

First report of naturally occurring recombinant non-coding DNA satellite associated with *Tomato yellow leaf curl Kanchanaburi virus* on eggplant in Indonesia

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Abstract. Kandito A, Hartono S, Sulandari S, Somowiyarjo S, Widyasari YA. 2019. First report of naturally occurring recombinant non-coding DNA satellite associated with *Tomato yellow leaf curl Kanchanaburi virus* on eggplant in Indonesia. *Biodiversitas* 20: 129-136. Begomovirus is a viral genus which is a major impediment in the cultivation of plants, especially Solanaceae. *Tomato yellow leaf curl Kanchanaburi virus* (TYLCKaV) is a member of the Begomovirus genus that is widely reported to infect eggplant and results in severe symptoms of chlorosis. In addition to the type of virus that infects, the severity of symptoms can also be caused by the presence of betasatellite associated with several begomovirus species. The presence of betasatellite that is related to several begomovirus species can lead to more severe symptoms. The aim of this study was to identify satellites associated with begomovirus in Indonesia. Eggplant samples from Bantul, Special Region of Yogyakarta show with mosaic and severe chlorotic symptoms due to Begomovirus infection. Total DNA samples were subject to PCR amplification using universal primer for begomovirus PALIV1978/PAR1C715 and specific primer for betasatellite $\beta 01/\beta 02$. The PCR amplification produced a DNA band measuring ± 1600 bp, identical size of begomovirus. The amplification of the betasatellite specific primer produced a DNA band featuring ± 1300 bp, identical size of betasatellites that were associated with begomovirus in the sample. The results of DNA sequencing, suggested that begomovirus in this study had a close relationship with TYLCKaV from Thailand and Indonesia. Characterization of the satellite-based on nucleotide sequence revealed the presence of stem-loop structures, satellite conservative regions, and adenine-rich regions that resembled structures present in the betasatellite, which were associated with *Tomato leaf curl virus* and *Ageratum yellow vein virus*, with no ORF found. These results indicated that the TYLCKaV could be associated with a non-coding satellite. This result is the first report regarding the TYLCKaV association with a non-coding satellite in Indonesia.

Keywords: Begomovirus, betasatellite, characterization, eggplant, TYLCKaV

INTRODUCTION

The Begomovirus genus is a member of the Geminiviridae family and is one of the major constraints for horticulture, especially on the Solanaceae family. Since 1999 Begomovirus has been reported to damage chili plantations in West Java and Special Region of Yogyakarta (Hidayat et al. 1999; Sulandari et al. 2001). The incidence of Begomovirus disease initially dominant in the lowlands has now been reported to occur in the highlands (Kusumaningrum et al. 2015). The disease incidence caused by begomovirus is still difficult to control because of the wide range of virus hosts and *Bemisia tabaci* as the vector, high viral genetic diversity, recombination among species of viruses, multiple infections, and the presence of satellite DNA which potentially exacerbate symptoms (Bhattacharyya et al. 2015).

Begomovirus may possess a single genome (monopartite) in the form of DNA-A, or a double genome (bipartite) consist of DNA-A and DNA-B. Monopartite begomoviruses found in Indonesia are *Ageratum yellow vein virus* (AYVV), *Tomato leaf curl virus* (ToLCV), and *Tomato leaf curl Java virus* (ToLCJaV), and bipartite

