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Ageratum yellow vein alpha satellite and tomato leaf curl Java beta satellite association with begomoviruses infecting crops and weeds in Indonesia

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Abstract: The symptomatic samples were taken from five districts in Yogyakarta and Central Java provinces of Indonesia to survey the genetic diversity of *Begomovirus*, α and β satellites. Fourteen samples tested positive by PCR for infections of tomato leaf curl New Delhi virus, pepper yellow leaf curl Indonesia virus, mungbean yellow mosaic India virus, and ageratum yellow vein China virus. For the first time, the Ageratum yellow vein alpha satellite (AYVA) was identified in Indonesia and the tomato leaf curl Java beta satellite (ToLCJaB) was detected in different hosts. The partial genome sequences of begomoviruses and complete sequences of the α and β satellites were obtained and deposited in NCBI GenBank (OP846592-OP846615). The phylogenetic study revealed that AYVA and ToLCJaB each had two separate lineages. Coherent with the other reports, the polymorphism and diversity analyses confirmed that the βC1 coding region of the ToLCJaB genome is highly conserved. The comparison of phylogroups 1 and 2 of ToLCJaB produced a high Fixation index (F_{ST}) value, indicating genetic isolation between them. This report could serve as a basis for future work on the poorly studied genetic variation of α and β satellites in Indonesia and other countries.

Keywords: gene flow; molecular detection; neutrality test; phylogenetic analysis; population study

The member species of the genus *Begomovirus* have circular single-stranded DNA genomes, which may be monopartite (having only a single genome: DNA-A) or bipartite (having two separate genomes in the same virion particle: DNA-A and DNA-B) (Fiallo-Olivé et al. 2021). Many *Begomovirus* species are associated with three types of satellites, namely, alpha, beta, and delta satellites, which may affect their pathogenicity and disease severity (Zhou 2013; Fiallo-Olivé et al. 2016; Zou et al. 2020). The alpha satellite genome and their corresponding helper viruses have similar replication genes. Therefore, they can replicate autonomously without their helper viruses. However, the beta satellite genome has the βC1 gene, suppressing the transcriptional gene silencing mechanism and jasmonic acid production, leading to increased disease severity. The delta satellites are non-coding satellites with a small genome size of 0.7 kbp (Zhou 2013).

The molecular diversity of *Begomovirus* genus members has been studied in Indonesia because of large yield losses in a wide range of important crops (Subiastuti et al. 2019; Lestari et al. 2022; Sidik et al. 2022). However, the reports on the genetic variation of the satellites are very scarce. A non-coding beta satellite isolate with a defective βC1 gene was identified together with a to-

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mato yellow leaf curl Kanchanaburi virus in eggplant (Kandito et al. 2020). Another non-coding beta satellite isolate with a defective βC1 gene was found to be associated with pepper yellow leaf curl Indonesia virus (PepYLCIV) in chilli pepper cultivated in one of the highlands in Indonesia (Kandito et al. 2021).

The accurate detection and effective control of viral pathogens may rely on understanding the virus population structure and its diversity (Gao et al. 2016; Coşkan et al. 2022). In this study, four districts in Yogyakarta province and one in Central Java province of Indonesia were surveyed. The symptomatic main crop and weed species were molecularly tested by PCR. The obtained partial genome sequences could provide the genetic diversity basis of the alpha- and beta satellites associated with begomoviruses in Indonesia. Subsequently, a small in silico analysis was performed to provide preliminary insight into the population structure of the most dominant satellite in the surveyed areas.

MATERIAL AND METHODS

Sample collection and PCR. The field surveys were conducted in horticultural production hubs in Sleman, Wates, Bantul, and Wonosari districts in Yogyakarta province and Magelang district in Central Java province of Indonesia during July–August 2022. A total of 40 leaf samples from 15 crops and 35 weeds were collected and brought to the plant virology laboratory at Universitas Gadjah Mada. All the samples showed conspicuous typical symptoms of begomovirus infection, such as leaf yellowing, leaf and fruit necrosis, and stunting.

