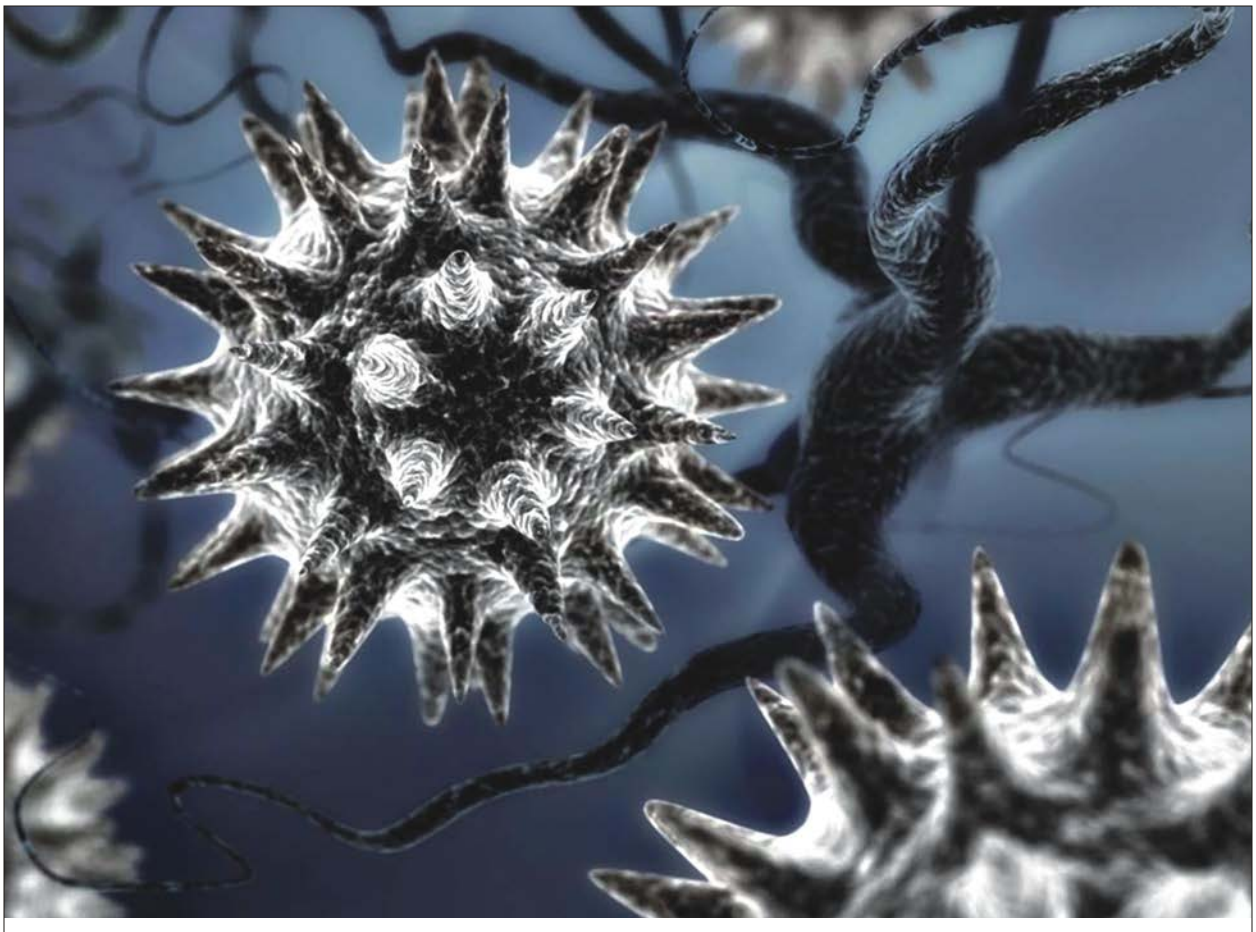

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Research Article
Serological and Molecular Characterization of a New *Badnavirus* Species in *Bougainvillea glabra* Plants in Egypt

