

## Detection and Sequence Diversity of Begomovirus Associated with Yellow Leaf Curl Disease of Pepper (*Capsicum annuum*) in West Sumatra, Indonesia

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Yellow leaf curl disease of pepper has become an emerging important disease in West Sumatra since early 2000. Several attempts have been made, including disease survey and detection, in order to identify the causal agent of the disease. Pepper (*Capsicum annuum*) plants showing yellow leaf curl from West Sumatra were analyzed for presence of Begomovirus employing Polymerase Chain Reaction (PCR) with degenerate primers pAL1v 1978 and pARc 715. A DNA fragment of 1.6 kb was successfully amplified and subjected to direct sequencing. A stem loop region was found in the nucleotide sequence obtained, which contains the conserved nucleotide signature sequence TAATATTAC present in begomoviruses. Based on the stem loop region comparisons and phylogenetic analysis, the virus isolates from West Sumatra showed the closest relationship to *Pepper yellow leaf curl Indonesia virus* (PYLVIV) and *Tomato yellow leaf curl Indonesia virus* (TYLCIV). The sequence was different from other Asia Begomoviruses reported earlier. These isolates were divided into three groups which were tentatively called *Pepper yellow leaf curl Indonesia virus*-West Sumatra-[group 1], -[group 2] and -[group 3] {PYLCIV-WS-[group1], -[group2], and -[group3]}.

Key words: begomovirus, pepper yellow leaf curl virus, sequence analysis

Begomovirus is a member of family Geminiviridae, which is largest group of plant viruses. The geminivirus is a group of plant viruses with distinct morphological characteristics. It's twinned isometric particles consist of circular single-stranded (ss) DNA genomes (Gutierrez 2000). Geminiviruses are subdivided into four genera on the basis of host range, insect vector and genome organization. The genus Begomovirus (sub group III) consists of viruses that are transmitted by whiteflies to infect dicotyledonous plants with a monopartite or bipartite arrangement (Fauquet and Stanley 2003). Begomovirus associated with pepper yellow leaf curl disease has become a major problem chillie pepper growing and causes crop damage in many tropical and subtropical regions worldwide (Varma and Malathi 2003). In Indonesia, a virus infection causing yellow mosaic, upward leaf curling and stunting was observed infecting pepper throughout production area in West Java, Central Java and Jogjakarta. Begomovirus-association with this disease was confirmed using polymerase chain reaction (PCR) method, and the virus was tentatively named *pepper yellow leaf curl virus* (PYLCV) (Sulandari 2004). Early studies conducted by Rusli *et al.* (1999) and Sulandari (2004) showed that PYLCV was not either seed or mechanically-transmitted, although the virus was easily transmitted through grafting and whitefly, *Bemisia tabaci* Genn. (Hemiptera: Aleyrodidae).

Characterization of a partial genomic sequence of the PYLCV was reported later (Sukanto *et al.* 2005; Hidayat *et al.* 2006). The begomovirus associated with yellow leaf curl disease in pepper in West Java (accession number AB246170) showed the highest sequence identity of 93 and 98% with

*pepper yellow leaf curl Indonesia virus* (PYLCIV) from *Capsicum annuum* (AB189845) and PYLCIV from *Lycopersicon esculentum* (AB189850), respectively.

Occurrence of pepper yellow leaf curl disease was reported from almost all pepper growing areas in Indonesia (Directorate of Plant Protection, personal communication), including West Sumatra. A preliminary survey in several regions in West Sumatra showed that disease incidence could reach 100 % (Syaiful 2005). In this paper, we reported sequence analysis of the intergenic region isolated from eleven begomovirus isolates infecting pepper plants collected from different location in West Sumatra.

