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SHORT COMMUNICATION

Molecular characterization and phylogenetic analysis of betasatellite molecules associated with okra yellow vein mosaic disease in Sri Lanka

Tharmila C. Jeyaseelan[1,2] · Emmanuel C. Jeyaseelan[1] · Devika M. De Costa[2,3] · Michael W. Shaw[4]

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Abstract

Okra production in Sri Lanka has been severely affected by okra yellow vein mosaic disease (OYVMD), which is caused by begomoviruses and associated betasatellites. These betasatellite molecules commonly determine the development and severity of the disease. Therefore, knowledge about the genetic variability of betasatellites associated with OYVMD could assist okra breeding programs in the selection of resistant varieties. The present study aimed to characterize the betasatellite DNA sequences associated with OYVMD in Sri Lanka and to determine their phylogenetic relationships. Betasatellite DNA of six virus isolates from widely separated geographical locations were sequenced and compared with already reported begomovirus betasatellites. The betasatellite molecules have features common to other betasatellite DNAs: a conserved nonanucleotide TAATATTAC, a coding sequence for the protein βC1, an adenine rich region and a satellite conserved region. Nucleotide diversity among the isolates was relatively low (π = 0.034). A recombination event was detected at a specific region in the genome of all isolates. The isolates shared >96% sequence identity with bhendi yellow vein betasatellites reported from India and phylogenetic analysis confirmed their genetic relationship.

Keywords *Abelmoschus esculentus* · Begomovirus · Plant virus · Virus recombination

Yellow vein mosaic disease of okra (*Abelmoschus esculentus*; local names bhendi/vendi/bandakka) causes great economic losses to yield and quality of okra in the Indian subcontinent (Pun et al. 2005). Earlier, it was considered that the disease was caused by a geminivirus called okra yellow vein mosaic virus (OYVMV) (Jose and Usha 2003) which belongs to the genus *Begomovirus* (Fauquet and Stanley 2005). Recent studies have revealed that an okra plant showing yellow vein mosaic disease (OYVMD) may carry mixed infections with bhendi yellow vein mosaic virus (BYVMV), okra enation leaf curl virus (OELCuV), alphasatellites and betasatellites (Priyavathi et al. 2016). Most OYVMV isolates are monopartite viruses with a single stranded genomic DNA (DNA-A-like) which is associated with a betasatellite molecule. The betasatellites have a gene which codes for a protein known as βC1 (Mansoor et al. 2003) which is essential for the development of typical symptoms in infected okra plants (Jose and Usha 2003), suppressing post-transcriptional gene silencing and up-regulating viral DNA levels *in planta* (Briddon et al. 2008). It has also been shown that OYVMD can be caused by a bipartite begomovirus known as bhendi yellow vein mosaic Delhi virus (BYVMDV-[India:Delhi:okra]). Although no betasatellite has been found associated with this begomovirus, infected okra plants show typical symptoms of OYVMD (Venkataravanappa et al. 2012). This complex mix of viruses associated with OYVMD suggests that identification and characterization of the causative virus and associated satellite molecules may help explain why OYVMD has become prevalent and more severe.

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✉ Tharmila C. Jeyaseelan
siva.tharmi3@gmail.com; stharmila@jfn.ac.lk

[1] Department of Botany, Faculty of Science, University of Jaffna, Jaffna, JA 40000, Sri Lanka

[2] Plant Protection Board of Study, Postgraduate Institute of Agriculture, University of Peradeniya, Peradeniya, KY 20400, Sri Lanka

[3] Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya, Peradeniya, KY 20400, Sri Lanka

[4] School of Agriculture, Policy and Development, University of Reading, Reading RG6 6BZ, UK

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In Sri Lanka, cultivated okra varieties such as TV8, MI5 and MI7 are greatly affected by OYVMD. So far, studies on this disease in Sri Lanka have mainly focused on determination of disease incidence and screening of resistant okra varieties. More recently, studies have attempted to detect the causal agent based on partial amplification of virus DNA by PCR (Senevirathna et al. 2016). In 2017, we identified the causal agent of OYVMD as BYVMV, based on whole genome sequencing of the virus isolates (Tharmila et al. 2017). The present study focused on genome sequence, organisation and phylogenetic relationships of betasatellite molecules associated with OYVMD in Sri Lanka.