begomoviruses are *Tomato leaf curl Kanchanaburi virus* (TYLCKaV), *Tomato leaf curl New Delhi virus* (ToLCNDV), and *Pepper yellow leaf curl virus* (PepYLCIV) (Kenyon et al. 2014). Each genome measures 2.7-2.8 kbp. The DNA-A consists of six open reading frames (ORF), namely AC1, AC2, AC3, AC4, AV1, and AV2. While the DNA-B consists of two ORFs, BC1 and BV1. Each ORF in begomovirus plays an essential role in the viral pathogenesis, symptom induction, also intracellular and intercellular transport. In addition, some Begomovirus species can also be associated with satellite DNAs such as alphasatellite, betasatellite, and deltasatellite. The size of the satellites can vary, ranged from 0.7-1.3kbp (Zhou 2013; Lozano et al. 2016). Alphasatellite is a satellite DNA that has a replication gene, resembling *Rep* gene in DNA-A of the helper virus, and it can replicate autonomously without the help of its helper. The presence of alphasatellite can interfere with the viral replication process, reduce viral accumulation, and attenuate symptoms (Idris et al. 2011). Betasatellite is satellite DNA that has the $\beta C1$ gene, which is known to suppress the mechanism of transcriptional gene silencing (TGS) and jasmonic acid production thereby increasing

disease severity (Dry et al. 1997; Briddon et al. 2001; Zhang et al. 2012; Zhou 2013). Deltasatellite is satellite DNA that does not encode certain proteins (non-coding satellite) and sizes approximately 0.7kbp (Zhou 2013).

Tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) is one of the species that belong to the Begomovirus genus mainly found infects Solanaceae in Indonesia, in addition to several other species namely *Pepper yellow leaf curl virus* (PepYLCIDV), *Ageratum yellow vein virus* (AYVV), *Tomato yellow leaf curl Java virus* (ToLCJaV), *Tomato leaf curl New Delhi virus* (ToLCNDV), and *Tomato leaf curl virus* (ToLCV) (Tsai et al. 2006; Kon et al. 2006; Kusumaningrum et al. 2015; Kenyon et al. 2014; Kintasari et al. 2013). In a single plant infected with Begomovirus, there might be found more than a single begomovirus. This caused the severity of the disease increases and has the potential to result in recombination between species (Sulandari et al. 2006). Mixed infection of begomovirus previously has been reported in Indonesia and caused severe symptoms in chili and chickpeas (Koeda et al. 2016; Sidik et al. 2017).

Satellite DNA commonly found associated with several monopartite Begomoviruses, including AYVV, ToLCJaV, and *Cotton leaf curl virus* (CuLCV), and the bipartite ToLCNDV. It is suggested that the association between satellites and Begomoviruses can affect the severity of the symptoms (Kon et al. 2006; Saunders et al. 2004; Nawaz-ul-Rehman et al. 2009; Agnihotri et al. 2018). However, there is no report of an association between other Begomovirus in Indonesia, such as TYLCKaV, with a satellite. This study aimed to detect and identify satellite DNA on eggplant plants that show severe symptoms.

MATERIALS AND METHODS

The diseased eggplant sample was obtained in Srigading, Bantul, Special Region of Yogyakarta, Indonesia (Figure 1), with symptoms of leaves in the form of mosaics accompanied by symptoms of yellowing and severe chlorosis suspected of being infected with Begomovirus (Figure 2). Leaf of eggplant was subjected to DNA extraction.

The DNA extraction was carried out using a total DNA extraction kit for plants (Geneaid Germany). Extraction steps follow instructions from the manufacturer. The total genome DNA extract was used as a template for amplification.

Polymerase Chain Reaction (PCR) was carried out using MyTaq Redmix Polymerase (Bioline), ddH₂O, forward primer, reverse primer, and DNA templates. The reaction is carried out in a volume of 50 μ L. The primers used in this study are in Table I. The PCR program for the primary pair PAR1C715/PALIV1978 which amplified the Begomovirus DNA-A region, i.e., pre-denaturation at 95°C for 3 minutes, followed 40 cycles of denaturation at 95°C for 1 minute, attaching (annealing) at 55°C for 30 seconds, extension for 1 minute 30 seconds. The final extension was at 72°C for 10 minutes. The PCR program for the β 01/ β 02 primer that amplified the full genome of the betasatellite was pre-denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 65°C for 30 seconds, an extension for 1 minute 45 seconds. The final extension was at 72°C for 10 minutes.