### MATERIALS AND METHODS

**Sample Collection.** Pepper plants showing typical symptoms of begomovirus infection (yellow mosaic, leaf curling, smaller leaflet, cupping and stunting) were collected from several pepper producing areas (18 areas, 6 district) in West Sumatra (Fig 1). Observation was conducted directly when the plants were at the flowering stage in the field with 300-500 m<sup>2</sup> area. Samples were taken with purposive-random-sampling according to the typical begomovirus symptom. Five samples were taken for each location that represent each typical symptom, thus the number of samples was 90. Samples were placed in plastic bags and carried to the laboratory for DNA extraction and to a greenhouse for virus isolation and propagation in the host plant. At the same time, disease incidence (%) was determined by counting the number of plants that showed the symptom from 10% of the plant population. The sample plants were determined according to systematic random sampling. However, disease severity was determined by using formula 1, with a modified scale of category according to Lapidot *et al.* (2001). Symptom

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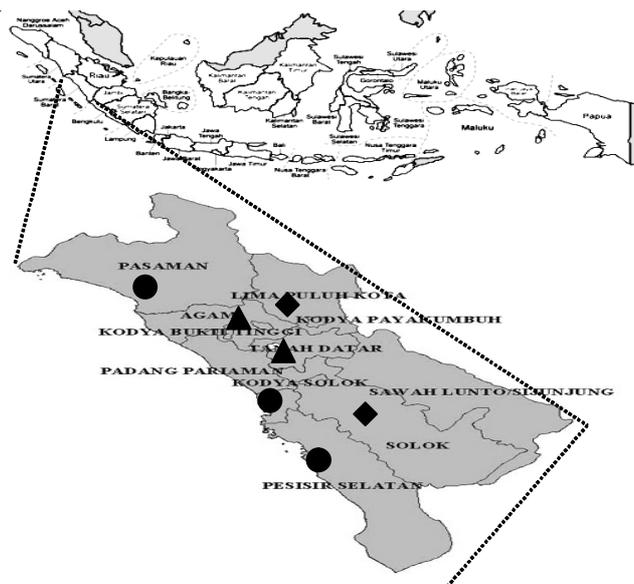


Fig 1 Sampling location in West Sumatra to collect pepper-infected begomovirus. ●, low altitude (< 400 m: Pasaman, Padang, Pesisir Selatan); ■, medium altitude (400-700 m: Solok, Limapuluh Kota); ▲, high altitude (>700 m: Tanah Datar, Agam).

development was evaluated according to the following scale: 0, no visible symptoms; 1, very slight yellowing of leaflet margin on apical leaf; 2, some yellowing and minor curling of leaflet ends; 3, a wide range of leaf yellowing, curling and cupping, with some reduction in size, yet plants continue to develop; and 4, very severe plant stunting and yellowing, pronounced leaf cupping and curling, plant growth stopped.

$$\text{Disease severity} = \frac{\sum(n_i \times V_i)}{Z \times N} \times 100\% \dots\dots\dots(1)$$

$n_i$ , sample plant in  $i$ ;  $V_i$ , value of symptom scale in  $i$ ;  $Z$ , value of highest symptom scale;  $N$ , number of observed sample plants

**DNA Extraction and PCR Analysis.** Total DNA was extracted from pepper leaves according to the method of Doyle and Doyle (1999) with slight modification. Leaf tissue was ground in a sterile mortar in 1.0 mL of extraction buffer. The extraction buffer used for the initial homogenization contained 100 mM Tris pH 8.0, 1.4 mM NaCl, 20 mM EDTA pH 8.0 and 0.2% (v/v)  $\beta$ -mercaptoethanol. The extraction buffer was autoclaved and 2% (v/v) polyvinylpyrrolidone (PVP) and 2% (w/v) CTAB were added immediately before use. Immediately after grinding, 500  $\mu$ L aliquotes were

transferred to a 1.5 mL microtube and incubated for 15 min at 65 °C with occasional mixing to avoid aggregation of the homogenate. To the extract was added 500  $\mu$ L of chloroform:isoamylalcohol (24:1) and the mixture was vortexed thoroughly. Each tube was then centrifuged for 15 min at 10 000 x g. The debris-free supernatant was then transferred to a new tube and proteins precipitated by adding 2.5 x volumes of absolute ethanol and washed twice with 70% ethanol (v/v). The pellet was dried and resuspended in 100  $\mu$ L of sterilized water. This DNA extract was stored at -20 °C for further use.

