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20   **Abstract**

21 In the past few years, okra growth and yield has been greatly affected in Sri Lanka by a

22 disease called okra yellow vein mosaic (OYVM). The disease is caused by begomovirus and

23 associated beta-satellites. The associated beta-satellite molecule determines the development

24 and severity of the disease. Therefore, knowledge of the sequence and diversity of the beta-

25 satellite molecules associated with OYVM disease would assist choice of virus isolates or

26 trial locations to use when selecting resistant varieties of okra. The present study aimed to

27 characterize the beta-satellite DNA sequences associated with OYVM disease in Sri Lanka

28 and determine their phylogenetic relationships. Beta-satellite DNA of six virus isolates from

29 widely separated geographical locations were sequenced and compared with already reported

30 begomovirus beta-satellites. The beta-satellite molecules have features common to

31 begomovirus beta-satellite DNAs: a conserved nonanucleotide TAATATTAC, a coding

32 sequence for the protein βC1, an adenine rich region and a satellite conserved region. er

33 Nucleotide diversity among the isolates was relatively low ($\pi = 0.027$). Recombination events

34 were detected at a specific region in the genome of all the isolates. The isolates shared

35 sequence identity >96 % with bhendi yellow vein beta-satellites reported from India. This is

36 further supported by phylogenetic analysis.

37

40

Introduction

Yellow vein mosaic (YVM) disease of Okra (*Abelmoschus esculentus*; local names Bhendi / Vendi / Bandakka) causes heavy economic losses, among pests and diseases that affect the yield and fruit quality of okra throughout the okra cultivating regions of the world (Pun et al. 2005). Leaves of infected plants are characterized by alternate green and yellow patches, vein clearing and a conspicuous yellow network of vein. In severe cases the chlorosis may extend to the inter-veinal area and may result in complete yellowing of leaves with stunting of plants (Taware et al. 2010). The disease is 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). In *Begomovirus*, single stranded, circular genomic DNA is encapsulated in quasi-isometric (''geminate'') virions and transmitted by whitefly (*Bemisia tabaci*) (Briddon et al. 2008).

Most OYVMV isolates are monopartite viruses with a single strand DNA (DNA A) of helper begomovirus which associated with a single strand beta-satellite molecule. The beta-satellites have a gene which codes a protein known as βC1 (Mansoor et al. 2003). It is essential for the development of typical symptoms in infected okra plants (Jose and Usha 2003), suppression of post-transcriptional gene silencing and up-regulation of viral DNA levels *in planta* (Briddon et al. 2008). Many isolates of the virus and associated satellite molecules have been characterized in various okra growing countries (Ghosh et al. 2008; Taware et al. 2010; Venkataravanappa et al. 2011). Recent studies have revealed that the OYVM disease can be caused by a bipartite begomovirus species known as Bhendi yellow vein mosaic Delhi virus [BYVDV-IN (India: Delhi: okra)]. This has both DNA-A and DNA-B molecules, but not beta-satellites, in okra plants which nonetheless have typical symptoms (Venkataravanappa et al. 2012).

66   In Sri Lanka cultivated okra varieties such as TV8, MI5 and MI7 are greatly affected by the

67   disease. Cultivation of the variety TV8 (locally known as 'Paal Vendi'), a popular variety

68   with a great market value in North and East parts of Sri Lanka, is vanishing because of

69   widespread disease. The variety 'Haritha' was recommended as resistant by the Department

70   of Agriculture, but in recent seasons the variety has suffered a high disease incidence,

71   especially in Northern Sri Lanka. So far, studies on OYVM disease in Sri Lanka have mainly

72   focusing on screening of resistant okra varieties, determination of disease incidence. More

73   recent studies have tried to identify the causative agent, based on partial amplification of virus

74   DNA by PCR (Senevirathna et al. 2016).

75   These studies have found that an okra plant showing yellow vein mosaic disease may carry

76   mixed infections with Bhendi yellow vein mosaic virus (BYVMV) DNA-A, Okra enation leaf

77   curl virus (OELCuV) DNA-A, beta- and alpha-satellites (Priyavathi et al. 2016). This

78   complex mix of virus genetic materials associated with OYVM disease suggests that

79   identification and characterization of the causative virus and associated satellite molecules

80   may help explain why OYVM disease has become more prevalent and severe recently. In

81   2016, we identified the causative agent of OYVM disease of the same samples as the

82   begomovirus OYVMV based on whole genome sequencing of the virus isolates (Tharmila et

83   al. 2017). The present study focused on genome sequence, organisation and phylogenetic

84   relationship of beta-satellite molecules associated with OYVM disease in Sri Lanka.

