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MOLECULAR DIAGNOSIS OF BEGOMOVIRUS ASSOCIATED WITH YELLOW VEIN MOSAIC DISEASE OF *URENA LOBATA*

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Abstract

Urena lobata L. is a commonly growing weed usually called as Caesar Weed, or Congo Jute. A begomoviral suspected disease was observed on many *Urena lobata* plants. The begomoviral association was confirmed by applying coat protein gene as well as betasatellites primers. The full length and betasatellites molecules were cloned and sequenced. The total sequence had 2742 nt in full length while betasatellites had 1340 nt. The analysis of full-length sequenses revealed the highest identities (99.8%) with Bhendi yellow vein mosaic virus-Okra from Tamil Nadu (FJ176236). The betasatellites sequences had identity (92.8%) with MYVMB-Aurangabad-Okra (GU111977) followed BYVMB-Okra isolates from Raichur (92.7%) as well as Varanasi (92.3%). The phylogenetic analysis of full genome showed the closest relationship with Bhendi yellow vein mosaic virus isolates from Tamil Nadu as well as New Delhi. The full betasatellites molecule clustered with Bhendi yellow vein mosaic betasatellites isolates from Coimbatore, Raichur, Aurangabad and Varanasi. Based on the results generated, the begomovirus causing disease of *U. lobata* was identified as a variant of BYVMV in India.

Key words: Begomovirus; *Urena lobata*; Betasatellites, BYVMV; Whiteflies; Yellow vein mosaic disease.

Introduction

The begomoviruses belongs to family *Geminiviridae* and has ss-DNA (Brown *et al.,* 2015). Currently, the family *Geminiviridae* has been divided in nine genera and designated as *Becurtovirus, Begomovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocovirus* and *Turncurotovirus* (Zerbini *et al.,* 2017). Begomovirus is the biggest genus containing both mono- and bipartite viruses causing the disease to dicotyledonous as well as to monocots (Varma & Malathi, 2003; Brown *et al.,* 2015; Ferro *et al.,* 2017). The DNA-A component transcribed into major six genes encoding for replication, encapsidation, and pathogenicity (Rojas *et al.,* 2005). DNA-B had 2 ORFs known as *BV1* and *BC1* and both are essential for disease development and symptom expression (Rojas *et al.,* 2005). The new world begomovirus contains only bipartite virus while, Old World comprises monopartite virus along with a satellite molecule known as DNA-1 satellites, alphasatellites, betasatellite and delta satellites (Hanley-Bowdoin *et al.,* 2013; Brustolini *et al.,* 2015; Fiallo-Olive *et al.,* 2016). A betasatellites molecules are ss-DNA (~1350 nt) are associated with monopartite begomoviruses (Briddon *et al.,* 2002; Bull *et al.,* 2003). Globally, the cultivated crops, as well as weeds, are reported as a reservoir and alternative hosts for whiteflies transmitted begomovirus facilitating the new recombinant virus strains/isolates emergence on new host range and diversified genome. Begomoviruses and associated alphasatellites and betasatellites are well known to cause disease on many plants resulting in severe economic loss (Basak, 2016). Currently many weeds as well as cultivated crops are known to be infected with various begomoviruses globally (Mahatma *et al.,* 2016; Sohrab, 2016; Sohrab 2017; Sayed, 2017; Sangeeta & Tiwari 2017; Sayed, 2018; Sohrab and Daur 2018a; Sohrab & Daur 2018b). Due to emerging whiteflies transmitted geminivirus problems, the characterization of begomoviruses infecting weeds and cultivated crops is urgently required to identify the crucial factors playing a significant role in the spreading of disease and their progress through insect vector to other cultivated plants infecting begomoviruses (Wyant *et al.,* 2011; Sohrab, 2016; Sohrab 2017; Ferro *et al.,* 2017). Currently, multiple wild plants in the family *Asteraceae, Caparaceae, Euphorbiaceae, Fabaceae, Labiatae, Malvaceae, Solanaceae* and *Sterculiaceae* are known as an alternative host of begomoviruses (Ferro *et al.,* 2017), causing the disease exhibiting variable disease symptoms including mosaic, yellow veinlets, chlorosis of complete leaves, stunting and leaf curling of plants (Hernandez-Zepeda *et al.,* 2007; Fiallo-Olive *et al.,* 2010; Jeske *et al.,* 2010; Fiallo-Olive *et al.,* 2012; Graham *et al.,* 2010; Tavares *et al.,* 2012; Mauricio-Castillo *et al.,* 2014; Alabi *et al.,* 2016; Sohrab, 2016; Sohrab, 2017).