Visualization of PCR products was carried out by agarose gel electrophoresis 1% (w/v) using 50V power for 50 minutes.

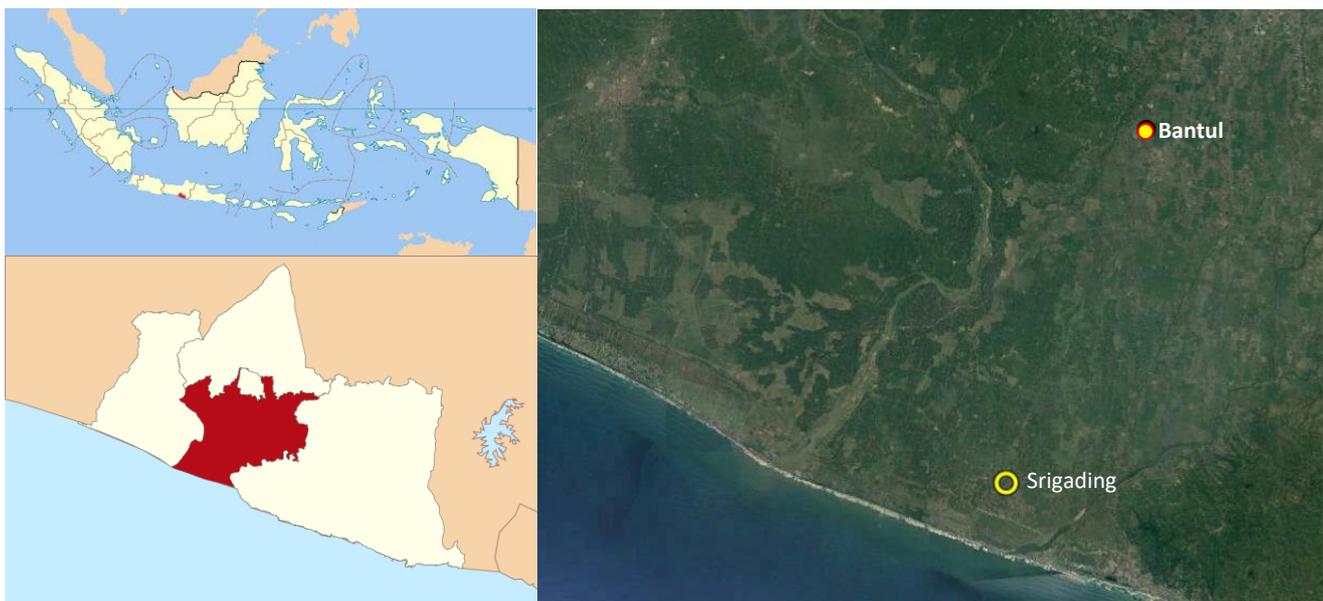
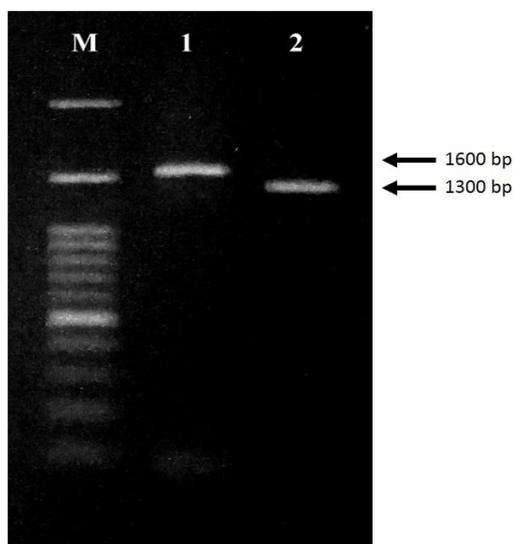


Figure 1. Map of sampling site in Srigading Village, Bantul District, Yogyakarta, Indonesia

