotton)	Cotton leaf curl mosaic diseases	CLCMEGV/FJ030874	LC, M, VE, VN	Giza	+	+	nd	
Hibiscus cannabinus# (Decan hemp) = (Mesta)	*Hibiscus cannabinus* yellow vein disease	???????	LC, YV	Giza	+	+	+	
Hibiscus sabdariffa# (Roselle)	*Hibiscus sabdariffa* yellow vein disease	???????	LC, YV	Giza	+	+	+	
Luffa aegyptiaca# (Luffa)	*Luffa aegyptiaca* leaf curl disease	???????	LC	Giza	+	+		
Hibiscus esculentus (Okra)	Okra leaf curl disease	OLCEGV/FJ030878	LC	Giza, Qalyubia	+	+	+	OLCEGB/FJ187796
Hibiscus esculentus (Okra)	Okra enation disease	OEEGV	LE		+	+	nd	
Hibiscus esculentus (Okra)	Okra yellow vein virus disease	OYVEGV	LC, YV	Giza	+	nd	nd	
Vegetable crops								
Solanceae								
Capsicum annuum# (Bell pepper)	*Capsicum annuum* leaf curl disease	CALCD	LC	Giza	+	nd	Nd	
Lycoperisicon esculentum (Tomato)	Tomato yellow leaf curl disease	TYLCEGV/FJ030876	LC, IY, S	Giza, Faiyum, Beni Suef, Aswan	+	+	+	OLCEGB/FJ187797
Cucurbitaceae								
Cucumis melo# (melon)	*Cucumis melo* leaf curl disease	???????	LC	Sharqia	nd	+	nd	
Cucumis sativus# (Cucuumber)	*Cucumis sativus* leaf curl disease	???????	LC, R	Giza	+	nd	+	
Cucurrbita pepo (Squash)$	Squash leaf curl virus	SLCEGV/FJ416866	LC, R	Giza, Qalyubia	+	+	+	OLCEGB/FJ455515
Citrullus lanatus# (Watermelon)	Squash leaf curl disease	SLCEGV/KT359351	LC, N	Giza,	+	+	nd	
Field crops								
Fabaceae								
Phseolus vulgaris#	Bean golden mosaic disease	???????	LC, GM	Giza	+	+	nd	
Glycine max# (Soybean)	Soybean crumple disease	???????	LCr	Giza	+	nd	nd	
Ornamental and weed crops								
Amaranthaceae								
Iresine herbstii#	*Iresine herbstii* leaf curl disease	???????	LC	Giza	+	+	+	
Asteraceae								
#	*Senecio macroglossus* leaf curl disease	???????	LC	Giza	+	nd	nd	
Chenopodiaceae								
Chenopodium album# (Fat hen)$	*Chenopodium album* leaf curl disease	SLCEGV/FJ455514	LC	Giza	+	+	+	SLCEGB/FJ436004
Euophrbaceae								
Ageratum conyzoides# (*Ageratum*)	*Ageratum conyzoides* leaf curl disease	SLCEGV/FJ030877	LC	Giza	+	+	nd	
Codiaeum variegatum# (Croton)	*Codiaeum variegatum* malformation disease	???????	Mal	Giza	+	nd	+	
Codiaeum variegatum# cv. goldfinger (croton)	*Codiaeum variegatum* cv. Goldfinger yellowing disease	???????	Y	Giza	+	nd	+	
Fabaceae								
Rhynchosia monophylla#	*Rhynchosia monophylla* leaf curl disease	???????	LC	Beni Suef	+	nd	+	
Laminceae								
Salvia splendens# (Scarlet sage)	*Salvia splendens* leaf curl disease	???????	LC	Giza	+	nd	nd	