85   Leaf samples of asymptomatic okra plants and okra plants showing yellow vein mosaic

86   symptoms were collected from six different widely separated locations across Sri Lanka

87   between May and July in 2015 (Table 1). In each location, three symptomatic and three non-

88   symptomatic leaf samples were collected from three different farmer's field or home gardens,

89   while the okra plants were at their post flowering stage. Total DNA was extracted from both

90   non-symptomatic and symptomatic okra leaf samples using a DNA extraction protocol

91   (Ghosh et al. 2009) with some modifications.

92   The extracted DNAs were PCR amplified with specific primers for the begomovirus genome.

93   Presence of begomovirus in symptomatic samples was confirmed by PCR amplification of

94   begomovirus DNA-A using a pair of degenerate primers (Deng et al. 1994). Full length beta-

95   satellite DNA was amplified with a pair of universal primers β01 and β02 as described by

96   Briddon et al. (2002). Six different PCR products of beta-satellite DNA, one from each

97   location sampled, were cloned and sequenced. The PCR products were purified using a spin

98   column PCR purification kit (NBS Biological, Huntingdon, Cambridgeshire) and cloned into

99   pCR®2.1 vector using a TA cloning kit (Invitrogen, USA) as described in the manufacturers'

100   protocols. The clones were sequenced by a commercial automated Sanger sequencing service

101   (Source Bioscience, UK). The complete nucleotide sequences of the beta-satellites were

102   deposited in GenBank database; accession numbers are given in Table 1.

103   Identity searches for the sequences were carried out by using the BLAST*n* program available

104   in the NCBI. Sequence alignments were performed using MUSCLE (Edgar 2004) and

105   pairwise identity scores were calculated with the Species Demarcation Tool (SDT) (Muhire et

106   al. 2014). The details of sequences retrieved from GenBank database are given in

107   Supplementary Table 1. Phylogenetic analysis was performed in MEGA 7 (Kumar et al.

108   2016) using the maximum likelihood algorithm with 1000 bootstrap replications. The mean

109   pairwise number of nucleotide differences per site (π) was estimated using DnaSP v. 5.10

110   (Rozas et al. 2003). Putative parental viruses and recombination breakpoints were determined

111   using Recombination Detection Program (RDP) v. 4.0 (Martin et al. 2015). Alignments were

112   analysed using default settings for the different methods and statistical significance was

113   defined as a *P* value less than the Bonferroni-corrected cut-off of 0.05.

114 PCR-mediated amplification of DNA extracts of all symptomatic samples with specific

115 primers for begomovirus DNA-A and beta-satellite yielded products with an approximate

116 length of 0.52 kb and 1.3 kb respectively (Data not shown). Amplifications from non-

117 symptomatic plants were uniformly negative with all primers. This shows the frequent

118 association of helper virus and beta-satellite with symptomatic plants.

119 The complete nucleotide sequences of the six beta-satellites ranged between 1318 and 1369

120 bp in size (Table 1). The sequences showed all the features typical of a beta-satellite (Briddon

121 et al. 2008), including a single ORF in a complementary sense strand which encodes a 118

122 amino acid protein and an adenine rich region. They also contain a nonanucleotide stem-loop

123 structure (TAATATTAC) and a satellite conserved region (SCR).

124

125 SDT based pairwise alignment shows that the Sri Lankan okra yellow vein mosaic beta-

126 satellites (OYVMB) had between 93.4 % to 99.2 % sequence identity. The most divergent

127 isolate OYVMB [LK:Kan:15] had 93.4 % to 94.0 % sequence identity with the rest of the Sri

128 Lankan OYVMB. The above isolates shared > 91 % of identity with some bhendi yellow vein

129 beta-satellites (BYVB), bhendi yellow vein mosaic beta-satellites (BYVMB) and

130 OYVMB[IN:Aur:08] from India. This classifies the sequences as coming from the same

131 species, according to the recently proposed beta-satellite species demarcation threshold of

132 91% (https://talk.ictvonline.org/files/ictv_official_taxonomy

133 _updates_since_the_8th_report/m/plant-official/6689). Sequence identity of Sri Lankan

134 OYVMB isolates was < 91% with sequences of okra leaf curl beta-satellites (OLCuB), bhendi

135 yellow vein India beta-satellites (BYVIB) and croton yellow vein mosaic beta-satellite

136 (CrYVMB [IN_Bhu_OYBHU_06]), all of which were also reported to be associated with

137 okra yellow vein mosaic disease in India (Venkataravanappa et al. 2011). Beta-satellite

138 ~~isolates associated with other begomovirus diseases in Sri Lanka had even lower identity (<70~~

139 ~~%) with the Sri Lankan OYVMB isolates.~~

140

141 ~~Nucleotide diversity (π) was measured in three subpopulations of beta-satellite isolates; Sri~~