Leaf samples of asymptomatic and symptomatic (displaying yellow vein mosaic) okra plants were collected from six widely separated locations across Sri Lanka between May and July in 2015 (Table 1). In each location, three symptomatic and three non-symptomatic leaf samples were collected from three different farmer fields or home gardens; a total of 18 symptomatic and 18 non-symptomatic leaf samples were collected. Total DNA was extracted from individual samples using the protocol described by Ghosh et al. (2009) with some modifications; the additional phenol-chloroform extraction step was omitted from the protocol. The presence of begomoviruses in symptomatic samples was confirmed by PCR amplification of a DNA-A fragment using a pair of degenerate primers (Deng et al. 1994). Full-length betasatellite genomes were amplified with the universal primer pair β01 and β02 described by Briddon et al. (2002). Six different PCR products of betasatellite DNA, one from each sampling location, were purified using a spin column PCR purification kit (NBS Biological) and cloned into the pCR2.1 vector using a TA cloning kit (Invitrogen) as described in the manufacturers' protocols. The clones were sequenced by a commercial automated Sanger sequencing service (Source Bioscience). The complete nucleotide sequences of the betasatellites were deposited in the GenBank database (accession numbers are given in Table 1).

Identity searches for the sequences were carried out using the BLAST*n* algorithm (Altschul et al. 1990) and the non-redundant GenBank database available in NCBI. Sequence alignments were performed using MUSCLE (Edgar 2004) and pairwise nucleotide identity scores were calculated with the Species Demarcation Tool v. 1.2 (Muhire et al. 2014). Details of sequences retrieved from GenBank are given in Supplementary Table S1. Phylogenetic analysis was performed in MEGA 7 (Kumar et al. 2016) using the maximum likelihood algorithm with general time reversible model and 1000 bootstrap replications. The mean pairwise number of nucleotide differences per site (π) was estimated for the complete genome of selected betasatellites using DnaSP v. 5.10 (Rozas et al. 2003). Putative parental sequences and recombination breakpoints were determined using Recombination Detection Program (RDP) v. 4.0 (Martin et al. 2015). Alignments were analysed using default settings for the different methods and statistical significance was defined as a *P* value less than the Bonferroni-corrected cut-off of 0.05. Only recombination events detected by at least five different methods were considered to be reliable.

Table 1 Characteristic features of betasatellites isolated from okra plants with okra yellow vein mosaic disease (OYVMD) from different locations in Sri Lanka

Districts in Sri Lanka	Betasatellite	Acronym	GenBank accession number	Length (bp)	GenBank sequence showing highest sequence identity (accession number, percent identity)
Vavuniya	Bhendi yellow vein mosaic betasatellite-[Sri Lanka:Vavuniya:2015]	BYVMB-[LK:Vav:15]	KX174318	1318	Bhendi yellow vein India betasatellite-[India:Jalna:Jal:2009] (KJ462078, 98.3%)
Jaffna	Bhendi yellow vein mosaic betasatellite-[Sri Lanka:Jaffna:2015]	BYVMB-[LK:Jaf:15]	KX174319	1334	Bhendi yellow vein India betasatellite-[India:Coimbatore:Co:2009] (KJ462077, 98.8%)
Kandy	Bhendi yellow vein mosaic betasatellite-[Sri Lanka:Kandy:2015]	BYVMB-[LK:Kan:15]	KX174320	1369	Bhendi yellow vein mosaic betasatellite-[India:Guntur:OY112:2006] (GU111969, 95.6%)
Matara	Bhendi yellow vein mosaic betasatellite-[Sri Lanka:Matara:2015]	BYVMB-[LK:Mat:15]	KX174321	1351	Bhendi yellow vein mosaic betasatellite-[India:Madurai:MKU-1:2014] (KR068483, 98.9%)
Trincomalee	Bhendi yellow vein mosaic betasatellite-[Sri Lanka:Trincomalee:2015]	BYVMB-[LK:Tri:15]	KX174322	1320	Bhendi yellow vein India betasatellite [India:Jalna:Jal:2009] (KJ462078, 98.8%)
Puttalam	Bhendi yellow vein mosaic betasatellite-[Sri Lanka:Puttalam:2015]	BYVMB-[LK:Put:15]	KX174323	1351	Bhendi yellow vein mosaic betasatellite [India:Aurangabad:OY165:2006] (GU111977, 97.3%)

All symptomatic samples were PCR-positive when using specific primers for begomovirus DNA-A and betasatellite, and yielded amplification fragments of approximately 0.52 kb and 1.3 kb, respectively (data not shown). No amplification was observed from asymptomatic plants. This shows the frequent association of helper begomoviruses and betasatellites with symptomatic plants.