The begomovirus genome was amplified by the PCR technique using two oligonucleotide degenerate primers for the geminivirus, pALv1978 (5'-GCATCTGCAGGCCCACTYGTCTTYCCNGT-3') and pARc715(5'-GATTTCTGCA GTT DATRITTYTCRTCCATCCA-3') (Rojas *et al.* 1993). PCR reactions were prepared in 25  $\mu$ L total volume, containing 10 x buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), dNTP mix (4 mM), 10 mM of each primer, 1 unit of Taq DNA polymerase and 2 ml of the DNA template. The amplification profile should be referred to Rojas *et al.* (1993).

**Nucleotide Sequencing and Phylogenetic Analysis.** The product of the PCR amplification, approximately 1.6 kb length, was sequenced by the dideoxy-nucleotide-chain termination method with ABI-Prims 3100-Avant Genetic Analyzer (Applied Biosystem, Foster City, CA, USA) at PT. Charoen Pokphand Indonesia, Tbk, Jakarta. Sequence data obtained was compared with those of other geminiviruses available in GeneBank using The National Center of Biotechnology Information basic local alignment search tool or NCBI BLAS (<http://www.ncbi.nlm.nih.gov/BLAST>). The sequences were aligned using Clustal W, while phylogenetic analysis was conducted using online tool facilities available at <http://www.genebee.msu.su/clustal/advaned.htm>1. Results of the analysis were used to construct a phylogenetic tree and the robustness of the internal branches of the tree was tested by bootstrap analysis using 100 replicates.

**RESULTS**

**Disease Symptoms.** Our survey was conducted in 18 pepper-producing areas covering 6 districts in West Sumatra from May to October 2007 (Fig 1). It was evident that the *pepper yellow leaf curl disease* occurred in all the surveyed regions, with the disease incidence ranging from 37.8 to 97.0%. Infection of the virus may cause various symptoms including yellowing, mosaic, curling, cupping and stunting.

Table 1 Isolate identity, observed symptoms on collected pepper samples and disease incidence of *pepper yellow leaf curl disease* in West Sumatra, Indonesia

Location (district)	Isolate identity	Observed symptoms on collected samples	Disease incidence (%)
Pesisir Selatan	PepPSS1-1	y, scr, scp	53.0-80.0
	PepPSS2-3	m, scr	
Pasaman Barat	PepPBST2-3	y, cr, cp, st	92.5-97.0
	Solok	PepSo2-1	
Limapuluh Kota	PepSo3-5	m, cr, cp, sl, st	63.3-81.7
	PepPYK1	y, scr, cp	
Tanah Datar	PepTD1-3	m, sc	37.8-71.7
	PepTD2-1	y, cr,cp, sl, st	
Agam	PepAg1-4	m, scr, cp, sl	86.7-95.0
	PepAg2-1	m, cr, scp, st	
	PepAg2-4	m, cr, cp, sl, st	

y, yellowing; scr, severe curling; cr, curling; scp, severe cupping; cp, cupping; m, mosaic; st, stunting; sl, smaller leaf.

Symptoms variability was found for each region (Table 1). Five samples were taken from each of the 18 locations, with different and varied symptoms, so that the total samples used was. Therefore, one isolate was selected for each symptom type for further detection and identification.

**Detection of Begomovirus by PCR and Sequence Characterization.** Specific DNA fragments of 1600 bp were successfully amplified from 90 leaf samples using geminivirus degenerate primers pALv1978 and pARc715 (Fig 2). The amplified DNA fragment, denoted as “top fragment”, include part of replicase gene, the full common region and part of the coat protein gene. Eleven isolates from 90 samples representing six different districts with specific symptoms were subjected to direct sequencing. These isolates consisted of two samples from Pesisir Selatan (*PepPSS1-1* and *PepPSS2-3*), one sample from Pasaman Barat (*PepPSBT-1*), two samples from Solok (*PepSo2-1* and *PepSo3-5*), one sample from Limapuluh Kota (*PepPY-1*), two samples from Tanah Datar (*PepTD1-3* and *PepTD2-1*) and three samples from Agam (*PepAg2-1*, *PepAg2-4* and *PepAg1-4*) (Table 1).