Urena lobata L. Belonging to the family Malvaceae is a common weed, grows well in in humid and hot climate in both tropical and sub-tropical areas. Many diseases affect the growth of this weed. In 2007, the begomovirus infection was identified with *U. lobata* causing yellow vein mosaic disease exhibiting symptoms like veins and veinlets yellowing, complete leaves

chlorosis and fusion of erratic chlorotic flakes and finally development of yellow net from Eastern India and this weed has already been reported as an alternative host for Okra mosaic virus in southern Ivory Coast (Chatterjee *et al.,* 2007). The weeds act as an alternative host and begomovirus infection in several plants like *Ageratum* spp., *Abelmoschus esculentus*, *Hibiscus cannabinus* and *Hibiscus sabdariffa* (Saunders *et al.,* 2000; Usha & Jose, 2000; Kashina *et al.,* 2003; Chatterjee *et al.,* 2005). The objective of the present study was to identify the virus causing disease in *U. lobata* plants growing. This work was performed to identify with the virus causing disease in *U. lobata* plants growing near agricultural area at Ballia, Uttar Pradesh, India.

Materials and Methods

Collection of samples: A field surveys was performed in the month of July-August 2016 in and around the vegetable growing agricultural field at Ballia, India and total nine infected as well as non-infected leaves were taken from *U. lobata* plant exhibiting yellow vein mosaic disease from various locations and used for the further experiment. For virus transmission, the pure culture of whiteflies (*Bemisia tabaci*), was raised from a single egg and sustained on Clatoria plant in green house. The whiteflies were allowed to feed on infected *U. lobata* leaves to acquire the virus for 24 hours and healthy seedling of *U. lobata* were inoculated with viruliferous whiteflies (10 whiteflies/plant) in triplicates by giving inoculation access period of 24-hours. Simultaneously, two plants were kept as a negative control by inoculating with non-viruliferous whiteflies. The inoculated seedlings were further grown in insect-proof cages and observed till 40 days for symptoms expression and disease development.

Isolation of DNA virus detection: The DNA was isolated from 100 mg freshly collected young leaves of symptomatic and non-symptomatic *U. lobata* plants by DN easy Plant Mini Kit (Qiagen, Valencia, CA, USA). The purified DNA was eluted in 50 µl nuclease free water. The purified DNA (100 ng) was used to confirm begomovirus infection by PCR amplification using begomovirus primers (Sohrab *et al.,* 2006; Briddon *et al*., 2002). The PCR reaction mixture contained template DNA, Taq polymerase (2.5 units) (MBI; Fermentas, USA), 10 x PCR buffer (5 µl), 10 mM dNTPs (0.5 µl), forward and reverse primers (0.5 µl) (10 pmol each) and remaining sterile distilled water in total 50 µl volume. The PCR condition for coat protein gene as well as betasatellites molecules were performed as per published protocol (Sohrab *et al.,* 2006). The PCR amplified products were visualized on Agarose gel (1%) stained with ethidium bromide by using an Ultraviolet transilluminator.

Rolling-circle amplification (RCA), viral genome cloning and sequencing: The viral genome was amplified by RCA technology using TempliPhi 100 Amplification Kit. The amplicon was further restricted with selected restriction enzymes (EcoRV and Hind III). The ~2.7kb restricted product was gel purified and cloned into a pUC-18 cloning vector. The PCR amplified betasatellites fragment (~1.4kb) was analyzed on 1% Agarose gel and cloned into the pGEMT-easy vector (Promega Life Corporation, USA). Initially, two clones were sequenced bi-directionally but only one clone sequence was used for further analysis and deposited into Gen Bank database.