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Abstract

Background and Objective: *Badnaviruses* are serious plant pararetroviruses affecting several economic plants resulting in heavy losses worldwide. No previous studies were available on the phenomenon of chlorotic spot in *Bougainvillea* in Egypt. This study aims to elucidate the virus nature infecting *B. glabra* in Egypt. **Materials and Methods:** Immunocapture PCR (IC-PCR) used local antiserum for *Banana Streak Virus* (BSV) and the Badna FP/RP specific primers for the RT and RNase H coding regions of ORF3 present in badnaviruses. Amplified DNAs were inserted into pTZ57R/T vector and cloned in *Escherichia coli* JM 109 cells. Obtained sequence was blasted with other badnaviruses using BLASTN programs. Genetic Distance (GD) and phylogenic studies for this virus and other described badnaviruses were analyzed with MEGA6 program. **Results:** The IC-PCR yielded 580 bp amplicons from *B. glabra*, banana and sugarcane infected with BSV and *Sugarcane Bacilliform Virus* (SCBV), respectively. Accession # KM821771.1 was assigned for the new badnavirus from *B. glabra*. The virus had a maximum nucleotide identity of 75% and GD>0.2 with three *Bougainvillea* infecting viruses from Taiwan, Brazil and India, respectively. In phylogenetic analysis, it clustered with badnaviruses infecting *B. spectabilis*. These data indicate that chlorotic spots associated with *B. glabra* in Egypt are caused by a new *Badnavirus* species. A tentative name of *Bougainvillea glabra Chlorotic Spot Virus-Egypt* (BgCSV-E) was suggested. **Conclusion:** Serologic, molecular and phylogenetic tools unveiled the viral nature of the badnavirus causing chlorotic spot in *Bougainvillea glabra* plants in Egypt. The spread of BgCSV-E over neighboring areas may be possible and could represent a potential reservoir of the virus for other susceptible crops.

Key words: *Badnavirus*, *Bougainvillea glabra*, *Banana Streak Virus*, *Sugarcane bacilliform* Virus, amplicons, sequence analysis, phylogenetic analysis, immunocapture polymerase chain reaction

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Competing Interest: The author has declared that no competing interest exists.

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

INTRODUCTION

Badnaviruses (Family: *Caulimoviridae*; Genus: *Badnavirus*) are pararetro plant viruses with non-enveloped bacilliform particles (30×120-150 nm) with a single, circular dsDNA of about 7.2-9.2 kb containing 3-7 open reading frames[1]. They are transmitted vertically by vegetative and/or pollen and seed transmission[1-3]. Horizontal transmission of the majority of *badnaviruses* is through various mealybug species[1,4]. Few badnaviruses; however, are aphid transmitted in a semi-persistent manner[5].

Badnaviruses with 32 known species are considered as serious pathogens affecting several economic plants all over the world, such as banana, sugarcane, cocoa, citrus, black pepper, taro, yam, canna, spiraea and *Bougainvillea*[1,6-9]. Estimated economic loss caused by the different species of badnaviruses in various crops varies[1] between 10 and 90%.

Some badnaviruses might act as endogenous viruses integrated into their host genomes. Biotic stress factors can activate these endogenous genomes into their episomal forms[1].

Bougainvillea plant species, native to Amazonian rainforests in South America, is an important ornamental and landscaping plant that is widely distributed worldwide. The genus *Bougainvillea* (*Nyctaginaceae*) has three species that are horticulturally important, namely *B. spectabilis*, *B. glabra* and *B. peruviana*. *Bougainvillea glabra* is an evergreen plant has elliptical green or variegated leaves, mostly glabrous (smooth and hairless). The petaloid bracts are triangular with many colors ranging from pink, red, gold, orange and white according to the variety. The flowers are white to cream[10].