Table 1: Continue

Host plant/family	Virus or virus-like disease*	Associated begomo-virus/ GenBank accession No.	Symptoms	Locations	PCR	IC-PCR	RCA	Associated betasaellites/ GenBank accession No.
Malvaceae								
Althea rosea (Hollyhock)	Hollyhock leaf crumple disease	HLCrEGV/FJ030873	LCr, E	Giza	+	+	+	HLCrEGB/FJ455516
Hibiscus piment[#] = syn., *Malvaviscus arboreus* (Wax mallow)	*Hibiscus piment* yellow vein chlorosis disease	???????	Chl, YV	Giza	+	nd	+	HLCrEGB/FJ436003
Hibiscus rosa[#] *sinensis*	*Hibiscus rosa-sinensis* leaf curl disease	???????	LC	Giza,	+	nd	+	
Nyctaginaceae								
Mirabilis jalapa[#]	*Mirabilis jalapa* leaf curl disease	???????	LC	Giza	+	nd	nd	
Oleaceae								
Jasminum grandiflorum[#] (Royal Jasmine)	*Jasminum grandiflorum* leaf curl disease	???????	LC	Assiut	+	nd	+	
Jasminum sambac[#] (Arabian jasmine)	*Jasminum sambac* rugosity disease	???????	R	Giza	+	nd	nd	
Rubiaceae								
Plumeria rubra acutifolia[#] (West Indian jasmine)	*Plumeria rubra acutifolia* mosaic disease	???????	Mos	Giza	+	nd	nd	
Solanaceae								
Cestrum fasciculatum[#] (Cestrum)	*Cestrum fasciculatum* leaf curl disease	???????	LC	Giza	+	+	nd	
Petunia hybrida[#] (Petunia)	*Petunia hybrida* leaf curl disease	???????		Giza	+	nd	nd	
Solanum rantonnetii[#] (Blue potato bush)	*Solanum rantonnetii* leaf curl disease	???????	LC	Giza	+	nd	nd	

*Virus disease phrase was added to the name of the host showing symptoms, Chl: Chlorosis, E: Enations, GM: Golden mosaic, IY: Interveinal yellowing, LC: Leaf curl, LCr: Leaf crumple, LE: Leaf enations, Mal: Malformation, Mos: Mosaic, N: Necrosis, R: Rugosity, S: Stunting, VE: Veinal enlargement, VN: Veinal necrosis, Y: Yellowing, [#]New sampled hosts for begomoviruses in Egypt, +: Positive results, ???????: Unknown begomovirus. Each sample was tested thrice in separate experiments with PCR and/or IC-PCR and RCA, nd: Not determined, [$]Represent presence of betasatellites with bipartite begomovirus. Betasatellite abbreviations included: OLCEGB: Okra leaf curl Egypt betasatellite, SLCEGB: Squash leaf curl Egypt betasatellite, HLCrEGB: Hollyhock leaf crumple Egypt betasatellite