Sequence and phylogenetic analysis: The generated begomovirus sequence data from both clones (full-genome and betasatellites) were searched primarily and compared for their identity by using NCBI-BLAST. The highly similar begomoviral sequences data were selected and retrieved from gene bank and further compared to their sequence similarity and identity matrix by using the BioEdit software. The full genome, as well as betasatellites, sequences were used for analyzing phylogenetic relationships with CYVMV isolates selected from various locations as well as from the Indian subcontinent by using MEGA7 software (Kumar *et al.,* 2016).

Results

Detection of virus: A survey was performed in number of agricultural fields in July-August 2016 and bright yellow vein mosaic disease as the natural infection was observed on many *U. lobata* plants (Fig. 1) growing in agriculture field in Ballia, India. The infected plants developed bright yellow vein mosaic symptoms and approximately 60-70% disease incidence was observed on *U. lobata* plants in the area surveyed. The virus transmission was successful to healthy *U. lobata* plant (24/30) and the transmission rate was found to be 80% in tested plants. There were no any symptoms developed on negative control plants. The inoculated plants developed similar symptoms as field infected plants after 19-21 days. The coat protein gene (~750 bp) (Fig. 2) and betasatellites molecule (~1.4 kb) were obtained from 8 symptomatic samples by PCR amplification Based on positive results obtained by PCR with expected amplicon size, the causative agent was identified as begomovirus with yellow vein mosaic disease in *U. lobata* in India.

Fig. 1. Naturally infection of *Urena lobata* plant exhibiting yellow vein mosaic disease.

Cloning and sequencing of viral genome: The full-genome was obtained by RCA technique by using purified DNA from naturally infected *U. lobata* plants. The amplicon was restricted with *Eco*RV and *Hind* III restriction enzymes. Many putative clones were obtained. The PCR amplified betasatellites molecule (~1.4 kb) was cloned into the pGEMT-Easy vector system. Finally, three clones from each was sequenced and analyzed. The full genome had 2742 nt while betasatellites had 1340 nt respectively and submitted to GenBank bearing accession numbers like KY612433-full length and KY612434-betasatellites and tentatively known as BYVMV-Urena-Ballia isolate.

Analysis of sequences and phylogenetic tree construction: The full genome of BYVMV from *U. lobata* (KY612433) had 2742 nt with six ORFs encoding for AV2 (119-484 nt pre-coat protein), and AV1 (279-1049 nt, coat protein) genes in the virion sense orientation. The AC1 (1498-2586 nt, replication associated protein), AC2 (1149-1601 nt, transcriptional activator protein, TrAP); AC3 (1052-1456 nt, replication enhancer protein, REn); AC4 (2130-2432 nt, C4 protein) genes and AC5 (627-983nt) were in the complementary sense. The BLAST analysis result of BYVMV-(KY612433) full genome showed the highest (99.8%) identities with BYVMV-Okra isolate from Tamil Nadu (FJ176236) followed by BYVMV-Okra-New Delhi (GU112063). The lowest (57.3%) identity was observed with *Okra leaf curl virus*-OLCV-Cameroon-Okra (NC-013017) isolate. Interestingly, only 86.6% identity was observed with BYVMV-Varanasi-Okra (HM590505) isolate. The overall identities were observed to be 92.7%-73.9% with selected BYMV isolates from Okra and Radish from various geographical regions of India and one Okra isolate from Pakistan (*Okra yellow vein mosaic virus*-OYVMV-AJ002451) showed 88.7 % identity while the sequence identities varied from other isolates reported from different crops like Okra, Malvastrum and Hollyhock at various countries like Cameroon (57.3%-OLCV-FM164726, 68.4%-*Okra yellow crinkle virus*-OYCrV-FM164724-Okra), Mali (68.6%-OYCrV-DQ902715), Egypt (68.4-68.5%- *Hollyhock leaf crumple virus*-HoLCrV-Hollyhock-AF014881), China (71.7%-*Malvastrum leaf curl virus*-MLCV-Malvastrum FJ712189) and Gezira (68.1%- *Cotton leaf curl virus* CLCuV-Gezira-okra FN554535) (Table 1).