142 ~~Lankan OYVMB (n=6), all the beta-satellite isolates reported from Sri Lanka (n=11) and~~

143 ~~some selected beta-satellites associated with okra (n=20). The lowest nucleotide diversity was~~

144 ~~noticed in Sri Lankan OYVMB (π=0.027) and the highest nucleotide diversity was in the total~~

145 ~~beta-satellite isolates reported from Sri Lanka (.π=0.245).~~

146

147 Briddon et al. (2003) reported two major clusters of beta-satellites corresponding to hosts in the

148 Malvaceae and non-Malvaceae. The Sri Lankan OYVMB clustered phylogenetically with

149 isolates from the Malvaceae and were completely separated from previously published beta-

150 satellite sequences from Sri Lanka, as expected (data not shown). Analysis of OYVMB and

151 selected beta-satellites associated with okra/bhendi clearly showed the close clustering of Sri

152 Lankan OYVMB with already reported Indian BYVB and BYVMB rather than okra enation

153 leaf curl beta-satellites (OELCuB) and OLCuB (Figure 1). The isolates OYVMB [LK:Put:15]

154 and OYVMB [LK:Kan:15] clustered separately from the rest of the Sri Lankan OYVMB

155 isolates.

156 Genetic recombination is an important process in the evolution of viruses and it is frequent in

157 begomoviruses and inter-species recombination is the major factor behind the emergence of

158 new begomovirus species (Lefeuvre et al. 2007). We looked for recombination events among

159 27 full-length genomes of different beta satellites associated with okra and other plants. A

160 recombination event was detected in the genome of all the test OYVMB isolates. The

161 recombination break point was detected immediately upstream of A-rich region and SCR

162 region. The major and minor parents were inferred to be ageratum yellow vein beta-satellite

163 (AYVB[LK:Age:03]) and Leucas zeylanica yellow vein beta-satellite (LZYVB[LK:Leu:06])

164 respectively (Table 2). These are associated with common weeds, *Ageratum* spp. and *Leucas*

165 *zeylanica* respectively, in Sri Lanka. The isolate OYVMB[LK:Kan:15] showed an additional

166 recombination event a few nucleotides downstream of the previous recombination.

167 OYVMB[IN:Aur:08] and AYVB[LK:Age:03] were inferred to be the minor and major

168 parents, respectively. One more recombination event was detected in the analysis, where the

169 isolates OYVMB[LK:Jaf:15] and OYVMB[LK:Kan:15] were parents of the recombinant

170 isolate OELCuB[IN:Jal-4/okr:15].

171 ~~In India, the beta-satellite molecules associated with okra segregated into four groups~~

172 ~~(Venkataravanappa et al. 2011): okra leaf curl beta-satellites (OLCuB); bhendi yellow vein~~

173 ~~beta-satellites (BYVB); bhendi yellow vein India beta-satellites (BYVIB); and a surprising~~

174 ~~distinct group called croton yellow vein mosaic beta-satellite (CrYVMB). In present study,~~

175 ~~the Sri Lankan beta-satellite isolates, except OYVMB[LK:Kan:15], were more closely related~~

176 ~~to BYVB (>96 %) than rest of the three groups.~~

177 Begomovirus may range over long distances with little differentiation in infectivity and

178 genome sequence. As expected, Sri Lankan isolates showed great similarities with Indian

179 isolates. The countries are close together and agricultural products are frequently exchanged,

180 so the isolates that associated with Sri Lankan OYVM disease might have arrived from India.

181 Sequence diversity among the Sri Lankan OYVM beta-satellites was lower than in previously

182 published isolates from India, possibly because the country is smaller and has less

183 geographical and environmental variation than India, or because the disease invaded recently.

184 **Acknowledgement**

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249

250 **Table 1** Characteristic features of beta-satellites isolated from OYVMD from different locations in Sri Lanka

Samples collected districts in Sri Lanka	Beta-Satellites	Abbreviation	Accession number in NCBI GenBank	Length of the DNA (bp)	GenBank sequence showing highest sequence identity in nucleotide BLAST search	
Vavuniya	Okra yellow vein mosaic beta-satellite [Sri Lanka: Vavuniya: 2015]	OYVMB [LK:Vav:15]	KX174318	1318	Bhendi yellow vein India beta-satellite [India:Jalna:Jal:2009] (KJ462078)	96%
Jaffna	Okra yellow vein mosaic beta-satellite [Sri Lanka: Jaffna: 2015]	OYVMB [LK:Jaf:15]	KX174319	1334	Bhendi yellow vein India beta-satellite [India :Coimbatore:Co:2009] (KJ462077)	98%
Kandy	Okra yellow vein mosaic beta-satellite [Sri Lanka: Kandy: 2015]	OYVMB [LK:Kan:15]	KX174320	1369	Bhendi yellow vein mosaic beta-satellite [India:Guntur:OY112:2006] (GU111969)	94%
Matara	Okra yellow vein mosaic beta-satellite [Sri Lanka: Matara: 2015]	OYVMB [LK:Mat:15]	KX174321	1351	Bhendi yellow vein mosaic beta-satellite [India:Madurai:MKU-1:2014] (KR068483)	98%
Trincomalee	Okra yellow vein mosaic beta-satellite	OYVMVB [LK:Tri:15]	KX174322	1320	Bhendi yellow vein India beta-satellite [India:Jalna:Jal:2009] (KJ462078)	96%