Table 1. Primers in this research

Primer	5' - 3'	Reference
PAR IC 715	GATTTCTGCAGTTDATRTTYTCRTCCATCCA	Rojas et al. (1993)
PAL IV 1978	GCATCTGCAGGCCACATYGTCTTYCCNGT	
β 01	GGTACCACTACGCTACGCAGCAGCC	Bridson et al. (2002)
β 02	GGTACCTACCTCCCAGGGGTACAC	

**Figure 2.** Eggplant showed severe yellowing and mosaic symptoms in Bantul, Yogyakarta, Indonesia**Figure 3.** Visualization on 1% agarose gel of PCR products using PARIC715/PALIV1978 (1) and β 01/ β 02 (2). (M) is a 100bp DNA Ladder.

Sequence analysis of begomovirus was conducted using the direct sequencing method of PCR products through the services of PT. Genetika Science. The sequence of the betasatellite was performed by the cloning method using the T7 plasmid. Analysis of sequence results was carried out with the MEGA v.7 program (Kumar et al. 2016), BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), Clustal Omega (<http://ebi.ac.uk/Tools/msa/clustalo/>), and ORFFinder (<https://www.ncbi.nlm.nih.gov/orffinder/>). The phylogenetic analysis uses the Neighbor-Joining method with 1000 bootstrap replicates (Efron et al. 1996) The recombinant analysis was performed using RDP v4 (Martin et al. 2015) Analysis of unique structures were performed using alignment analysis. The betasatellite sequence then submitted to GenBank with accession number MN510674.

RESULTS AND DISCUSSION

Amplification of total DNA by PCR using the primer pair PAR1c715/PAL1v1978 targeting a portion of DNA-A begomovirus produced a target DNA measuring ~ 1600bp. These results indicated that a positive sample is infected by Begomovirus. Total DNA amplification with the primer pair β 01/ β 02, which was a specific primer, produced ~ 1300bp DNA bands. The PCR results of the two targets are then sequenced. Sequence analysis with a target of 1600bp showed the highest homology with TYLCKaV. On the other hand, the 1300bp target showed the highest homology with ToLCJAV-betasatellite and PepYLCIV DNA-B components (Figure 3).

The results of DNA samples sequenced using the direct sequencing method showed that the samples were positively infected with TYLCKaV with identity values in the BLAST program up to 99% with various TYLCKaV isolates in GenBank. Phylogenetic analysis using Neighbor-Joining method with 1000 bootstraps in MEGA v7 program (Figure 4) showed that the sample is in the same group as Thailand and Indonesian TYLCKaV from tomato and eggplant, while TYLCKaV from Thailand and Indonesia are in different groups with TYLCKaV from Vietnam and Cambodia.

TYLCKaV is a member of the Begomovirus genus in the Old World, and most are reported to attack eggplant, tobacco, tomatoes, and chili (Kintasari et al. 2013; Kusumaningrum et al. 2015; Koeda et al. 2016). TYLCKaV infection in eggplants induces chlorotic, yellowing, and mosaic symptoms. While the infection in tomato is known to cause apical shoots to permeate and dwarf. In addition, infection in tobacco causes the leaves to curl yellow and dwarf (Tang et al. 2014; Widarta et al. 2017).