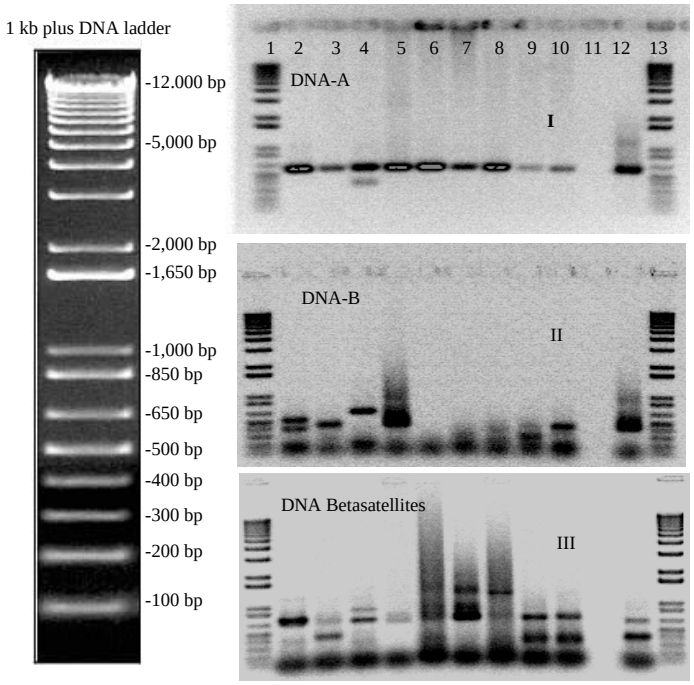

Fig. 2: PCR amplicons amplified from DNAs products of RCA of some samples infected with begomoviruses. RCA-DNA-Products were amplified with primers for I: AV/AC core, II: DNA-B and III: Betasatellites. Tested samples were in the same order in the all tested gels. Tested samples are as follow, 1: 13: 1 kb plus DNA Ladder, 2: *Hibiscus piment* (Wax mallaw), 3: *Chenopodium album*, 4: *Iresine herbstii* (Herbs), 5: *Cucurbita pepo* (Squash), 6, 7: *Althea rosea* (Hollyhock), 8: *Hibiscus esculentus* (Okra), 9: *Hibiscus sabdariffa* (Roselle), 10: *Hibiscus cannabinus* (Mesta), 11: Negative control (H_2O) and 12: Positive control (TYLCEGV)

Leaf curl symptoms were observed on other ornamentals, detected as positive for begomovirus infection including *Ageratum conyzoides*, *Iresine herbstii*, *Salvia splendens* and Senecio *macroglossus* (Fig. 1g-j). Only *A. conyzoides* was turned out to be infected with SLCEGV; while for the other ornamentals in Table 1 further studies are needed to identify the associated begomovirus(s).

Jasminum sambac (Arabian jasmine) was found positive to begomovirus presence with PCR analysis in the present study (Fig. 1k, l) as well as in associated *Trialeurodes ricini* whitefliy (Fig. 1m). Idriss *et al.*[48] reported the ability of *T. ricini* to transmit TYLCV in Egypt. Interestingly, *J. sambac* was found as host for Sonchus yellow mosaic virus in India[49].

The PCR and/or RCA results detected begomovirus-coat protein in croton plants (Fig. 1n, o) and in its parasitized dodder, *Cuscuta* sp. (Fig. 1p). Begomovirus-coat protein was detected) in both the threads and fruits of dodder plant. This shows the importance of dodder in transmitting begomoviruses as stated by Narayana *et al.*[50].

The leaf curl symptoms developed on chili pepper (*Capsicum annuum*) was also attributed to begomovirus infection. Similar results reported the infection of chili pepper with tomato leaf curl New Delhi virus (TLCNDV) in India[51] and cotton leaf curl Multan virus (CLCuMV) in Pakistan[52].

RCA, PCR and/or IC-PCR analysis: Virus diversity was further investigated using RCA, PCR and/or IC-PCR analysis. Using RCA products, in 9 tested samples, as the PCR templates (Fig. 2), DNA-A fragments of ~580 bp were amplified from all samples as previously shown for begomoviruses[53]. The presence of DNA-B components were confirmed in most tested samples with a range between 400-650 bp indicating contaminations with the DNA-B component of bipartite begomovirus(s) probably the newly introduced squash leaf curl virus from the new world[9,10]. No DNA-B components; however were recovered from RCA of *A. rosea* sample number 6. Betasatellites were detected in all samples (Fig. 2). Only full length satellites at ~1300 bp were detected in lanes 6 and 7 for HLCrEGB and lane 8 for OLCEGB. Shorter betasatellites (700-1000 bp) were however, present in all tested samples (Fig. 2). It appears that most of betasatellites in the Nile valley are shorter than the Asian betasatellites[11,18]. The presence of betasatellites associated with the bipartite genome of SLCEGV was observed by PCR in squash and chenopodium plants