Bougainvillea plants are vegetatively propagated. This allows the spread of viruses worldwide. Although little is known about viruses infecting *Bougainvillea* species, *Impatiens Necrotic Spot Virus*, *Tospovirus*, was reported in Iran[11]. *Clerodendron Yellow Mosaic Virus*, *Begomovirus* was reported to infect *B. peruviana* in India[12]. A *Badnaviruses* was first reported to infect *B. spectabilis* in São Paulo (SP), Brazil[13,14]. Symptoms of chlorotic spots and mild mosaic were detected on *B. glabra* in SP, Brazil[15]. Chlorotic spot symptoms on *Bougainvillea* plants were also described in India[8] and Taiwan[16]. The name *Bougainvillea Chlorotic Vein Banding Virus* (BCVBV) was accepted by the International Committee on Taxonomy of Viruses (ICTV)[17].

Recently, chlorotic spot symptoms were observed on *B. glabra* in the gardens of the Faculty of Agriculture, in Cairo University in Egypt. Serology and molecular tools including polymerase chain reaction, cloning, DNA sequencing and phylogeny were employed to elucidate the nature of the causing disease agent.

MATERIALS AND METHODS

Study area: This study was carried out at the Plant Pathology Department, Virology Lab, beginning from November, 2014- December 2017.

Virus isolates: Samples of *B. glabra* leaves with symptoms of chlorotic spots were used to detect the virus understudy. Other virus isolates included an Egyptian isolates of *Banana Streak Virus* (BSV-E) from banana[18] and a *Sugarcane Bacilliform Virus* (SCBV-E) isolate from sugarcane[4].

Immunocapture PCR (IC-PCR): The IC-PCR was performed using a rabbit polyclonal antiserum prepared for BSV-E[19]. Positive serologic relationships between BSV and some badnaviruses were reported by several authors[4,18,20].

The IC-PCR procedure was followed[18]. Sterile polypropylene thin-walled 0.2 mL microfuge tubes were coated overnight at 4°C with 25 µL of BSV polyclonal antiserum, diluted 1/100 in ELISA coating buffer (pH 9.6). Tubes were then washed 3 times with 100 µL of PBST washing buffer, pH 7.4 and incubated overnight at 4°C with 25 µL of sap extract. Plant extracts were diluted 1/10 (w/v) with citrate extraction buffer (0.05 M sodium citrate, pH 7.0, containing 0.5 mM EDTA, 1% (w/v), skim milk powder, 0.5% glycerol, 0.05% Tween-20 and 0.05% of 2-mercaptoethanol). Plant extracts were then clarified by centrifugation at 14,000 g for 5 min. The tubes were washed as before with washing buffer. To circumvent any possible presence of integrated DNA viral sequence[18] and assure the episomal nature of BgCSV-E, tubes were incubated at 37°C for 30 min with 3 U of DNase I (Invitrogen) in a total reaction volume of 50 µL. The DNAse I was removed by washing tubes with 100 µL washing buffer. The tubes were then rinsed with 100 µL sterile water. The complete PCR mixture was added directly to the tube using degenerate primers Badna FP (5'ATGCCITTYGGIITIAARAAYGCICC3') and Badna RP (5'CCAYTTRCAIACISCICCCCAICC3') designed based on the consensus sequence of the RT and RNase H coding regions to amplify a 580 bp product[21].

The PCR reaction mixture of 25 µL contained 5 µL of 5X Go*Taq* DNA polymerase reaction buffer (Cat No. M8301, Promega, Madison, WI, USA), 2.5 mM $MgCl_2$, 0.2 mM for each dNTP base, 0.4 µmol for each forward and reverse primers, 1.25 U *Taq* polymerase (M8301, Promega). Sterile bi-distilled water was added at a final volume of 25 µL. The PCR cycle conditions, as described by Yang *et al.*[21], included an initial denaturation step at 94°C for 4 min, followed by 40 cycles (94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec) followed by

a final elongation step of 5 min at 72°C. Ten microliters of PCR amplicons were analyzed by electrophoresis with 1% agarose gel prepared in TAE buffer and stained with 0.5 μg mL^{-1} ethidium bromide.