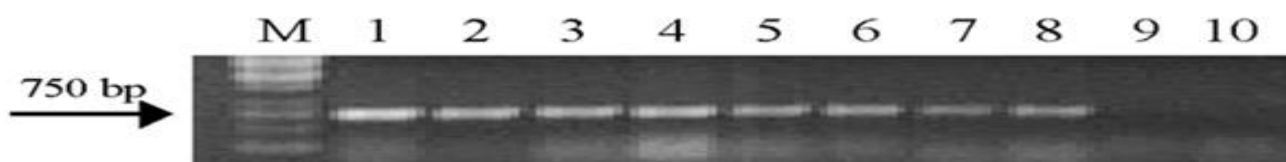

Fig. 2. PCR detection of begomovirus by coat protein gene specific primers from infected *U .Lobata*) M: 1kb ladder, 1-9 :Naturally infected samples from *Urena lobata* and 10 :Negative control.

Table 1. Sequence identity matrix of full genome of begomovirus from *Urena lobata* (KY612433) with selected begomoviruses.

S. No.	Accession No.	Acronyms	Hosts	Locations	% Identity
1.	NC_003418	BYVMV	Okra	Tamil Nadu	92.7
2.	AF241479	BYVMV	Okra	Tamil Nadu	92.7
3.	FJ176236	BYVMV	Okra	Tamil Nadu	99.8
4.	GU112022	BYVMV	Okra	Tamil Nadu	87.7
5.	JQ326267	BYVMV	Okra	Maharashtra	91.4
6.	JX181786	MeYVMV	Okra	Punjab	81.0
7.	GU112064	BYVMV	Okra	Bangalore	92.1
8.	GU112030	BYVMV	Okra	Aurangabad	87.5
9.	GU112006	BYVMV	Okra	Sonipat	91.4
10.	GU112063	BYVMV	Okra	New Delhi	94.2
11.	GU112027	BYVMV	Okra	Guntur	87.9
12.	GU112025	BYVMV	Okra	Kolkata	87.2
13.	HM590505	BYVMV	Chilli	Varanasi	86.6
14.	AJ002451	OYVMV	Okra	Pakistan	88.7
15.	GQ245760	OLCV	Okra	New Delhi	91.0
16.	NC_014894	OELCV	Okra	Haryana	80.9
17.	FM164726	OLCV	Okra	Cameroon	57.3
18.	DQ902715	OYCrV	Okra	Mali	68.6
19.	NC_013017	OLCV	Okra	Cameroon	57.3
20.	AF014881	HoLCrV	Hollyhock	Egypt	68.4
21.	AY036009	HoLCrV	Hollyhock	Egypt	68.5
22.	FM164724	OLCrV	Okra	Cameroon	68.4
23.	FJ712189	MLCV	China	Malvastrum	71.7
24.	FN554535	CLCV	Okra	Gezira	68.1
25.	EF175733	RaLCV	Radish	Varanasi	74.9
26.	HQ257375	RaLCV	Okra	Bihar	73.9

Table 2. Sequence identity matrix of associated betasatellites from *Urena lobata* (KY612434) with the selected begomoviruses.