The complete nucleotide sequences of the six betasatellites ranged between 1318 and 1369 bp in size (Table 1). The sequences showed all the typical features of a betasatellite (Briddon et al. 2008), including an adenine rich region and a single ORF in the complementary sense which encodes a putative 118 amino acid protein. They also contain a nonanucleotide stem-loop structure (TAATATTAC) and a satellite conserved region (SCR).

Pairwise sequence comparisons showed the Sri Lankan betasatellites had 93.4 to 99.2% nucleotide identity among them (Fig. 1). The most divergent isolate, LK:Kan:15, showed 93.4 to 94.0% identity with the other Sri Lankan isolates. The above isolates shared >91% identity with a number of bhendi yellow vein betasatellite (BYVB), bhendi yellow vein mosaic betasatellite (BYVMB), bhendi yellow vein India betasatellite (BYVIB) and okra yellow vein mosaic betasatellite (OYVMB) isolates. According to the recently proposed betasatellite species demarcation threshold of 91% (https://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/plant-official/6689), all these sequences belong to the same species, *Bhendi yellow vein mosaic betasatellite*. Sequence identities of Sri Lankan BYVMB isolates were < 91% when compared to sequences of okra leaf curl betasatellites (OLCuB) and croton yellow vein mosaic betasatellite (CrYVMB), all of which were also reported to be associated with okra yellow vein mosaic disease in India (Venkataravanappa et al. 2011). The remaining