Total DNA was extracted from the samples using a Genomic DNA Mini Kit (Genaid, Taiwan) following standard manufacturer protocols. The DNA obtained was used in PCRs with universal primer pairs to amplify the partial AV1 gene of begomoviruses (Revill et al. 2003), DNA101/DNA102 and UN101/UN102 primer pairs (Bull et al. 2003) to amplify the full genome of the associated alpha satellites, β01/β02 (Briddon et al. 2002), and CLB36F/CLB37R primers (Kumar R. et al. 2018) to amplify beta satellites. Each PCR reaction was prepared in a total volume of 50 µL and contained 25 µLof MyTaq HS Red Mix (Bioline, Germany), 2.5 µL (10 pmol/µL) each of the forward and reverse primers, 5 µL of template DNA, and 15 µL of PCR-grade ddH_2O. The presence of the target band was observed under a UV transilluminator (Optima Inc., Japan) following agarose gel electrophoresis. The successfully amplified PCR products were submitted to 1st BASE (Singapore) for bidirectional sequencing via the Sanger method. The recovered raw sequences were assembled and checked for errors in CLC Main Workbench (version 20) and then deposited in GenBank (NCBI).

Phylogenetic analysis. The obtained nucleotide (nt) sequences were tested using Blastn online algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify their relation with other nt sequences in NCBI GenBank. They were then aligned with the sequences of several isolates of their respective virus species available in GenBank by applying the ClustalW algorithm in the software MEGA X (version 10.2.4) (Kumar S. et al. 2018). A total of five phylogenetic trees were constructed based on the partial genome of begomovirus, full genome and complete replication gene of alpha satellite, and full genome and complete βC1 gene of beta satellite using maximum likelihood algorithms in MEGA X software (version 10.2.4) with a uniform rate among sites and complete deletion of missing data treatment applied with the Tamura–Nei parameter models (Tamura & Nei 1993). The statistical significance of branches was estimated by using 1 000 bootstrap replicates.

Population structure of beta satellite. The important population structure-related parameters, namely, number of haplotypes (h), haplotype diversity (Hd), total number of mutations (η), number of variable sites (S), nt diversity (per site) (π), average number of nt differences between sequences (k), and transcriptional selection pressure (dN/dS = ω), and the three neutrality tests; Fu and Li's D^* and F^* (Fu & Li 1993), and Tajima's D (Tajima 1989), were estimated for the beta C1 gene of the obtained beta satellite genome using the DnaSP software (version 6.12.03) (Rozas et al. 2017).

The K_S, Z, S_{nn}, and F_{ST} values (Hudson et al. 1992; Hudson 2000) were also estimated via DnaSP software (version 6.12.03) to understand the level of genetic differentiation and gene flow between the obtained betasatellite phylogroups based on the beta C1 gene. The fixation index (FST) value could be between 0 for panmixia and 1 for fully distinct populations (Hudson et al. 1992). Therefore, F_{ST} value > 0.33 usually suggests a rare gene flow and expanding genetic separation among tested populations (Santosa & Ertunç 2021).

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RESULTS

Molecular identification by PCR. The PCR revealed that 14 samples, including main crops and two weed species, tested positive for begomovirus infection. The symptoms of the negative samples were probably attributed to abiotic factors such as nutrition, especially in weeds, or infection with other viruses. Further tests using DNA101/DNA102 and UN101/UN102 primer pairs on these 14 samples detected alpha satellite infection only in sample No. 1Btl-bgw. The beta satellite infection was also confirmed in 1Btl-bgw and eight other samples using β01/β02 and CLB36F/CLB37R primer pairs (Table 1). The successfully amplified amplicons were then submitted for nt sequencing. Blastn analysis on the obtained 552 nt long sequences of begomoviruses identified two isolates of tomato leaf curl New Delhi virus (ToLCNDV), five isolates of PepYLCIV, one isolate of mungbean yellow mosaic India virus (MYMIV), and six isolates of ageratum yellow vein China virus (AYVCNV). Moreover, the full genome sequences (±1300 nt) of one isolate of ageratum yellow vein alpha satellite (AYVA) and nine isolates of tomato leaf curl Java beta satellite (ToLCJaB) were recovered. These 24 sequences were deposited in NCBI GenBank under accession No. OP846592-OP846615 (Table 1).