S. No.	Accession No.	Acronyms	Locations	Hosts	% Identity
1.	NC_009903	MYVMB	Barrack pore	*U. lobata*	53.5
2.	HM590506	BYVMB	Varanasi	Okra	92.3
3.	NC_003405	BYVMB	Tamil Nadu	Okra	88.8
4.	AJ308425	BYVMB	Tamil Nadu	Okra	88.8
5.	GU233520	OYVMB	Aurangabad	Okra	87.1
6.	EF417919	BYVMB	Barrack pore	Okra	87.7
7.	EU188922	BYVMB	Barrack pore	Okra	68.2
8.	AJ316039	OYVMB	Egypt	Hibiscus	46.3
9.	AJ810094	SiYVMB	China	Sida	43.5
10.	AJ744882	MYVMB	China	Ageratum	52.0
11.	GU111995	CroYVMB	Bhubaneswar	Okra	41.1
12.	GU111990	BYVMB	Pandarahalli	Okra	79.5
13.	GU111974	BYVMB	Pandarahalli	Okra	87.5
14.	GU111988	BYVMB	Aurangabad	Okra	87.6
15.	GU111962	BYVMB	Sonipat	Okra	81.1
16.	GU111969	BYVMB	Guntur	Okra	88.8
17.	GU111966	BYVMB	Raichur	Okra	92.7
18.	GU111970	BYVMB	Trichy	Okra	91.9
19.	GU111975	BYVMB	Coimbatore	Okra	85.2
20.	GU111977	BYVMB	Aurangabad	Okra	92.8
21.	GU111984	BYVMB	Jalgaon	Okra	87.9
22.	GU111991	BYVMB	Bangalore	Okra	91.6
23.	AJ542498	AgeYVMB	Sri Lanka	Ageratum	46.2
24.	JF792241	OLCuB	Hyderabad	Okra	90.9
25.	GQ245761	OLCuB	New Delhi	Okra	89.0
26.	GU111961	OELCuB	Sonipat	Okra	87.3
27.	EU081883	ToLCNDB	New Delhi	Okra	89.0
28.	AJ316030	OLCuB	Pakistan	Hibiscus	87.6
29.	EU024121	OLCuB	Mali	Okra	48.2
30.	FM164731	OlCuB	Cameroon	Okra	46.3
31.	KJ396939	OlCuB	Jordan	Tomato	46.0
32.	HM146307	CLCuB	Rajasthan	Cotton	52.7
33.	GU732207	ToLCBB	Bihar	Okra	44.2
34.	EF190215	PeLCB	Varanasi	Chilli	42.5
35.	AY438558	ToLCuB	Rajasthan	Tomato	44.5
36.	DQ343289	ChiLCuB	Lucknow	Chilli	44.2
37.	AM712313	CLCuB	Pakistan	*G. annumalum*	44.0
38.	AM412239	AgYLCB	Pakistan	Ageratum	43.7
39.	DQ136001	TYLCB	Mali	Tomato	48.0

The phylogenetic tree results based on the complete nucleotides sequences of BYVMV-(KY612433), clustered with BYVMV-Tamil Nadu-Okra- FJ176236 isolate following additional two isolates from Tamil Nadu only while the Okra isolate from Varanasi formed a separate cluster with Radish isolate. Interestingly, the isolate from Pakistan formed the closed cluster with other BYVMV isolates from India. The isolates from other locations like China, Egypt, Cameroon, Mali, and Gezira formed another cluster (Fig. 3).

Sequence and phylogenetic analysis of betasatellites molecule: The BLAST results based on betasatellites molecules (KY612434-BYVMB) from *U. lobata* with selected betasatellites, the highest identity (92.8%) was observed with *Bhendi yellow vein mosaic betasatellites (*BYVMB) from Aurangabad (BYVMB-EU604296) followed by BYVMB (GU111966-Raichur). The lowest identity (42.5%) was found with *Pepper leaf curl virus satellites*-PeLCB-Varanasi (EF190215) causing disease in Chili. The overall identity among BYVMB, OYVMB, and *Okra leaf curl betasatellites* (OLCuB) varied from 92.3%-53.5% and other isolates causing different disease reported from multiple locations showed variable sequence identity (Table 2). Interestingly, the sequence identity (53.5%) was observed with begomovirus identified on *U. lobata* from Barrack pore. This shows the diversity of betasatellites as many reports have been published about the diversity of betasatellites from diverse crops from various geographical regions. The phylogenetic analysis of BYVMB-Ballia-*U. lobata* isolate (KY612434) showed the closest relationship with Okra isolates of BYVMB-Coimbatore (GU111975-Okra) and Raichur (GU111966-Okra) followed by BYVMB-Aurangabad (GU111977), Varanasi (HM590506), Trichy (GU111970), Hyderabad (JF792241) and Bangalore (GU111991) and shared the closed relationships with many other BYVMB isolates reported from India and distinct clustering was observed with other betasatellites reported from different locations (Fig. 4).