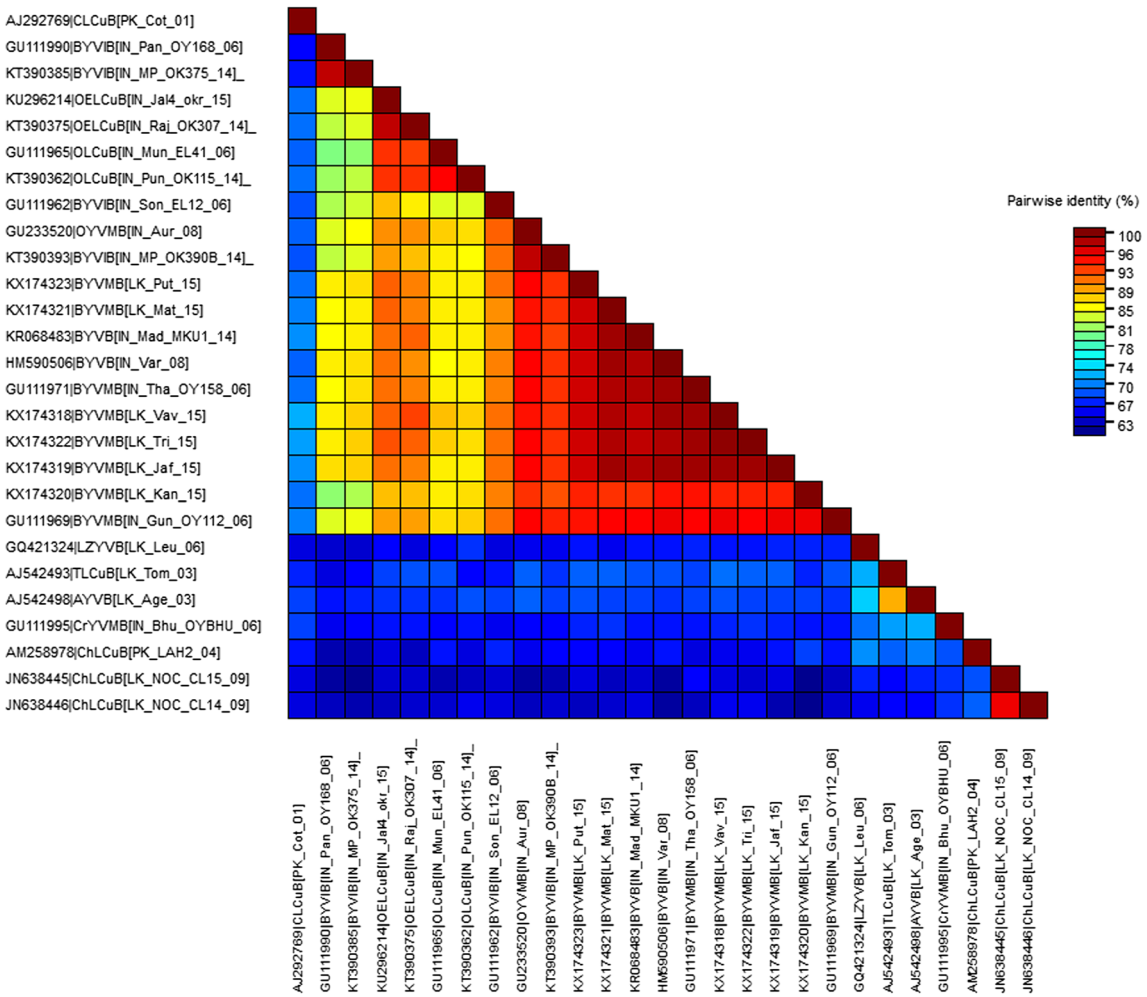

Fig. 1 Colour coded pairwise identity matrix generated from 27 betasatellite DNAs, including the six betasatellites described in this work, five betasatellites associated with different begomovirus diseases in Sri Lanka, 14 beta-satellites associated with okra/bhendi and two betasatellites associated with chilli and cotton. See Supplementary Table S1 for details on the compared sequences. Each coloured cell represents a percent identity score between two sequences. The coloured key indicates the correspondence between pairwise identities and the colours displayed in the matrix

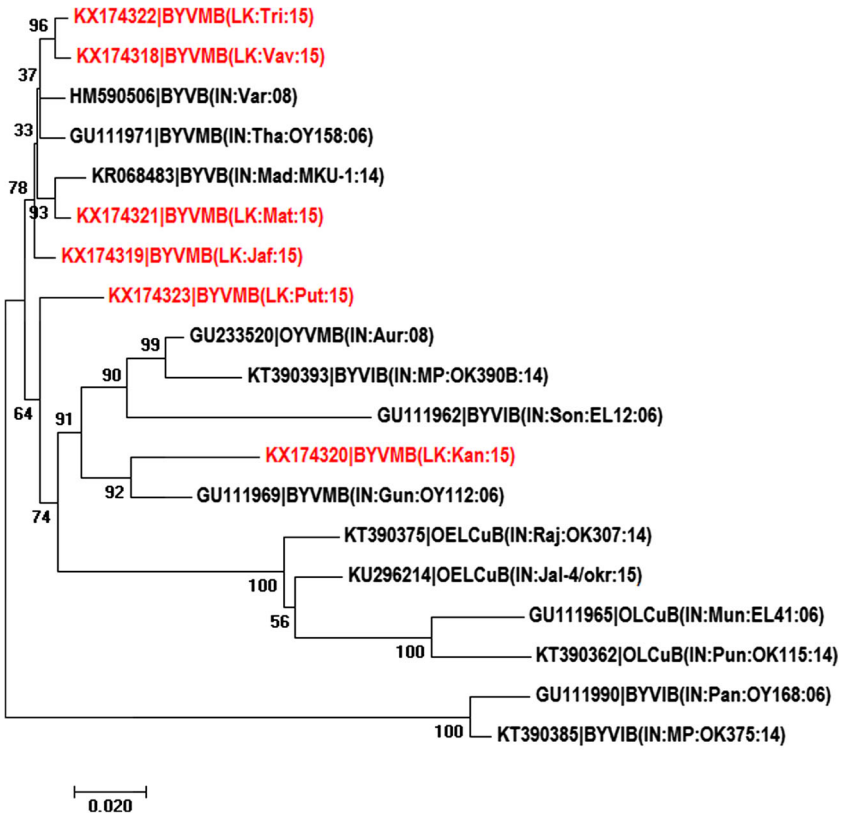

Fig. 2 Maximum likelihood phylogenetic tree based on complete betasatellite genomes. Indian and Sri Lankan isolates are denoted IN and LK, respectively, followed by the isolate code. Sri Lankan BYVMB isolates are highlighted in red

betasatellite isolates associated with different begomovirus diseases in Sri Lanka revealed relatively low identity values (<70%) with the Sri Lankan BYVMB isolates. Nucleotide diversity (π) was measured in two subpopulations of betasatellite isolates: Sri Lankan BYVMB ($n = 6$) and some selected Indian BYVMB ($n = 15$). Sri Lankan BYVMB showed lower nucleotide diversity ($\pi = 0.034$) compared to Indian BYVMB ($\pi = 0.072$).