Phylogenetic analysis. In the constructed phylogenetic tree, four species of *Begomovirus* were clustered with the isolates of their respective species. The six Indonesian AYVCNV isolates from two weed species (*Ageratum conyzoides* and *Amaranthus spinosus*) formed a cluster that was separated from other tested

Table 1. *Begomoviruses* and associated alpha and beta satellites identified in this study

No.	Sample code	Location	Host	Isolate name (NCBI GenBank Acc. No.)*		
				DNA-A	Alpha	Beta
1.	9Wat-cu	Wates, Yogyakarta	Cucumber (*Cucumis sativus*)	9Wat-cu-ToLCNDV (OP846603)	–	–
2.	10Wat-cu	Wates, Yogyakarta	Cucumber	10Wat-cu-ToLCNDV (OP846604)	–	–
3.	7Smn-pep	Sleman, Yogyakarta	Chili pepper (*Capsicum annuum*)	7Smn-pep-PepYLCIV (OP846606)	–	7Smn-pep-ToLCJVB (OP846598)
4.	8Smn-yb	Sleman, Yogyakarta	Yardlong bean (*Vigna unguiculata* ssp. *sesquipedalis*)	8Smn-yb-MYMIV (OP846592)	–	8Smn-yb-ToLCJVB (OP846599)
5.	10Smn-pep	Sleman, Yogyakarta	Chili pepper	10Smn-pep-PepYLCIV (OP846607)	–	10Smn-pep-ToLCJVB (OP846600)
6.	6Wno-spamr	Wonosari, Yogyakarta	Spiny amaranth (*Amaranthus spinosus*)	6Wno-spamr-AYVCNV (OP846610)	–	6Wno-spamr-ToLCJVB (OP846601)
7.	7Wno-spamr	Wonosari, Yogyakarta	Spiny amaranth	7Wno-spamr-AYVCNV (OP846611)	–	7Wno-spamr-ToLCJVB (OP846602)
8.	8Wno-spamr	Wonosari, Yogyakarta	Spiny amaranth	8Wno-spamr-AYVCNV (OP846612)	–	–
9.	9Wno-spamr	Wonosari, Yogyakarta	Spiny amaranth	9Wno-spamr-AYVCNV (OP846613)	–	–
10.	1Btl-bgw	Bantul, Yogyakarta	Billygoat weed (*Ageratum conyzoides*)	1Btl-bgw-AYVCNV (OP846614)	1Btl-bgw-AYVA (OP846593)	1Btl-bgw-ToLCJVB (OP846594)
11.	2Btl-bgw	Bantul, Yogyakarta	Billygoat weed	2Btl-bgw-AYVCNV (OP846615)	–	–
12.	5Btl-pep	Bantul, Yogyakarta	Chili pepper	5Btl-pep-PepYLCIV (OP846595)	–	5Btl-pep-ToLCJVB (OP846595)
13.	6Btl-pep	Bantul, Yogyakarta	Chili pepper	6Btl-pep-PepYLCIV (OP846596)	–	6Btl-pep-ToLCJVB (OP846596)
14.	3Mgg-pep	Magelang, Central Java	Chili pepper	3Mgg-pep-PepYLCIV (OP846605)	–	3Mgg-pep-ToLCJVB (OP846597)

*ToLCNDV – tomato leaf curl New Delhi virus; PepYLCIV – pepper yellow leaf curl Indonesia virus; MYMIV – mungbean yellow mosaic India virus; AYVCNV – ageratum yellow vein China virus; AYVA – ageratum yellow vein alphasatellite; ToLCJVB – tomato leaf curl Java betasatellite

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isolates, indicating high genetic variation in Indonesian isolates. The five new Indonesian PepYLCIV isolates showed close relationships with other Indonesian isolates available in GenBank. However, the two isolates from Bantul and two isolates from Sleman districts of Yogyakarta province were located in a cluster that was separated by one isolate (3Mggpep-PepYLCIV) from the Magelang district of Central Java province, suggesting that the isolates, even those from relatively close sampling locations, have high diversity (Figure 1).

The two Indonesian ToLCNDV isolates shared a basal node with two other Indonesian isolates and one Thai isolate, showing that they were genetically closer to several isolates from Southeast Asia than those from South Asia (Bangladesh, India, and Pakistan). In addition, the only new MYMIV isolate was shown to be closely related to two other Indonesian isolates from legumes: soybean (*Glycine max*) and yardlong bean (*Vigna unguiculata* ssp. *sesquipedalis*) (Figure 1).

Currently, the molecular data on AYVA available in GenBank are very limited. The only six AYVA isolates with full genomes included three from Nepal and three from Pakistan. These isolates formed two separate clusters based on their respective origin countries. The only Indonesian AYVA isolate in this study was shown to have a closer relationship with Nepalese isolates than with Pakistani isolates, as shown by full genome and Rep gene comparisons (Figure 2).