KY817991_CLCuV_betasatellite_Cotton_India	MTTSGTNNKGVRFIVDVRIMENMKIFIHMRILSTKSPSLIKYEGIIQYRYEDIHVFPD--	58
KX951462_CLCuV_betasatellite_Cotton_India	MTTSGTNNKGVRFIVDVRIMENMKIFIHMRILSTKSPSLIKYEGIIQYRYEDIHVFPD--	58
AB307732_ToLCPHV_betasatellite_Tomato_Phillippines	MTITYKNTKGMTFVINVRLKENSISVEIDLFLTKKPFLLHKEQFIIPYGHQGIITPFD--	58
AB308071_ToLCPHV_betasatellite_Tomato_Phillippines	MTITYKNTKGMTFVINVRLRRENSISVEIDLFLTKKPFLLHKEQFIIPYGHQGIITPFD--	58
AB162142_ToLCJav_betasatellite_Ageratum_Indonesia	MTISYSNNRGMFIIQVRLSSEIQTIVLEMYSTRSPALVRHKFSIPYGHNGIIPPFN--	58
AB113651_ToLCV_betasatellite_Tomato_Indonesia	MTISYSNSKGMFLIINIKFRSEIQFTVEVEVFSTRSPALVKHKFSIPYGHNGIIPPFN--	58
KC282642_ToLJav_betasatellite_Papaya_Nepal	MTISYSNSKGMFIIINIKFRSEIQFTVEVEVFSTRSPALVKHKFSIPYGHNGIIPPFN--	58
KC677734_ToLCJav_betasatellite_Tomato_Japan	MTISYRNNQGMVFIINVRKSEMLADEVEMLSTRSPAVVKHKFSIPYGHNGIIPPFN--	58
KY780620_AYVW_betasatellite_Ageratum_China	MTISYTNDKGVLFIIINVRLRSELKFTVDVEVFSTRSPALVKHKFSIPYGHNGIIPPFN--	58
AJ542495_ToLCV_betasatellite_Tomato_Taiwan	MTISYTNDKGVLFIIINVRLRSELKFTVDVEVFSTRSPALVKHKFSIPYGHNGIIPPFN--	58
AB306522_AYVW_betasatellite_Tomato_Japan	MTISYTNDKGVLFIIINVRLRSELKFTVDVEVFSTRSPALVKHKFSIPYGHNGIIPPFN--	58
AM691551_ToLCV_betasatellite_Ageratum_China	MTISYTNDKGVLFIIINVRLRSELKFTVDVEVFSTRSPALVKHKFSIPYGHNGIIPPFN--	58
AB100306_ToLCV_betasatellite_Tomato_Indonesia	MTISYSNNKGISFIIQVRLSSEIQTIVLEMFSTRSPALVKHKFSIPYGHNGIIPPFN--	58
MN510674_TYLCKaV_satellite_Eggplant_Bantul	ISFFLEHHKGMVFIINVKFGSEIQFTVEVELFSTRSPALVKHKFSIPYGHNGIIPPFN--	59
	:: : * : * : *	
KY817991_CLCuV_betasatellite_Cotton_India	-----FNGFEGSIIANFLFAYNEAMIEEIEFIEDIVHRLDILVLENPEILGINV	106
KX951462_CLCuV_betasatellite_Cotton_India	-----FNGFEGSIIANFLFAYNEAKIEEIEFIEDIVHRLDILVLENPEILGINV	106
AB307732_ToLCPHV_betasatellite_Tomato_Phillippines	-----FNDIEEGIKGIINIMYADSPAEEFRHEDMVEAIDILMMNEAPVVEINI	106
AB308071_ToLCPHV_betasatellite_Tomato_Phillippines	-----FNDLEEGIKGLINIMYADSPAEEFRHEDMVEAIDILMMNEAPVVDINI	106