The sequence of the “top fragment” contains the nonanucleotide sequence 5'-TAATATTAC-3' as a part of a hairpin loop structure, and repetitive sequences known as an iteron. In his study, the hairpin loop structure was found in all sequence of begomovirus samples from West Sumatra. Variability in the structure and the length of hairpin loop region were observed among West Sumatran samples with only three samples having similar structure, i.e. *PepPYK1*, *PepAg2-1* dan *PepAg2-4* (Fig 3). The shortest hairpin loop region was 27 bases (*PepSo3-5*) whereas the longest was 33 bases (*PepTD1-3* and *PepAg1-4*). The isolates with similar

hairpin loop length, contain different structure of GC and AT pairs. Similar repetitive sequence 5'-GGAGACA-3' was found in all of the samples although their relative position to common region varies (Table 2).

**Phylogenetic Analysis.** Relationship between isolates of begomovirus from West Sumatra and other selected begomovirus in GeneBank (Table 3) was evaluated based on “top fragment” sequences. Eleven begomovirus isolates determined in this research could be differentiated into three groups (Fig 4). The first group consisted of 6 isolates (*PepTD1-3*, *PepAg1-4*, *PepSO3-5*, *PepAG2-4*, *PepAG2-1* and *PepPSS1-1*). The second group consisted of 4 isolates (*PepPBST2-3*, *PepTD2-1*, *PepPSS2-3* and *PepSO2-1*), where as the third group consisted of a one isolate, i.e *PepPYK1*. It is interesting to notice that all isolates collected from Agam (*PepAG2-1*, *PepAG2-4* and *PepAG1-4*) belong to the same group 1, where as other isolates collected from same location belong to different group, for instance *PepTD1-3* and *PepTD 2-1*, *PepPSS1-1* and *PepPSS2-3*, *PepSO3-5* and *PepSO2-1*. Begomovirus infecting pepper in Indonesia that have been reported earlier (*PYLCIV-Bgr*, *PYLCIV-To*, *PYLCIV-Lbg*, and *PYLCIV-ageratum*) was not belong in the same group with the begomovirus identified in the present research. However, they are closely related with group 2 of the begomovirus identified from West Sumatra.

In general begomovirus isolates from West Sumatra were tentatively called *pepper yellow leaf curl Indonesia virus-West Sumatera*-[group1], -[group2] and -[group3] {*PYLCIV-WS*-[group1], -[group2] and -[group3]}, although sequence diversity occurred among eleven isolates from West Sumatra.

**DISCUSSION**

Pepper yellow leaf curl disease is considered an important new emerging disease in Indonesia which causes significant damage to plants. Association of begomovirus with the disease was first reported in 1999 in West Java, but by early 2003 the disease ha been found in almost all pepper growing areas in Java (Sulandari 2004). In the same time, Syaiful (2005) reported the incidence of the disease in West Sumatra, and since 2005 epidemics occurred in pepper causing up to 100% disease incidence (Trisno *et al.* 2005). The type of symptoms associated with the disease in West Sumatra is different from

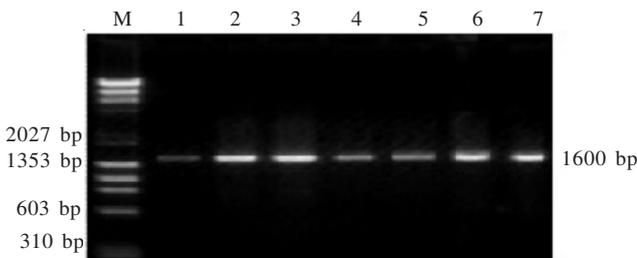


Fig 2 Agarose gel electrophoregram of DNA amplified by PCR using degenerate primers PAL1v 1978 dan PAR1c 715. M, marker (1kb Ladder), Lanes 1-7: begomovirus infecting pepper.