Briddon et al. (2003) reported two major clusters of betasatellites, corresponding to hosts in the Malvaceae and non-Malvaceae. The Sri Lankan BYVMB clustered phylogenetically with isolates from the Malvaceae and were completely separated from previously published betasatellite sequences from Sri Lanka (Supplementary Fig. S1). Analysis of BYVMB and selected betasatellites associated with okra/bhendi clearly showed the close clustering of Sri Lankan BYVMB with previously reported Indian BYVB and BYVMB rather than okra enation leaf curl betasatellites (OELCuB) and OLCuB (Fig. 2). The isolates BYVMB-[LK:Put:15] and BYVMB-[LK:Kan:15] clustered separately from the other Sri Lankan BYVMB isolates.

Table 2 Putative recombination events detected within betasatellites associated with okra yellow vein mosaic disease (OYVMD) and betasatellites reported from Sri Lanka, based on full-length betasatellite sequences

Event	Breakpoints		Recombinant	Parents		Methods[a]	*P*-Value[b]
	Begin	End		Major	Minor		
1	1076	1316	BYVMB-[LK:Vav:15]	AYVB-[LK:Age:03]	LZYVB-[LK:Leu:06]	RGBMCS3	$3.16E^{-06}$
	1092	1332	BYVMB-[LK:Jaf:15]	AYVB-[LK:Age:03]	LZYVB-[LK:Leu:06]	RGBMCS3	$3.16E^{-06}$
	1154	1367	BYVMB-[LK:Kan:15]	AYVB-[LK:Age:03]	LZYVB-[LK:Leu:06]	RGBMCS3	$3.16E^{-06}$
	1109	1349	BYVMB-[LK:Mat:15]	AYVB-[LK:Age:03]	LZYVB-[LK:Leu:06]	RGBMCS3	$3.16E^{-06}$
	1078	1318	BYVMB-[LK:Tri:15]	AYVB-[LK:Age:03]	LZYVB-[LK:Leu:06]	RGBMCS3	$3.16E^{-06}$
	1109	1349	BYVMB-[LK:Put:15]	AYVB-[LK:Age:03]	LZYVB-[LK:Leu:06]	RGBMCS3	$3.16E^{-06}$
2	998	1127	BYVMB-[LK:Kan:15]	OYVMB-[IN:Aur:08]	AYVB-[LK:Age:03]	RGBMCS3	$5.93E^{-11}$
3	52	812	OELCuB-[IN:Jal-4/okr:15]	BYVMB-[LK:Jaf:15]	BYVMB-[LK:Kan:15]	RGBMCS3	$2.95E^{-15}$

[a] R, RDP; G, GeneConv; B, Bootscan; M, MaxChi; C, Chimera; S, SisScan; 3, 3SEQ

[b] The reported *P*-values are for the methods underlined, and are the lowest *P*-values calculated for the region in question

Genetic recombination is an important process in the evolution of viruses. It is frequent in begomoviruses and interspecies recombination is a major factor behind the emergence of new begomovirus species (Lefeuvre et al. 2007). We looked for recombination events among 27 full-length genomes of different betasatellites associated with okra and other plants. A recombination event was detected in the genome of all BYVMB isolates. The recombination breakpoint was detected immediately upstream of the A-rich region and SCR region. The major and minor parents were inferred to be ageratum yellow vein betasatellite (AYVB-[LK:Age:03]) and a tentative betasatellite named *Leucas zeylanica* yellow vein betasatellite (LZYVB-[LK:Leu:06]), respectively (Table 2). These betasatellites are associated with *Ageratum* spp. and *Leucas zeylanica*, respectively, in Sri Lanka. The isolate BYVMB-[LK:Kan:15] showed an additional recombination event a few nucleotides downstream of the previous recombination event. OYVMB-[IN:Aur:08] and AYVB-[LK:Age:03] were inferred to be the minor and major parents, respectively. One more recombination event was detected, where the isolates BYVMB-[LK:Jaf:15] and BYVMB-[LK:Kan:15] were parents of the recombinant isolate OELCuB-[IN:Jal-4:okr:15].

Begomoviruses may be disseminated over long distances with little differentiation in infectivity and genome sequence. Sri Lankan isolates showed great similarities with Indian isolates. These countries are geographically close and agricultural products are frequently exchanged, so the isolates associated with Sri Lankan OYVMD might have arrived from India. Sequence diversity among the Sri Lankan BYVMB was lower than in previously published isolates from India, possibly because the country is smaller and has less geographical and environmental variation than India, or more likely because the disease reached it more recently.

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