AB162142_ToLCJav_betasatellite_Ageratum_Indonesia	-----FNALIEEGIKNLLRIMYKDSIIEEIKAEADMVEIIDMMMQQEAVIDIQV	106
AB113651_ToLCV_betasatellite_Tomato_Indonesia	-----FNALIEEGIKNLLRIMYKDSIIEEIKAEADMVEIIDMMMQQEAVIDIQV	106
KC282642_ToLJav_betasatellite_Papaya_Nepal	-----FNALIEEGIKNLLRIMYKDSIIEEIKAEADMVEIIDMMMQQEAVIDIQV	106
KC677734_ToLCJav_betasatellite_Tomato_Japan	-----FNALIEEGIKNLLRIMYKDSIIEEIKAEADMVEIIDMMMQQEAVIDIQV	106
KY780620_AYVW_betasatellite_Ageratum_China	-----FNALIEEGIKNLLRIMYKDSIIEEIKAEADMVEIIDMMMQQEAVIDIQV	106
AJ542495_ToLCV_betasatellite_Tomato_Taiwan	-----FNALIEEGIKNLLRIMYKDSIIEEIKAEADMVEIIDMMMQQEAVIDIQV	106
AB306522_AYVW_betasatellite_Tomato_Japan	-----FNALIEEGIKNLLRIMYKDSIIEEIKAEADMVEIIDMMMQQEAVIDIQV	106
AM691551_ToLCV_betasatellite_Ageratum_China	-----FNALIEEGIKNLLRIMYKDSIIEEIKAEADMVEIIDMMMQQEAVIDIQV	106
AB100306_ToLCV_betasatellite_Tomato_Indonesia	-----FNALIEEGIKNLLRIMYKDSIIEEIKAEADMVEIIDMMMQQEAVIDIQV	106
MN510674_TYLCKaV_satellite_Eggplant_Bantul	RNGVHHRQVQRNTHS*DRHVFN*VYRPS-*A*FISEDMVEIIDILVQQQAQVVEIHW	114
	* : . : * : : : * : * : : : : : : : : : : : : : : : : : : : *	
KY817991_CLCuV_betasatellite_Cotton_India	MEPYTFNKKFTV*	118
KX951462_CLCuV_betasatellite_Cotton_India	MEPYVFNKKFTV*	118
AB307732_ToLCPHV_betasatellite_Tomato_Phillippines	GEKFNVYSTTNI*	118
AB308071_ToLCPHV_betasatellite_Tomato_Phillippines	GEKFNVYSTTNI*	118
AB162142_ToLCJav_betasatellite_Ageratum_Indonesia	NDEYRVYKHVTV*	118
AB113651_ToLCV_betasatellite_Tomato_Indonesia	NDEYRVYKHVTV*	118
KC282642_ToLJav_betasatellite_Papaya_Nepal	NDEYHVKWTV*	118
KC677734_ToLCJav_betasatellite_Tomato_Japan	NDEYHMYKSVSV*	118
KY780620_AYVW_betasatellite_Ageratum_China	NDEYHVKWTV*	118
AJ542495_ToLCV_betasatellite_Tomato_Taiwan	NDEYHVKWTV*	118
AB306522_AYVW_betasatellite_Tomato_Japan	NDEYHVKWTV*	118
AM691551_ToLCV_betasatellite_Ageratum_China	NDEYHVKWTV*	118
AB100306_ToLCV_betasatellite_Tomato_Indonesia	NDEYRVYKHVTV*	118
MN510674_TYLCKaV_satellite_Eggplant_Bantul	NDEYHVKWTV*	126
	: : . . . : *	