Only three ToLCJaB isolates with complete genomes were found in GenBank prior to this study. Seven new Indonesian isolates were in the same cluster as another one from Billygoat Weed (NC_005497). The remaining two new Indonesian isolates were from spiny amaranth and clustered with isolates from Nepal (KC282642) and Japan (KP777735) (Figure 3).

Betasatellite population structure. The only complementary-sense ORF in the ToLCJaB genome encodes the βC1 protein, which actively participates in disease pathogenicity. Therefore, performing diversity and demographic analyses on this region and comparing the results with the findings on other beta satellites are of interest. The 357 nt-long βC1 gene was estimated to be highly conserved, as demonstrated by its high value of H_d and low values of other parameters (S, η, k, and π). Interestingly, the coding region had dN/dS > 1, indicative of positive selection constraints (Table 2). The three neutrality test methods assigned negative values to the overall and group 1 populations but provided positive values to the group 2 population (Table 2). This result showed that different selection patterns acted on each phylogroup.

The comparison between groups 1 and 2 generated high values of K_S = 2.6624, K_{ST} = 0.1496, Z = 2.7391, and S_{nn} = 0.9167 with significant statistical support. Additionally, the result of F_{ST} = 0.4202 implied a rather large genetic separation between the two populations.

DISCUSSION

The ToLCNDV, MYMIV, PepYLCIV, and AYVCNV viruses have been molecularly detected in different main crops cultivated in several provinces of Indonesia (Wilisiani et al. 2019; Lestari et al. 2022). The current study presented the first report of AYVCNV infection in two weeds commonly found in Indonesia: spiny amaranth (*Amaranthus spinosus*) and billygoat weed (*Ageratum conyzoides*). The weeds' role as reservoir hosts of begomoviruses was highlighted once more in this study.

An AYVCNV *A. conyzoides* isolate (1Btl-bgw-AYVCNV) was found to be associated with a ToLCJaB isolate (1Btl-bgw-ToLCJVB) and the only alpha satellite isolate (1Btl-bgw-AYVA) detected in this study. This work reported AYVA in Indonesia and detected ToLCJaB in chili pepper,

Table 2. Summary of genetic diversity, polymorphism analysis, and demography test statistics of the βC1 gene (357 nt) of tomato leaf curl Java beta satellite from two phylogroups

Phylogroups	N	h	H_d	S	η	k	π	dS	dN	ω	Fu and Li's D*	Fu and Li's F*	Tajima's D
All isolates	12	11	0.985	87	96	27.955	0.0783	0.0592	0.0828	1.3986	–0.64074 ns	–0.70723 ns	–0.56192 ns
Group 1	8	8	1.000	48	52	16.464	0.0461	0.0392	0.0478	1.2194	–1.01166 ns	–1.11619 ns	–0.96415 ns
Group 2	4	3	0.833	49	50	27.667	0.0775	0.0505	0.0841	1.6653	0.21395 ns	0.21492 ns	0.15034 ns

N – number of isolates; *h* – number of haplotypes; *Hd* – haplotype diversity; *S* – number of variable sites; η – total number of mutations; *k* – average number of nt differences between sequences; π – nt diversity (per site); dN – nonsynonymous nt diversity; dS – synonymous nt diversity; ω – dN/dS; ns – not significant