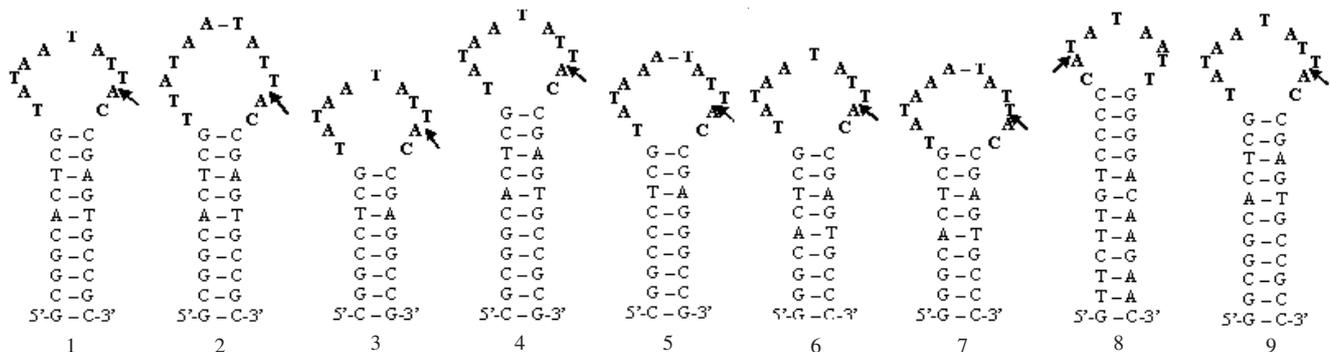


Fig 3 Comparison of the hairpin loop structure of pepper begomovirus. 1, the 31 base hairpin-loop region in *PepPYK1*, *PepAgam2-1*, *PepAgam2-4*, and *PepYLCIDV*; 2, the 32 base hairpin-loop region in *PepSolok2-1*; 3, the 27 base hairpin-loop region in *PepSolok3-5*; 4, the 33 base hairpin-loop region in *PepT.datar1-3*; 5, the 29 base hairpin-loop region in *PepT.datar 2-1*; 6, the 29 base hairpin-loop region in *PepPSS1-1*; 7, the 30 base hairpin-loop region in *PepPSS2-3*; 8, the 30 base hairpin-loop region in *PepPasBt 1*; 9, the 33 base hairpin-loop region in *PepAgam1-4*. The conserved nonanucleotide sequence TAATATTAC is in bold, and the nick site is marked by an arrow.

Table 2 Comparison of iteron upstream of the C1 gene TATA Box in 11 begomovirus isolates from West Sumatra with PepYLCIV-pepper yellow leaf curl Indonesia virus (AB246170.1), TLCIV-tomato leaf curl Indonesia virus (AB100304) and TLCJAV-tomato leaf curl Java virus (AB162141)

Isolate	Iteron (5'-3')	Position <sup>x</sup>
PepYLCIV <sup>y</sup>	GGAGACA	-86 s/d -80, -93 s/d-87, -122 s/d-116
TLCIV <sup>y</sup>	GGAGACA	-134 s/d -128, -196 s/d -190
TLCJAV <sup>y</sup>	GGGTCTCAA	-102 to -94, -137 to -129
PepPYK1	GGAGACA	-82 s/d -76, -89 s/d-83, -118 s/d-112
PepSo2-1	GGAGACA	-89 s/d -83, -97 s/d-91, -125 s/d-119
PepSo3-5	GGAGACA	-84 s/d -78, -91 s/d-85, -119 s/d-113
PepTD1-3	GGAGACA	-87 s/d -81, -94 s/d-88, -123 s/d-117
PepTD2-1	GGAGACA	-83 s/d -77, -90 s/d-84, -117 s/d-111
PepPSS1-1	GGAGACA	-88 s/d -82, -95 s/d-89, -122 s/d-116
PepPSS2-3	GGAGACA	-85 s/d -79, -93 s/d-87, -121 s/d-115
PepPBST2-3	- <sup>z</sup>	-
PepAg1-4	GGAGACA	-90 s/d -84, -97 s/d-91, -125 s/d-119
PepAg2-1, PepAg2-4	GGAGACA	-89 s/d -83, -96 s/d-90, -124 s/d-118

<sup>x</sup>Nucleotide numbering starts from the A adjacent to the nicking site within the conserved nonanucleotide (TAATATT↓AC), <sup>y</sup>Database GeneBank, <sup>z</sup>Nona data.