Cloning and sequencing: The PCR amplicons were cut from the gel, purified using QIAEX II gel extraction kit (Qiagen) and then cloned into pTZ57R/T vector using InsT/Aclone PCR product cloning kit #1214 (Fermentas). Plasmids were transformed in *E. coli* JM 109 cells. Colonies were tested for the presence of PCR inserts in plasmids using Badna F/P primers. Three plasmids were purified from positive bacterial clones using Qiaprep Spin Miniprep Kit (Cat. No. 27104). Extracted DNAs were sequence in both directions and sequences were submitted to GenBank.

Data analysis: Percentage nucleotide identities between BgCSV-E and other badnaviruses were performed using the Blastn tool of the GenBank. Phylogenetic analysis by Maximum Likelihood method based on the Tamura-Nei model[22] was followed. Evolutionary analysis were conducted in MEGA6[23]. Genetic distance analysis were conducted using the Maximum Composite Likelihood model[24].

RESULTS

Biological studies

Symptomatology: Symptoms observed on infected *Bougainvillea* plants with BgCSV-E include yellow chlorotic spots that coalesce to form larger chlorotic areas which might turn necrotic afterwards (Fig. 1a, b). Sometimes the developed chlorotic spots may enlarge to circumvent the whole leaf areas except the veins giving rise to chlorotic vein banding appearance (Fig. 1c, d). Various types of secondary symptoms were also observed on the diseased leaves including leaf distortion, wrinkling and even loss of flower setting.

Fig. 1(a-d): Symptoms of chlorotic spots developed on two varieties of *B. glabra*, (a) White bracts, (b) Pink bracts, upon infection with BgCSV and (c-d), progressive development of chlorotic vein banding symptoms on infected leaves

Table 1: BgCSV-E and other badnaviruses used in comparative Blastn*DNA sequence analysis of the RT and RNase H coding regions (ORF3)

Badnavirus/Isolates	Country	Virus isolate/strain	Virus acronym in relation to its country	GenBank account number	Host	Nucleotide identity (%) with BgCSV-E
Bougainvillea glabra Chlorotic Spot Virus-Egypt	Egypt	Giza	BgCSV-E***	KM821771.1	*Bougainvillea glabra*	100
Bougainvillea spectabilis Chlorotic Vein Banding Virus	Taiwan	Not reported**	BsCVBV-Tai	EU034539.1	*B. spectabilis*	75
Bougainvillea spectabilis Chlorotic Vein Banding Virus	India	Tirupati	BsCVBV-In	GQ254410.1	*B. spectabilis*	75
Bougainvillea spectabilis Chlorotic Vein Banding Virus	Brazil	Not reported**	BsCVBV-Br	AY532653.1	*B. spectabilis*	75
Banana streak OL *virus*	Tanzania	Tz112	BSOLV-Tn	KF545103.1	*Musa* sp. cv. cavendish	67
Citrus yellow mosaic virus	India	PM	CYMV-In	EU489745.1	*C. maxima*	77
Dioscorea bacilliform virus	Benin	SN	DiBV-Bn	DQ822073.1	*Dioscorea cansibarensis*	81
Piper yellow mottle virus	India	Not reported**	PYMoV-In	DQ836237.1	*Piper longum*	80
Sugarcane bacilliform virus	China	WZ2-5	SCBV-Ch	KX944303.1	*Saccharum officinarum*	68

*Search nucleotide data using a nucleotide query, **Not reported in sequence information by GenBank, ***Viruses were arranged alphabetically after BgCSV-Egypt, BgCSV-E