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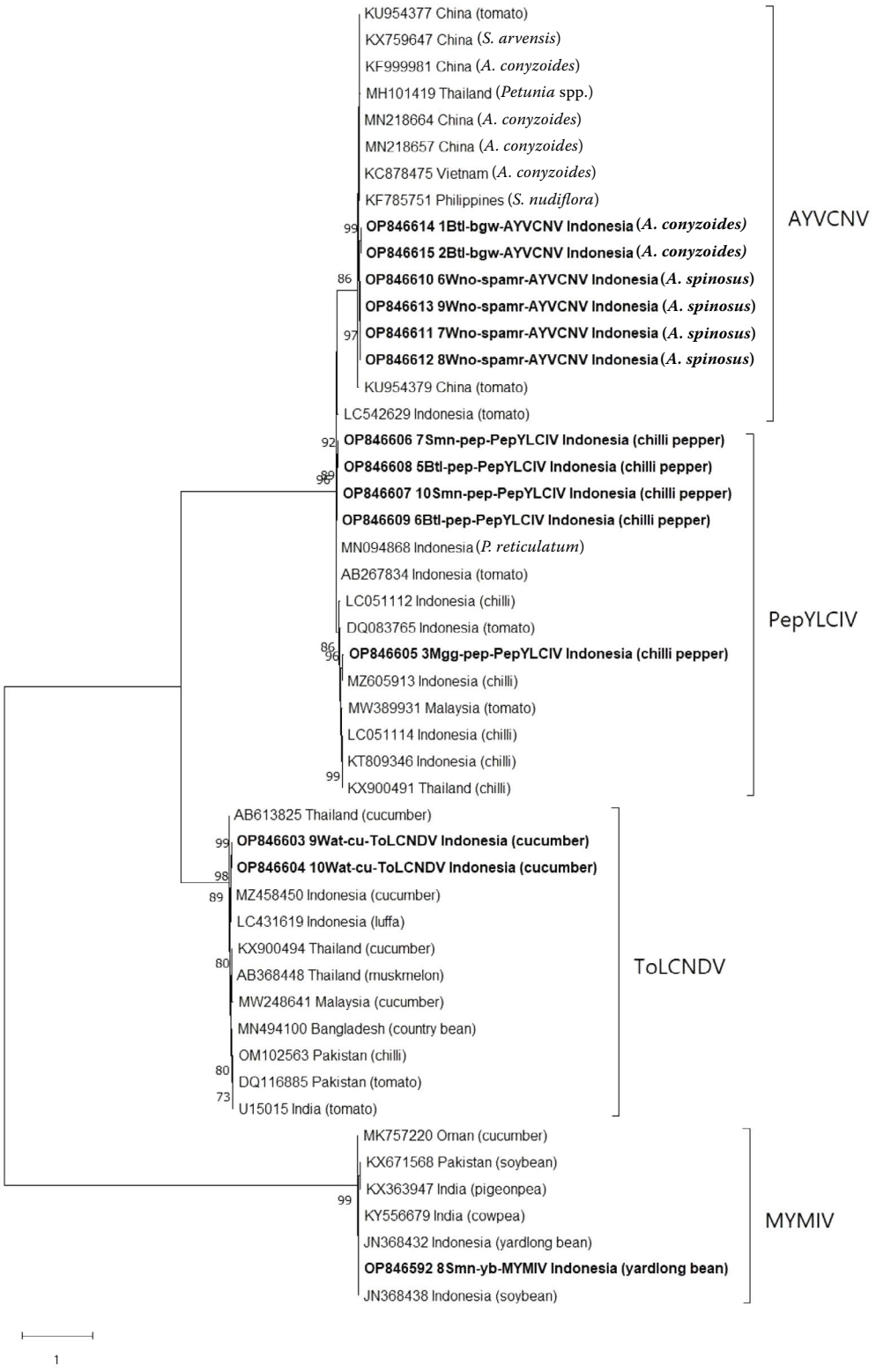

Figure 1. Phylogenetic tree based on the partial nt sequence of the AV1 gene (552 nts) of four Begomovirus species: AYVCNV, PepYLCIV, ToLCNDV, and MYMIV

This tree was generated by using the maximum likelihood method with the Tamura-Nei parameter model and uniform rates among sites implemented in MEGA X. Branching confidence was tested by using 1 000 bootstrap replicates. Only values > 70% are shown. New Indonesian isolates are presented in bold