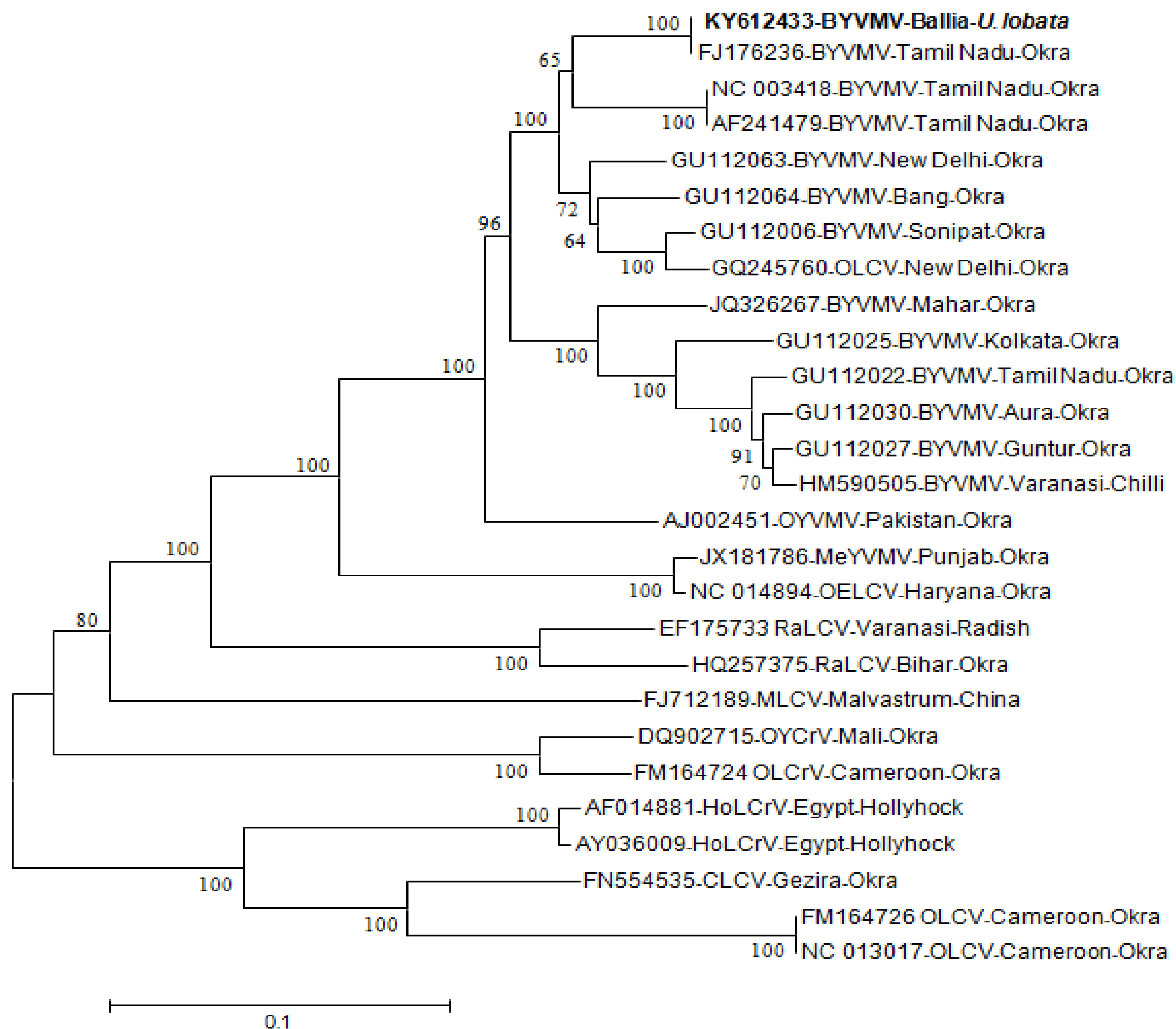

Fig. 3. Phylogenetic analysis of full genome of BYVMV (KY612433) associated with yellow vein mosaic disease of *Urena lobata* with selected begomoviruses.