Figure 6.B. Amino acid translation of predicted βC1 ORF between TYLCKaV satellite with other betasatellites obtained from GenBank. Translation from TYLCKaV satellite showed several stop codons (*) along the sequence (no. 60, 79, 86, 93, and 95)

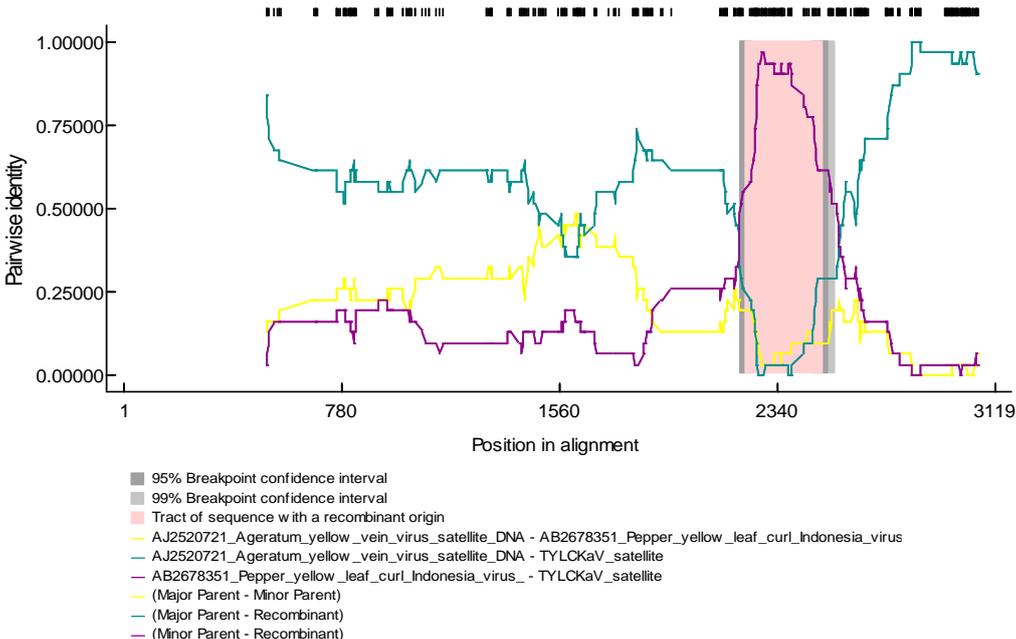


Figure 7. Recombinant analysis using RDP v4. The recombination point highlighted in pink

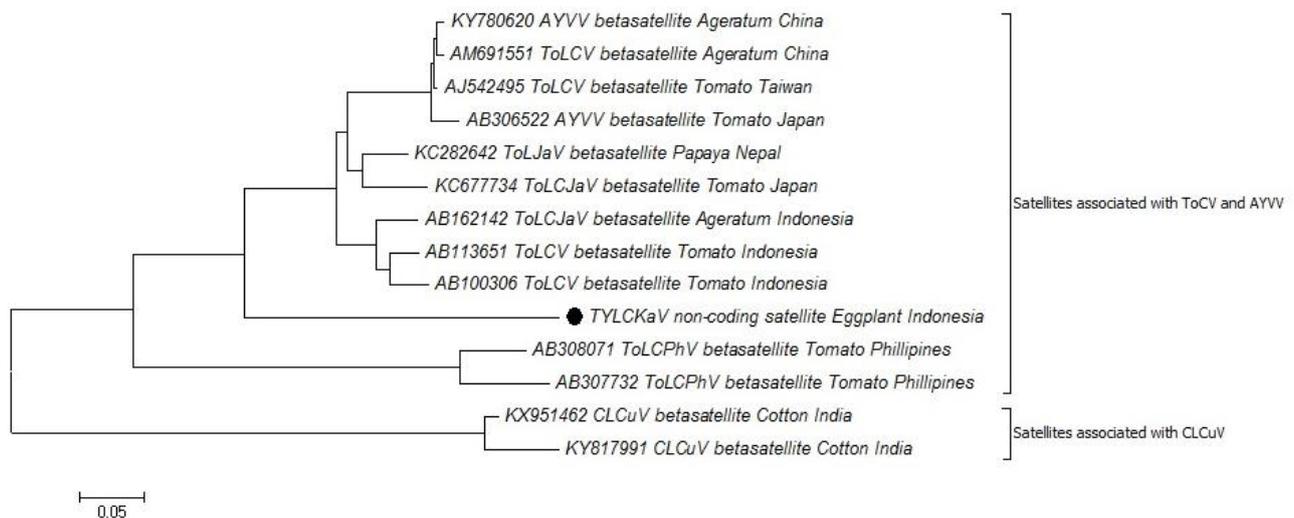


Figure 8. The phylogenetic tree of the TYLCKaV satellite shows a relationship with other satellites associated with ToLCV obtained from GenBank. CLCuV betasatellites are selected as outgroup