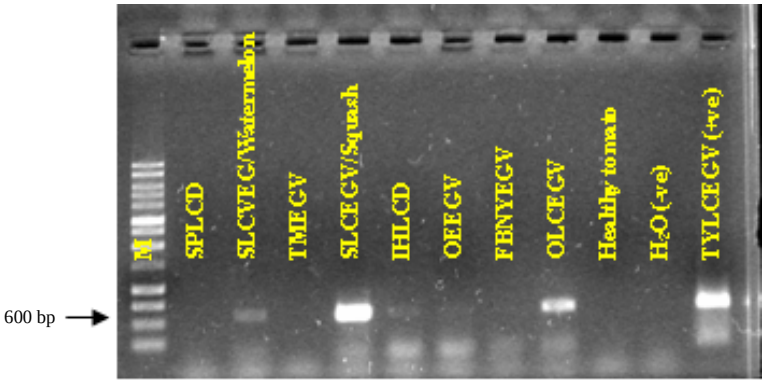

Fig. 3: 0.7% agarose gel electrophoresis in TAE buffer showing the migration of IC-PCR amplicons of different infected samples entrapped with a cocktail of polyclonal antisera and visualized with ethidium bromide, M: Benchtop 1 kb DNA ladder, SPLCD: Sweet potato leaf curl disease with leaf curling symptoms, SLCEGV/Watermelon: An isolate of SLCEGV from watermelon, TMEGV: Tobacco mosaic Egypt virus, SLCEGV/Squash: An isolate of SLCEGV from squash, IHLCD: A begomovirus infecting *Iresine herbstii* causing leaf curl disease, OEEGV: Okra enation Egypt virus, FBNYEGV: Faba bean yellows Egypt virus, OLCEGV: Okra leaf curl Egypt virus. Negative controls (-ve) involved sap from healthy tomato, H_2O and positive control (+ve), TYLCEGV: Tomato yellow leaf curl Egypt virus

infected with SLCEGV (Table 1). Likewise, in India[23] have shown that 50% of tested cucurbitaceous, solanaceous and leguminaceous plant hosts for infection with the bipartite TLCNDV had betasatellites associated with their viral entities. No alphasatellites were detected in the nine tested samples. Alphasatellites frequency was very low in all samples. Only two samples were positive for alphasatellites.

The IC-PCR results illustrated in Fig. 3 indicated the high capability of this technique in differentiation between begomo, tobamo, nanoviruses and badaviruses. Variability in DNA band intensities of begomoviruses could be attributed to the variability of the virus concentrations, DNA-primer binding and antigen-antibody interactions in the different tested hosts. For example DNA band intensities in SLCEGV isolates from watermelon and squash differed drastically. Rampersad and Umaharan[25] and Abdel-Salam[42] have shown the significance of IC-PCR in detection of begomoviruses in plant extracts of the families Solanacese, Malvaceae and Fabaceae.

RCA-RFLP: The RCA-RFLP analysis detected the presence of begomoviruses from experimental and natural host plant sources (Table 2). The lengths of the resulting fragments in most of the tested samples were within the known full length genome of begomoviruses. Restriction Digestion (RD) pattern varied in samples due to the absence or presence of restriction sites for the tested restriction enzyme. Most of the found viruses gave RD patterns of monopartite begomoviruses already existed in Egypt, (code#, AS1, A48, BS41, BS44, T22, T24, 43 and 46). Sample AS9 is not probably a geminivirus. Sample C84 gave RD pattern typical for BBTV, *Babuvirus*, with 1.1 kbp for each of its six ORFs[54].

Sample shaded with grey (code#, A47, C56, C75, 21) with RD patterns ≥5 kbp may represent known bicomponent begomovirus as in sample code# 21 and C75 for SLCEGV or unknown bipartite begomovirus, or may contain additional some of the short Nile basin DNA satellites described by Briddon *et al.*[11] and Idris *et al.*[10]. It is known that phi 29 DNA polymerase co amplify circular DNA satellites with virus genomic DNAs[29]. Future testing of these samples through DNA sequencing and Southern hybridization using probe for DNA-B is needed.

Based on DNA sequence identities of identified begomoviruses in Egypt, the present study indicates that diversity in begomoviruses species in Egypt is low to moderate. Most of the detected begomoviruses in vegetables, field crop and ornamentals were restricted in TYLCEGV, OLCEGV/CLCuEGV, HLCrEGV, OYVEGV) and the newly introduced bipartite SLCEGV. Some new begomoviruses are possibly present but not yet identified. Results based on field observation of new symptoms of leaf crumpling associated with distinctive yellow chlorotic spots on squash and cucumber, as described by Brown *et al.*[32] give a strong indication of the presence of the bipartite cucurbit leaf curl virus, CuLCV, (also known as cucurbit leaf crumple virus, CLCrV)[55]. Therefore, one would dedicate that the RCA-RFLP analysis of tomato (Assiut-Egypt) samples with RD of a ≥5 kbp (Fig. 4, Table 2) would be attributed to contamination with bipartite begomoviruses.