Discussion

There are few reports available about the natural infection of viruses on *U. lobata.* Begomovirus causing yellow vein mosaic disease are known (Chatterjee *et al.,* 2007). In this study, during field survey and sample collection, many *U. lobata* plant growing in and around agricultural field in Ballia, India were observed to develop yellow vein mosaic disease symptoms and the presence of begomovirus vectors as whiteflies were also observed in and around the field and led to suspect as begomovirus infection in *U. lobate* plants. During the whitefly transmission studies; the virus transmission was successful by whiteflies from *lobate infected to* healthy *U. lobata* plants and developed similar disease symptoms as observed in the natural field. Many attempts were made to amplify DNA-B, but no positive amplicon was obtained which indicated the monopartite nature of associated begomovirus. The PCR results showed the diversity and prevalence of begomovirus in Eastern India.There may be multiple begomovirus strains causing disease in both weeds as well as economically important crops with variable symptoms which requires extensive study.

The full genome sequence of BYVMV-Urena isolate (KY612433) had 2742 nucleotides (nt) and total 1340 nt was found in betasatellite molecules (KY612434). The full genome sequences showed the highest identities with BYVMV-Okra from Tamil Nadu (FJ176236) followed by with BYVMV-New Delhi-Okra (GU112063) India.There are many other begomoviruses have been identified from diverse crops with multiple locations. Additionally, an isolate of BYVMV-Okra showed identity 87.2% reported from Kolkata. The prevalence of diversified begomovirus isolates in Eastern India showed major diversity as compared to BYVMV-Urena isolate. Many diverse and recombinant begomovirus isolates with various associated satellites from with BYVMV as well as ToLCNDV across the India are known. The variability among these begomoviruses favors the emergence of new virus isolates in wide geographic distribution with their extended hosts and due to genetic diversity with extended host an alarming condition may arise for the economic loss of important crops in this region.