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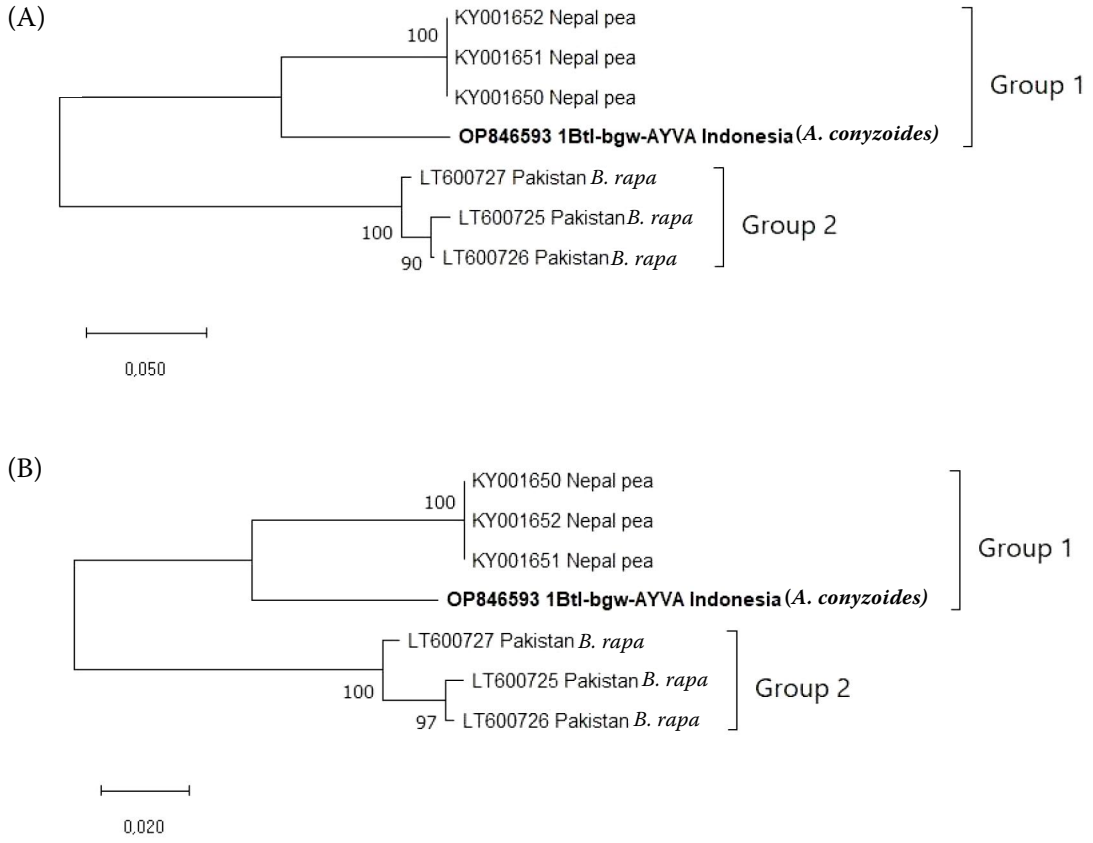

Figure 2. Phylogenetic trees based on the nt sequences of (A) the full genome and (B) the Rep gene (948 nt) of AYVA generated by using the maximum likelihood method with the Tamura-Nei parameter model and uniform rates among sites implemented in MEGA X

Branching confidence was tested by using 1 000 bootstrap replicates, and only values > 50% were shown. New Indonesian isolate is printed in bold

yardlong bean, *A. spinosus*, and *A. conyzoides* for the first time in Indonesia. ToLCJaB was widespread in the surveyed districts and associated with MYMIV, PepYLCIV, and AYVCNV in different crops and weeds. According to a previous study in Japan, ToLCJaB was identified in a tomato plant harboring ageratum yellow vein virus (Shahid et al. 2014).

Phylogenetic analysis based on the complete genome sequence of the seven isolates revealed the existence of two main AYVA lineages, which were separated based on their geographic locations: Nepal and Pakistan. The Indonesian isolate had relatively large genetic differences from the three Nepalese isolates but was closer to the Nepalese cluster than the Pakistani one (Figure 2). Similarly, the phylogeny of ToLCJaB based on the nt sequence of either the full genome or βC1 gene consisted of two lineages. Interestingly, two ToLCJaB *A. spinosus* isolates were in group 2 and were separate from the other seven Indonesian isolates clustered in group 1 (Figure 3). Thus, the phylogrouping could be more related to hosts than the origins.

The βC1 coding region of the beta satellite genome is highly conserved (Saunders et al. 2004), and the diversity analysis performed in the current study confirmed low genetic variation in the βC1 gene of ToLCJaB. Interestingly, the DnaSP analysis estimated that the βC1 gene has an evolutionary rate > 1, indicating that it is undergoing diversification under positive selection pressure. Indeed, the three neutrality tests provided negative values for the overall population and group 1, indicating that these two populations are expanding due to population growth without further subdivision.