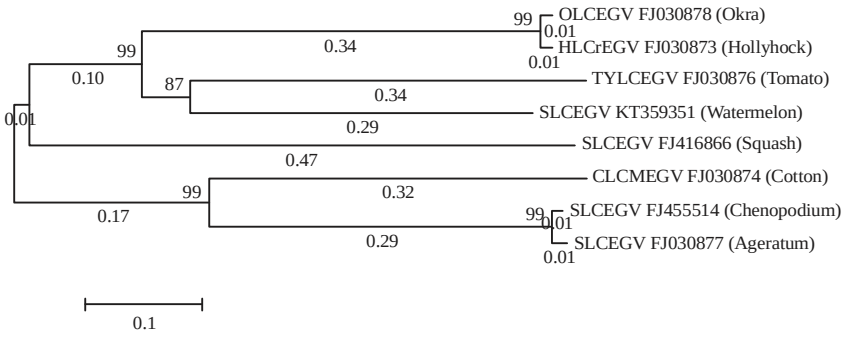

Fig. 4: Phylogenetic tree depicting the relationships between the described begomoviruses in this study based on amino acid sequences using the neighbor-joining method. The dendrogram was boot strapped 500 times (scores are shown on nodes). The distance scale represents number of amino acid differences. The analysis involved 8 amino acid sequences. Names of begomoviruses, hosts and their accession numbers in the GenBank were depicted in Table 1. Evolutionary analysis were conducted in MEGA6

Table 2: Restriction Digestion (RD) of RCA-DNA products of begomovirus samples collected from different location in Egypt

No. of Samples	Code No.	Locations	Virus/host	RFLP (kbp) *SspI	NcoI	BamHI	ClaI	SacI	EcoRI
1	AS1	Aswan	TYLCEGV/tomato	2.7	2.2	(-)	1.6, 1.2	2.7	2.7
2	AS9	Aswan	?/Cucumber	(-)	(-)	(-)	(-)	(-)	(-)
3	A47	Assiut	?/Tomato	2.7, 2.4, 1.0, 0.4 (6.5)#	2.4	2.4	2.7	2.7	1.2
4	A48	Assiut	?/Jasmine	1.65, 1.0	(-)	(-)	1.0	1.0, 0.60	(-)
5	BS41	Beni Sweif	TYLCEGV/Tomato	2.7	2.7	2.4	2.7	(-)	2.2, 1.3
6	BS44	Beni Sweif	?/Rhynchosia	1.8	2.5	(-)	1.0, 0.80	2.1	(-)
7	T22	Toukh	OLCEGV/Okra	1.5, 1.2, 0.85, 0.2	(-)	(-)	(-)	2.7	(-)
8	T24	Toukh	OLEGV/Okra	1.5, 1.65, 0.85	1.8, 1.0	2.7	2.7	(-)	(-)
9	C56	Giza	?/*Hibiscus rosa-sinensis*	3.0, 2.5 (5.5)#	2.7	(-)	(-)	3.1, 2.0 (5.1)#	(-)
10	C75	Giza	?/Cucumber	2.5, 1.65, 0.85 (5)#	1.8, 0.80	(-)	2.7	2.7	2.7, 2.3 (5)#
11	C79	Giza	?/Croton	(-)	(-)	2.7	(-)	(-)	(-)
12	C81	Giza	?/Croton	(-)	(-)	(-)	(-)	(-)	2.7
13	C84	Giza	BBTEGV/Banana	1.1	(-)	(-)	1.1	(-)	(-)
14	43	Giza	?/Roselle	3.0	nd	nd	nd	nd	nd
15	46	Giza	?/Mesta	1.65 and 1.30	nd	nd	nd	nd	nd
16	21	Giza	SLCEGV/Squash	2.5, 1.65, 0.85, 0.65 (5.65)#	nd	nd	nd	nd	nd