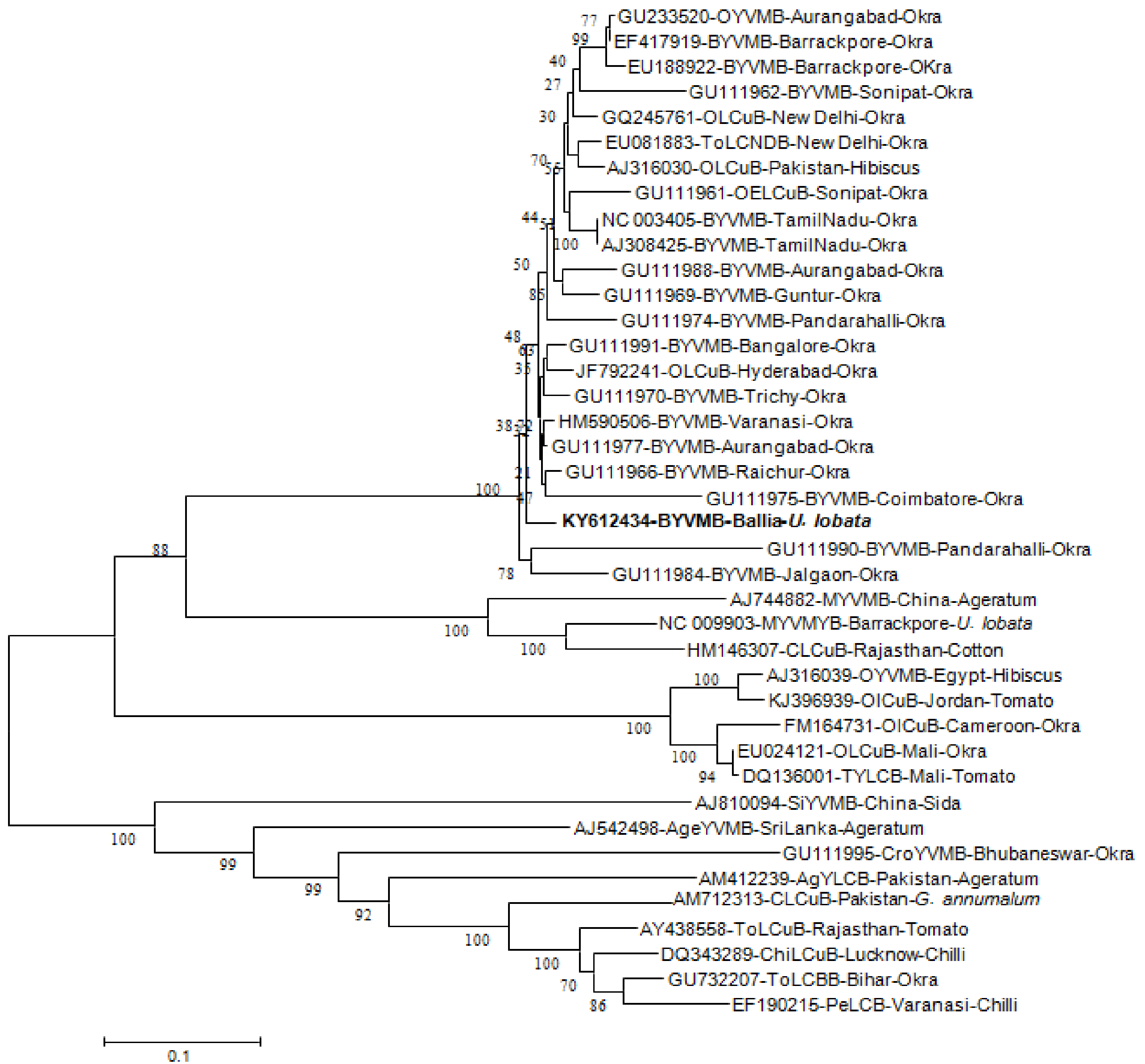

Fig. 4. Phylogenetic analysis associated betasatellites molecule with *Urena lobata* (KY612434) selected begomoviruses.

Interestingly, the phylogenetic analysis of BYVMV-Urena isolates based on full genome sequences with selected begomovirus isolates from multiple locations, the other BYVMV isolates formed closed cluster while other begomoviruses reported from different crops and locations formed separate clusters and the same situation was also observed in the phylogenetic tree constructed by using selected betasatellites from various locations and other plants species. The closed clustering of BYVMV-Urena isolate based on full genome with selected begomoviruses indicated the genetic diversity among the reported begomoviruses in Eastern part of India. The BYVMV and RalCV isolates from Varanasi infecting Chili and Radish formed very far clustering with BYVMV-Urena isolate. Additionally, a diverse clustering was also observed based on betasatellites genome among many isolates from multiple locations and crops and these results again supporting the genetic diversity among begomovirus with extended hosts in diversified geographic locations. The diversity of betasatellites could be due to mutations in the genome sequences. Based on literature survey and published information only a few viruses are reported to cause disease on *U. lobata* (Chatterjee *et al.,* 2007). Our result is strongly supported based on many published reports about the begomovirus full genome as well as betasatellites sequences diversity causing disease in many crops with variable symptoms from different geographical locations (Sohrab *et al.,* 2012; Venkataravanappa *et al.,* 2013; Sayed *et al.,* 2014; Rishishwar *et al.,* 2015; Sohrab *et al.,* 2015; Priyavathi *et al.,* 2016; Roy *et al.,* 2017; Moriones *et al.,* 2017). Results obtained from PCR detection, a full length as well as betasatellites sequence identity and phylogenetic tree was constructed using full genome and associated betasatellites with other begomoviruses reported from other geographical regions. The virus identified from *U. lobata* was found to be a variant strain of BYVMV and tentively known as BYVMV-Urena Ballia isolate. The information generated in this study will be highly valuable and beneficial for researchers as well as crops and vegetable growers to design and development of an effective disease management strategy.

Conclusion

The results obtained leads to a conclusion that the virus causing disease on *U. lobata* is a variant strain of BYVMV causing disease in many crops in India. This plant may serve as an alternative host for BYVMV to disease spread; and infect crops growing in India and elsewhere.

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