Table 3 List of geminivirus used for phylogenetic analysis (Database Genebank)

Genebank Accession Number	Organism	Nukleotide's length (bp)	Geography origin	Host plant	Acronim
AB246170.1	pepper yellow leaf curl Indonesia virus	2760	Indonesia, Bogor	<i>C. annuum</i>	PYLCVI-Co
AB267838	pepper yellow leaf curl Indonesia virus	2760	Indonesia, Bogor	<i>Ageratum conyzoides</i>	PYLCVI-Ag
AB189845	pepper yellow leaf curl Indonesia virus	1563	Indonesia, Bandung, Lembang	<i>L. esculentum</i>	PYLCVI-T2
DQ083764	pepper yellow leaf curl Indonesia virus-Bogor	2743	Indonesia, Bogor	<i>Capsicum annuum</i>	TYLCVI-Bgr
NC_008267	tomato yellow leaf curl Indonesia virus-Lembang	2762	Indonesia, Bandung, Lembang	<i>L. esculentum</i>	TYLCVI-Lbg
AF189018	tomato yellow leaf curl Indonesia virus-Lembang	2762	Indonesia, Bandung, Lembang	<i>L. esculentum</i>	TYLCVI-LbA
NC_004005	tomato yellow leaf curl virus	2781	Almeria, Spain	<i>L. esculentum</i>	TYLCV
NC_005347.1	pepper yellow vein mali virus	2786	Mali	-	PYLVMiV
EU585781.1	pepper leaf curl Yunnan virus	2747	China	<i>C. annuum</i>	PLCYuV
EU487040	tomato leaf curl philippines virus	2755	Philippines, Batanga	<i>L. esculentum</i>	TLCPhiV
AB162141	tomato lea curl Java virus	1562	Indonesia, Magelang	<i>L. esculentum</i>	TLCJAV
AF327436.1	tomato leaf curl Malaysia virus	2754	Malaysia, Klang	<i>L. esculentum</i>	TLCVMal
DQ116884.1	tomato leaf curl Pakistan virus	2759	Pakistan	<i>L. esculentum</i>	TLCPaV
DQ641697.1	tomato leaf curl virus Vietnam	2745	Vietnam, Hanoi	<i>L. esculentum</i>	TLCVit
NC_000869.1	tomato yellow leaf curl China virus	2734	China	<i>L. esculentum</i>	TYLCVCi
EU249457.1	tomato yellow leaf curl Thailan virus	2743	Thailan	<i>C. annuum</i>	TYLCTaiV
AJ495813.1	ageratum yellow vein China virus	2768	China, Hainan	<i>Ageratum conizoides</i>	AYVCiV
AB306314.1	ageratum yellow vein virus-Ishigaki	2753	Japan, Okinawa	<i>L. esculentum</i>	AYLCVishi
EF544600	ageratum yellow vein Taiwan virus	2753	China Fujian	<i>Ageratum conizoides</i>	AYVTaiV
AB189913.1	ageratum yellow vein virus-Indonesia	1557	Indonesia, Bandung, Lembang	<i>Ageratum conizoides</i>	AYLCIndV
X74516.1	ageratum yellow vein virus	2741	China	<i>Ageratum conizoides</i>	AYVV

those found in Java, which commonly caused bright yellow colouring (Sulandari 2004). The differences in symptoms type of the disease between West Sumatra and Java may be caused by the difference of: (i) the type of cultivar used, where Peper cultivars that are generally planted in West Sumatra is the local cultivar “lado keriting darek” (from direct interview with farmers). Meanwhile, the cultivar that is generally planted in Java are an introduction cultivar, such as TM 999, TM 888 and Jatilaba (Sulandari 2004), (ii) the strain of geminivirus; this research showed the different strains of geminivirus existed between West Sumatra and Java (Fig 4). Idris and Brown (1998) stated that there are different virus strains that cause plant disease with different symptoms. Since high genetic diversity of begomoviruses has been previously reported, it is important to determine the identity of begomovirus infecting pepper crops in West Sumatra.