*Samples 1-13 were collected from Egypt, while samples 14 and 15 were from Sudan, Samples 16-18 were from Egypt and digested with *SspI* only, (-): Negatvie reaction, nd: Not determined, ?: Unknown virus, AS: Aswan, A: Assiut, BS: Beni Swuif, T: Toukh and C: Cairo. Positive controls included TYLCEGV (code No. AS1), monopartite begomovirus, SLCEGV (code No. 21), bipartite begomovirus, BBTEGV (code No. C84), a babuvirus, #Represent DNAs with ≥5 kbp, **SspI* is specified for the nonanucleotide invariant sequence 5'-TAA↓TATTAC-3' present in the potential hairpin intergenic region in geminiviruses and betasatelltes

On the other hand, the described begomoviruses in Egypt showed high biodiversity in pathology, especially the newly introduced SLCEGV. The SLCEGV has widened up its hosts and infected different plant families). For instance SLCEGV was detected in *Ageratum conyzoides* and *Chenpodium album* (Fig. 1, Table 1). Additionally, new isolates of SLCEGV was reported in the present study to infect watermelon (Table 1). Similarly, in India, TLCNDV had a diverse host range from different families and was recently isolated from *Eclipta prostrate*, an ornamental[56]. Recently TLCNDV was isolated from cucurbits in Taiwan[57], Spain[58] and Tunisia[59]. Additionally, a begomovirus isolated from *Vinca minor* (periwinkle) in Pakistan had 90% sequence homology with the coat protein of tomato leaf curl Joydebpur begomovirus[60]. Further, a given begomovirus infecting an ornamental species can infect another ornamental species. For instance *Pedilanthus* leaf curl begomovirus infecting *Pedilanthus tithymaloides* was recently isolated from *Vinca minor*[61]. Similarly recent results have reported the infection of TYLCV to *Lamium amplexicaule* herb in Korea[62].

Phylogeny and interactions between begomoviruses and betasatellites in Egypt: In the present study, phylogeny between begomoviruses in Fig. 4 was based on amino acid substitution of coat protein gene of monopartite begomoviruses (OLEGV, HLCrEGV, CLCEGV, TYLCEGV) and the newly introduced bipartite squash leaf curl virus (SLCEGV).

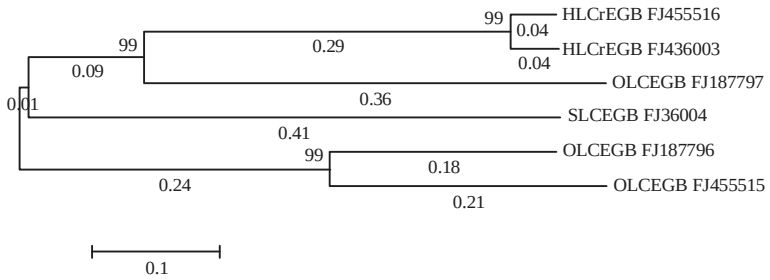

Fig. 5: Phylogenetic tree depicting the relationships of the begomoviru-associated betasatellites using the neighbor-joining method. The dendrogram was boot strapped 500 times (scores are shown on nodes. The distance scale represents number of amino acid differences. Evolutionary analysis was conducted in MEGA6 program. The abbreviations are according to Briddon *et al.*[12]

Table 3: Characteristics of studied betasatellite DNA sequences

Betasatellites/host plant	Accession No.	Length (bp)	Closest hit	Accession No. of closest hit	Coverage (%)	Max identity (%)
OLCEGB/okra	FJ187796	668	OLCVEGB	AF397215	83	98
OLCEGB/tomato	FJ187797	714	HLCrEGB	FJ436003	60	98
OLCEGB/squash	FJ455515	704	HLCrEGB	FJ436003	59	97
SLCEGB/chenopodium	FJ436004	421	Begomovirus-DNA-II sat. satellite	AY836366	7	94
HLCrEGB/hollyhock	FJ455516	676	HLCrEGB	AF397214	72	99
HLCrEGB/wax mallaw	FJ436003	534	HLCrEGB	AF455516	80	98

Begomoviruses clustered in two clades. In clade 1, SLCEGV from watermelon and squash clustered with OLCEDV, HLCrEGV and TYLCEGV. The SLCEGV from squash clustered, however, on a separate branch in clade 1 which indicates amino acid differences in their coat protein. Indeed this latter drawn conclusion is fortified with IC-PCR experiment results (Fig. 3) where the two isolates of SLCEGV showed difference of epitope-paratopes binding and hence different intensity in DNA patterns. In clade 2, SLCEGV from chenopodium and ageratum clustered with the monopartite CLCMEGV which indicates again that SLCEGV isolates from these latter hosts are different from the corresponding isolates from watermelon and squash.