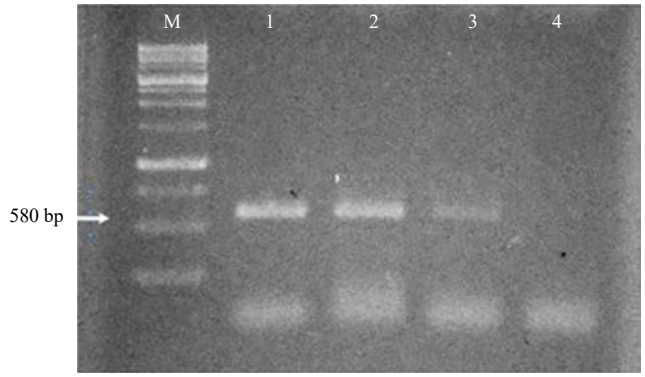

Fig. 2: Agarose gel (1%) of Immunocapture-PCR of three badnaviruses using BSV antiserum
Lane 1: Amplicon from BgCSV-E/from *B. glabra*, Lane 2: Amplicon of BSV/banana, Lane 3: Amplicon from SCBV/sugarcane, Lane 4: Healthy *B. glabra*, M: 1 kb DNA ladder (Promega)

Molecular studies

Immunocapture PCR (IC-PCR): The IC-PCR was successful in elucidating the nature of the badnavirus of BgCSV-E. All the three tested badnaviruses, namely: BgCSV-E, BSV-E and SCBV-E migrated equally in agarose gel to the 580 bp positions specific for the tested degenerate Badna primers (Fig. 2).

Cloning, sequencing and phylogenic studies: An accession number KM821771 was assigned by the Genbank for BgCSV-E from *B. glabra*. Blastn analysis of BgCSV-E with other badnaviruses described in GenBank in Table 1, indicated a highest and lowest nucleotide identity of 81 and 67% with DiBV and BSOLV, respectively.

Percentage of Nucleotide Pairwise Sequence Identity (NPSI) between BgCSV-E (Account number KM821771.1) and other BsCVBV from India (Account number GQ254410.1), Brazil (Account number AY532653.1) and Taiwan (Account number EU034539.1) was 75% (Table 1, 2). Percentage of NPSI ranged 73-81% between BsCVBV(s) from India, Brazil and Taiwan. Only BsCVBV-Br and BsCVBV-Tai had NPSI of 81% (Table 2).

The nucleotide Genetic Distances (GD) between the tested badnaviruses ranged between 0.21-0.91 (Table 3). The estimated overall average of GD was 0.56 between the nine tested DNA sequences. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 312 positions in the final dataset. Details of nucleotide sequence information are mentioned in Table 1.

Phylogenetic analysis of BgCSV-E and other badnaviruses indicated the presence of two separate monophyletic groups (Fig. 3). The tree with the highest log likelihood (-2529.7283) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 312 positions in the final dataset. Group one involved all badnaviruses infecting *Bougainvillea* with similarity value of 75%, including BgCSV-E (Account number KM821771.1), BsCVBV(s) Taiwan (Account number EU034539.1), Brazil (Account number AY532653) and India (Account number EU489745.1). Within the monophyletic group 1, however, each of BgCSV-E and BsCVBV-In branched alone. The BsCVBV(s) from Taiwan and Brazil (similarity, 85%) clustered separately in a second subgroup. The second group involved two sub-groups where