	[Sri Lanka: Trincomalee: 2015]					
Puttalam	Okra yellow vein mosaic beta-satellite [Sri Lanka: Puttalam: 2015]	OYVMVB [LK:Put:15]	KX174323	1351	Bhendi yellow vein mosaic beta-satellite [India:Aurangabad:OY165:2006] (GU111977)	97%

251

252 **Table 2** Putative recombination events detected within beta-satellites associated with okra and beta-satellites reported from Sri Lanka, based on

253 full-length beta-satellite sequences

Event	Breakpoints		Recombinant	Parents		Methods[1]	P-Value[2]
	Begin	End		Major	Minor		
1	1076	1316	OYVMB[LK:Vav:15]	AYVB[LK:Age:03]	LZYVB[LK:Leu:06]	RGBMC<u>S</u>3	3.16E-06
2	1092	1332	OYVMB[LK:Jaf:15]	AYVB[LK:Age:03]	LZYVB[LK:Leu:06]	RGBMC<u>S</u>3	3.16E-06
3	1154	1367	OYVMB[LK:Kan:15]	AYVB[LK:Age:03]	LZYVB[LK:Leu:06]	RGBMC<u>S</u>3	3.16E-06
4	1109	1349	OYVMB[LK:Mat:15]	AYVB[LK:Age:03]	LZYVB[LK:Leu:06]	RGBMC<u>S</u>3	3.16E-06
5	1078	1318	OYVMB[LK:Tri:15]	AYVB[LK:Age:03]	LZYVB[LK:Leu:06]	RGBMC<u>S</u>3	3.16E-06
6	1109	1349	OYVMB[LK:Put:15]	AYVB[LK:Age:03]	LZYVB[LK:Leu:06]	RGBMC<u>S</u>3	3.16E-06
7	998	1127	OYVMB[LK:Kan:15]	OYVMB[IN:Aur:08]	AYVB[LK:Age:03]	RG<u>B</u>MCS3	5.93E-11
8	52	812	OELCuB[IN:Jal-4/okr:15]	OYVMB[LK:Jaf:15]	OYVMB[LK:Kan:15]	RGBMCS<u>3</u>	2.95E-15

254 [1] *R* RDP, *G* GeneConv, *B* Bootscan, *M* MaxChi, *C* Chimera, *S* SisScan, *3* 3SEQ

255 [2] The reported *P* values are for the methods by underline, and they are the lowest *P* values calculated for the region in question

256

257

258   **Figure legends**

259   **Fig. 1** Colour coded pairwise identity matrix generated from 27 different beta-satellite DNAs,

260   including 6 beta-satellites described in this work, 5 beta-satellites associated with different

261   begomoviral diseases in Sri Lanka, 14 different beta-satellites associated with okra/bhendi

262   and 2 other beta-satellites associated with chilli and cotton. See Supplementary Table 1 for

263   details on the compared sequences. Each coloured cell represents a percentage identity score

264   between two sequences. The coloured key indicates the correspondence between pairwise

265   identities and the colours displayed in the matrix.

266   **Fig. 2** Molecular Phylogenetic analysis by Maximum Likelihood method

267   The evolutionary history was inferred by using the Maximum Likelihood method based on

268   the General Time Reversible model. The tree with the highest log likelihood (-10077.7604) is

269   shown. The percentage of trees in which the associated taxa clustered together is shown next

270   to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying

271   Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the

272   Maximum Composite Likelihood (MCL) approach, and then selecting the topology with

273   superior log likelihood value. A discrete Gamma distribution was used to model evolutionary

274   rate differences among sites (5 categories (+G, parameter = 0.9163)). The tree is drawn to

275   scale, with branch lengths measured in the number of substitutions per site. The analysis

276   involved 30 nucleotide sequences; Indian and Sri Lankan isolates are denoted IN and LK

277   respectively within the parentheses following the isolate code, . All positions containing gaps

278   and missing data were eliminated. There were a total of 806 positions in the final dataset.

279   Evolutionary analyses were conducted in MEGA7.

280

281