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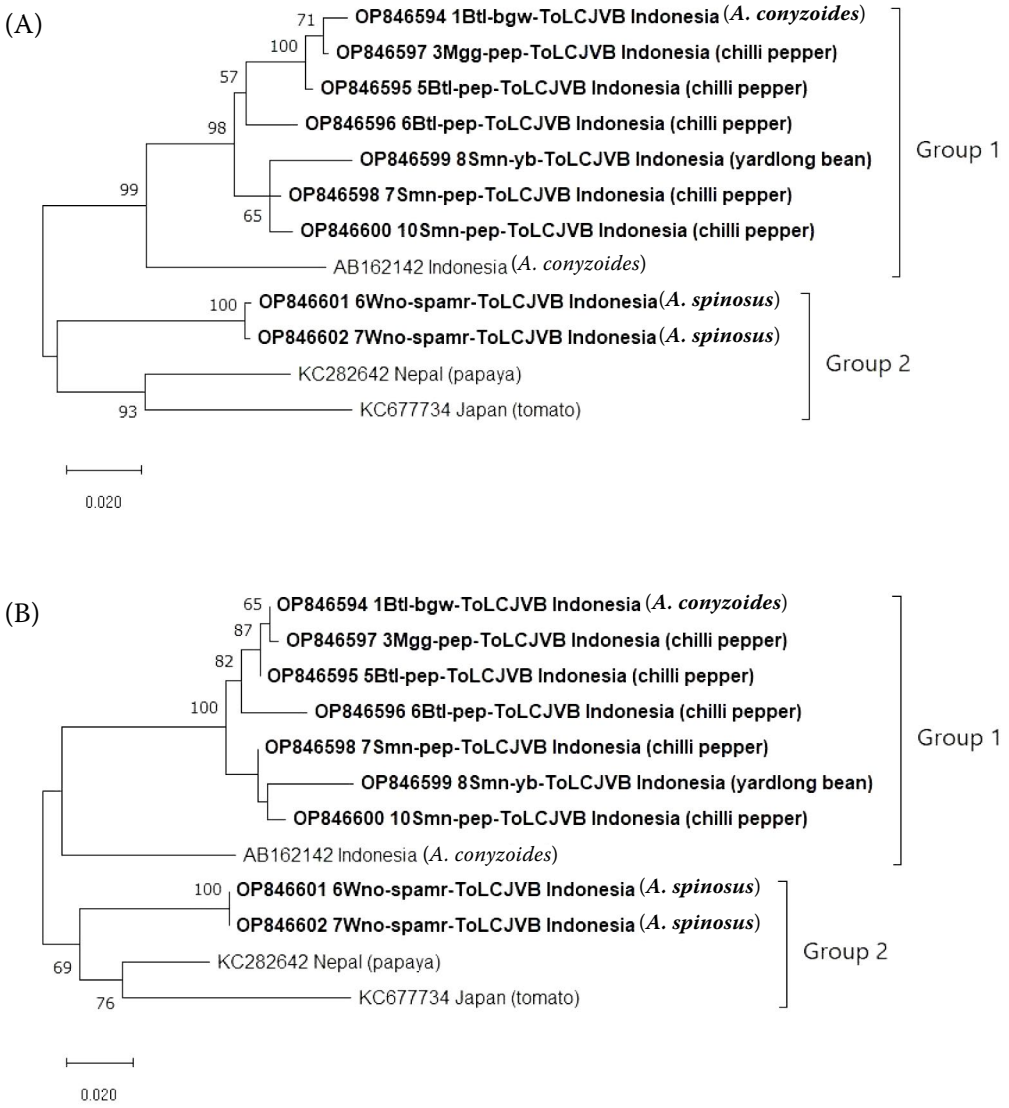

Figure 3. Phylogenetic trees based on the nt sequences of the (A) full genome and (B) beta C1 gene (357 nts) of ToLCJaB generated by using the maximum likelihood method with the Tamura-Nei parameter model and uniform rates among sites implemented in MEGA X

Branching confidence was tested by using 1 000 bootstrap replicates, and only values > 50% are shown. New Indonesian isolates are presented in bold

Therefore, other evolutionary forces might retain the high identities of the βC1 region because, contrary to group 1, group 2 consistently presented positive neutrality values.

The high values of the four genetic separation parameters and high F_{ST} value demonstrated minimal "gene flow" and increased genetic isolation between groups 1 and 2. These results supported the phylogenetic tree clustering of the isolates and suggested that the short and conserved βC1 gene is sufficient to resolve the phylogeny of ToLCJaB. These promising findings from relatively small areas could incite further studies to uncover additional diverse begomovirus satellites in Indonesia and other countries.

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