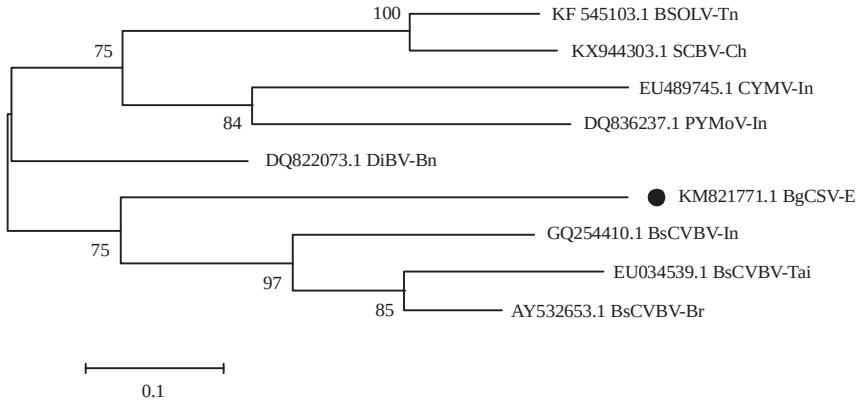

Fig. 3: Molecular phylogenetic analysis by maximum likelihood method showing the evolutionary history of the tested badnaviruses using the maximum likelihood method based on the Tamura-Nei model[22]
BgCSV from Egypt was marked with circle

Table 2: Pairwise comparison (%) of nucleotide sequences* of RT/RNase H regions among BgCSV-E and badnaviruses infecting bougainvillea

DNA sequence/Virus acronym	1	2	3	4
1. KM821771.1 BgCSV-E		75.00	75.00	75.00
2. GQ254410.1 BsCVBV-In	75.00		73.00	74.00
3. AY532653.1 BsCVBV-Br	75.00	73.00		81.00
4. EU034539.1 BsCVBV-Tai	75.00	74.00	81.00	

*Details of virus names and sequence information are mentioned in Table 1

Table 3.Estimates of evolutionary divergence among tested badnaviruses based on nucleotide sequences* of RT/RNase H regions

DNA sequence/Virus acronym	1	2	3	4	5	6	7	8	9
1. KM821771.1 BgCSV-E									
2. EU034539.1 BsCVBV-Tai	0.67								
3. GQ254410.1 BsCVBV-In	0.69	0.33							
4. AY532653.1 BsCVBV-Br	0.55	0.20	0.32						
5. KF545103.1 BSOLV-Tn	0.72	0.61	0.60	0.50					
6. EU489745.1 CYMV-In	0.91	0.63	0.61	0.57	0.53				
7. DQ822073.1 DiBV-Bn	0.57	0.50	0.52	0.45	0.49	0.58			
8. DQ836237.1 PYMoV-In	0.82	0.67	0.58	0.68	0.54	0.50	0.49		
9. KX944303.1 SCBV-Ch	0.72	0.61	0.56	0.54	0.21	0.58	0.47	0.56	

*Details of virus names and sequence information are mentioned in Table 1, number of base substitutions per site from between sequences are shown, analysis were conducted using the maximum composite likelihood model[24]

DiBV clustered on a separate branch from the other tested monophyletic badnaviruses, viz. BSOLV-Tn, SCBV-Ch, CYMV-In and PTMoV-In.

DISCUSSION

In the present study, BgCSV-E infecting *B. glabra* induces symptoms typical to those induced by BsCVBV described in Taiwan, Brazil and India. This similarity in symptom expression on a given host by badnavirus infections is a common phenomenon and does not represent a key factor in pinpointing the nature of the infecting virus. For instance symptoms of banana streak disease on banana may be expressed by several divergent badnaviruses infecting banana[18,25,26]. In addition, infection with a badnaviurs is usually composed of mixed populations of this virus[27]. Therefore, identification of BgCSV-E was solely based on serologic and molecular tools for virus diagnosis.

The IC-PCR detected BgCSV-E, BSV-E and SCBV-E. Such results confirmed the serologic relatedness between some badnviruses as previously reported by several investigators[4,18,20]. The involvement of DNase I in the IC-PCR protocol exclude the presence of integrated genomic badnaviruses in the three tested viruses and confirm their episomal nature as intact badnaviruses[18].

Two major-taxonomic markers are used for species demarcation within the genus *Badnavirus* based in differences in nucleotide identity and genetic distance values in the RT/RNase H genes within the ORF3. The first demarcation criterion is >20% nucleotide variation as proposed by Fauquet *et al*.[28] and Geering and Hull[29] or nucleotide identity <80% as proposed by King *et al*.[30]. The second demarcation criterion is the threshold for nucleotide genetic distance value[31] of >0.2.