BLAST result of this sequence showed a region similar to the PepYLCIV DNA-B component. This indicates that satellites associated with TYLCKaV probably are a non-coding satellite perhaps as the result of recombination between betasatellite and PepYLCIV DNA-B. This result confirmed using the Recombination Detection Program (RDP v4) and showed recombination events occur between betasatellite and PepYLCIV (Figure 7). The possibility of recombinant events probably caused by similar stem-loop TAATATTAC between DNA-A, DNA-B, and satellites, considering both DNA-B and satellites do not have a *Rep* gene. The replication of DNA-B or satellite depends on its DNA-A, and it also hypothesized that DNA-B previously was a satellite, captured by DNA-A, and then evolve and become an integral part of the genome component (Bridson et al. 2010). The recombinant DNA satellites indicated as the result of a recombination event between a complex of DNA satellites and begomoviruses, while the existence of recombinant satellite could be maintained as well as other satellites by begomovirus (Tao and Zhou 2008; Huang et al. 2013).

The phylogenetic tree analysis (Figure 8) shows that the satellite associated with TYLCKaV is grouped with satellites associated with Indonesian AYVV and ToLCV, although it is located in a different branch. This indicates that the satellites associated with TYLCKaV, ToLCV, and AYVV are originated from the same complex. At one time, transreplication could occur, while the presence of betasatellites could be acquired by one of these species. Acquisition of a betasatellite by another virus, as well as the occurrence of recombination between betasatellite and other satellites or other viruses, can be suspected because of the motive recognized by the Rep (Rep-binding site) protein (Fontes et al. 1994; Qing and Zhou 2009).

Betasatellite is a sub-viral agent whose replication depends on the begomovirus as its helper. Betasatellite is half the genome of the helper virus (~ 1.3kb) and is circular

in shape and has an ORF measuring 118 amino acids that encode the β C1 protein, an adenine-rich region (A-rich region), and the TAATATAC stem-loop structure. Between the begomovirus genome technique, both DNA-A and DNA-B did not have significant homology except for the TAATATAC loop stem (Zhou 2013; Lozano et al. 2016).

Betasatellite associations are needed by some Begomoviruses to induce symptoms. AYVV and CLCMuV infections without the association of betasatellites have been reported to produce no symptoms (Saunders et al. 2004; Tan et al. 1995, Bridson et al. 2002). The betasatellite association with TYLCV was reported to increase symptom severity (Ito et al. 2009). In Indonesia, the betasatellite is associated with *Tomato leaf curl Java virus* (ToLCJaV) and *Ageratum yellow vein virus* (AYVV) (Kon et al. 2006). But there have been no reports of betasatellite or other satellite association with other begomoviruses species in Indonesia. The result of this research is the first report of an association between satellite and TYLCKaV in Indonesia.

The association of viruses with betasatellites generally occurs in monopartite begomovirus, where the betasatellites act like DNA-B in bipartite begomovirus. In some Begomovirus DNA-A infections alone often do not cause symptoms. Symptoms can occur if there is a betasatellite that accompanies the Begomovirus. Betasatellites which are associated with begomovirus are known to increase symptom severity. This is caused by the expression of the β C1 gene, which can suppress plant resistance mechanisms in the form of *transcriptional gene silencing* (TGS) and jasmonic acid production (Sharma et al. 2010; Zhang et al. 2012; Amin et al. 2011). The expression of the β C1 gene can also help transfer virus particles from one cell to another. This indicates that the presence of a betasatellite is one of the symptom severity determinants in addition to several genes in the helper

(Saunders et al. 2004; Briddon and Stanley 2006; Saeed et al. 2007).

In conclusion, based on the analysis of the nucleotide sequence from eggplant, which showed symptoms of yellow leaves and mosaics, there was an association between TYLCKaV and a non-coding satellite that was thought to be the result of recombination between betasatellites and PepYLCIV DNA-B. The existence of satellites associated with the TYLCKaV can induce extreme symptoms. This is the first report of non-coding recombinant DNA satellite associations with the *Tomato yellow leaf curl Kanchanaburi virus*.

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