Tiendrebeogo *et al.*[63] described a new recombinant bipartite African Cassava Mosaic Virus (ACMV) from Burkina Faso (ACMBFV) with major parents related to the West African isolates of ACMV and minor parents related to the monopartites tomato leaf curl cameroon virus (ToLCCV) and cotton leaf curl Gezira virus. The ACMBFV clustered with ToLCCV confirming the recombination nature of ACMBFV. Similarly, the clustering of the bipartite SLCEGV isolates with the monopartite begomoviruses, added to the presence of DNA-B component in many tested monopartite begomovirus in Fig. 2, may suggest the incidence of recombinant events between the existing monopartite begomoviruses and the bipartite SLCEGV since it is known that wild types of monopartite and bipartite begomoviruses are clustered separately.

All betasatellites showed high nucleotide sequence homology over the 78% taxonomic threshold[12] (Table 3, Fig. 5). The SLCEGB associated with the bipartite SLCEGV differed from the other betasatellites and clustered on a separate branch (Fig. 5). The SLCEGB had 32 nt homology with DNA-II satellite (AY836366), associated with cassava mosaic disease (Table 3). The present results in Table 1 indicated possible movement of betasatellites across plant species, being promiscuous as reported for the Asian and African betasatellites[4,15-17]. For instance OLCEGB was isolated from okra infected with OLCEGV from tomato infected with TYLCEGV and from squash infected with SLCEGV. Similarly SLCEGB was isolated from chenopodium (fat hen) infected with SLCEGV. In addition to its association with its helper virus in hollyhock, HLCrEGB was also isolated from wax mallow infected with unknown begomovirus. Such movements of betasatellites between hosts dramatically increase begomovirus diversity in disease-symptom expression.

The introduction of the bipartite SLCV into Egypt marked the first incidence of a new world begomovirus causing disease in the old world. With the presence of beogomvirus-associated betasatellites in the old world and their being promiscuous has possibly led to their association with SLCEGV too and leading to the presence of DNA-A and DNA-B viral component with DNA betasatellites. In our case, OLCEGB and SLCEGB were associated with SLCEGV (Table 1). This, in turns is expected to increase biodiversity of this virus dramatically in Egypt. Similar studies have detected the

association of betasatellites with bipartite begomoviruses in the old world[21-23,64] and recently in the new world[65]. With the introduction of watermelon chlorotic stunt (WCSV), a new bipartite begomovirus comer from the new world to Israel, Jordan and Lebanon[31] and TLCNDV from the Indian subcontinent to Spain[58] and Tunisia[59] one would expect that these bipartite begomoviruses would be recorded in Egypt in a matter of few years from now.

CONCLUSION

The present study tackles for the first time the presence of begomoviruses and their associated satellites infecting vegetable, fiber, weeds and ornamental crops in Egypt. Ten years ago, the sole known begomovirus in Egypt was TYLCV. Therefore, this study is adding new information on the presence of other begomoviruses in Egypt. Although the interaction between begomoviruses and their associated satellites is known worldwide, no information was available on such subject in Egypt before carrying out the present research. Due to the limited number of described begomoviruses and associated satellites one would be compelled to describe the biodiversity of these entities, in terms of found viruses, as low to moderate. However, with the presence of big number of unidentified begomoviruses and associated satellites one would expect that diversity regarding pathology of these viruses would be high. The betasatellites described in this study with being promiscuous in nature, the introduction of SLCV into Egypt and the coming threats of arrival of other bipartite begomoviruses as WCSV, CLCrV and TLCNDV to Egypt pose a serious threat to crop production and hence food security in Egypt.

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