In the present study BgCSV-E and BsCVBV-In had NPSI of <80 and >20% difference with each other as well as with the other *Bougainvillea* viruses from Brazil and Taiwan. This indicated that each of BgCSV-E and BsCVBV-In are new *Badnavirus* species according to the criteria set by Fauquet *et al*.[28] and Geering and Hull[29]. BsCVBV(s) from Brazil and Taiwan had NPSI of >81 indicating that they are probably different isolates for the same virus species or very close species as previously suggested by Baranwal *et al*.[8]. BgCSV-E had NPSI of 80% and 81% with PYMoV-In and DiBV-Bn, respectively. However the latter viruses as well as BSV and SCBV do not infect *Bougainvillea* species. This is due to the fact that *Bougainvillea* plants are not palatable hosts for mealybugs transmitting badnaviruses[16]. Yet the un-answered question remains of how *Bougainvillea* species acquired badnaviruses.

Both BgCSV-E and BsCVBV-In had GD(s) >0.2 indicating that they are new species of *Badnaviruses* according to the criterion of Bousalem *et al*.[31]. The BsCVBV(s) from Brazil and Taiwan had GD of 0.20 indicating very close genetic relationships since their NPSI >80% according to Baranwal *et al*.[8], Fauquet *et al*.[28] and Geering and Hull[29].

Inverse correlation was observed between the values of GD and NPSI. For instance, GD between BsCVBV-Tai and BsCVBV-Br, with 81% NPSI was 0.20; whereas, GD between BsCVBV-Tai and BgCSV-E, with 75% NPSI was 0.67. Similarly, GD of 0.72 was observed between BgCSV-E and both of BSOLV-In and SCBV-Ch with each of NPSI with BgCSV-E of 67 and 68%, respectively. Both DiBV-Bn and BgCSV-E with, 81% NPSI had GD of 0.57.

Phylogenetic analysis indicates that all the studied *Bougainvillea* badnaviruses, in the present study, are driven from common ancestors since they clustered in one monophyletic group. However, from the evolutionary standpoint, BsCVBV-In appears to have more proximity with BsCVBV-Ta and BsCVBV-Br since they have similarity value of 97% comparing to a comparable value of 75% with BgCSV-E. Similar results by Baranwal *et al*.[8] confirmed the close evolutionary proximity between BsCVBV(s) from India, Brazil and Taiwan. Phylogenetic analysis, in the present study, also confirm the genetic diversity between *Bougainvillea* badnaviruses and the other tested badnaviruses as they cluster separately from other badnaviruses as previously found by Baranwal *et al*.[8] and Alexandre *et al*.[32]. This study has also an implication in measuring diversity among badnaviruses infecting *Bougainvillea* spp., in several countries and should be useful in quarantine certification of plant materials in exchange programs.

CONCLUSION

The present study confirmed the presence of a new badnavirus species causing chlorotic spots in *Bougainvillea glabra* plants in Giza, Egypt. This conclusion was solely deduced from the obtained serologic, molecular, DNA sequence and phylogenic analysis on the virus under-study. As *Bougainvillea* plants are vegetatively propagated, the spread of BgCSV-E over neighboring areas may be possible and could represent a potential reservoir of the virus for other susceptible crops.

SIGNIFICANCE STATEMENT

The present study describes the presence of a new badnavirus in ornamentals. Due to the high diversity in the member of the genus *Badnavirus*, diagnosis based of symptomatology should not be considered since diverged badnaviruses may induce similar symptoms. Little is known about infection of ornamentals with badnaviruses. Therefore, this study should enrich our knowledge in this concept. Further investigation about the mode of badnavirus transmission in *Bougainvillea* plants is needed.

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