The identity of begomovirus infecting pepper in West Sumatra was determined based on their hairpin-loop structure and repetitive sequence (iteron) found in the common region. These hairpin loop structure has been found in all geminivirus sequenced so far (Zhou *et al.* 2003) and iteron is known as the specific-binding site of the geminivirus replication-associated proteins (Ribeiro *et al.* 2006). Both the hairpin-loop structure and iteron have been found in all geminivirus sequenced to date and this might be used for analysis of their genetic diversity. It was known that differences in the common region may indicate a different begomovirus strain (Hidayat *et al.* 1999; Chatchawankanphanich and Maxwell 2002; Sukamto *et al.* 2005; Ribeiro *et al.* 2006; Santoso *et al.* 2008). It was found in our present research that three isolates of begomovirus infecting pepper in West Sumatra (PepPYK1-1, PepAg2-1 and PepAg2-4) have a hairpin loop

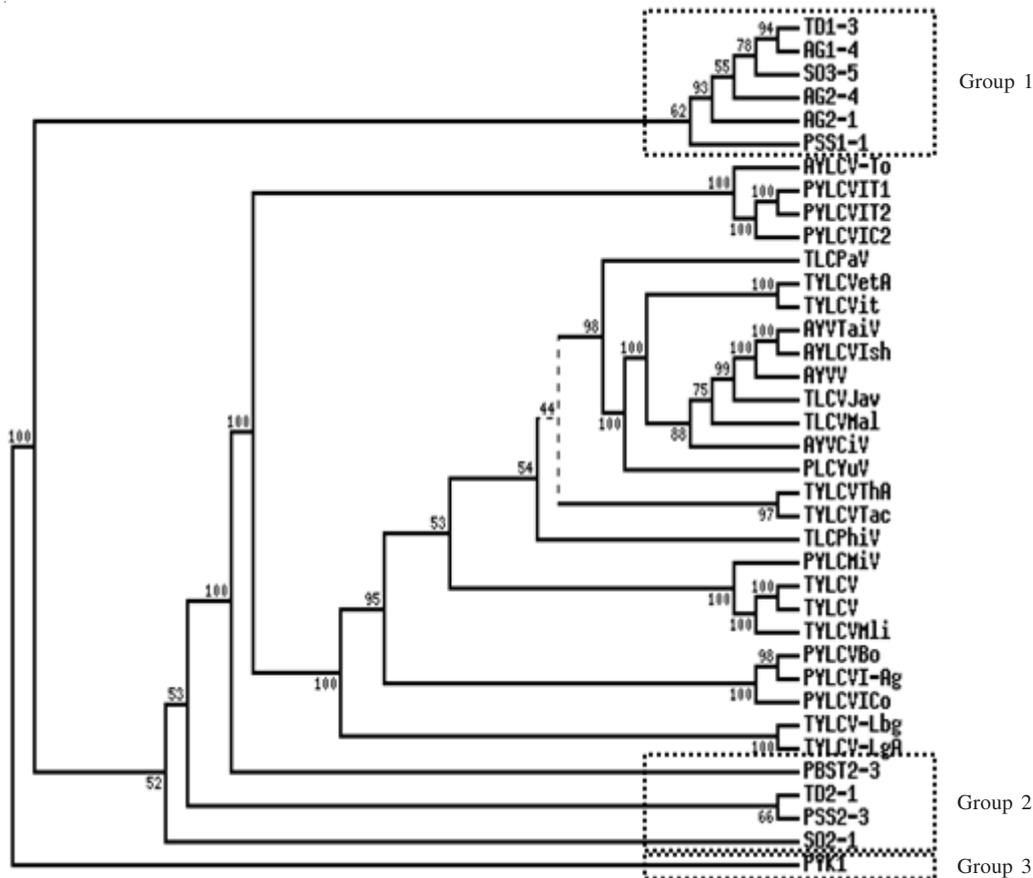


Fig 4 Phylogenetic tree based on the alignments of the common region sequences of pepper-infesting begomovirus from West Sumatra with other selected begomovirus (GeneBank database). Squares ( ) show position of the samples.

structure similar to that of PYLCIV-Bogor (Hidayat *et al.* 2006), whereas the isolates from the rest of West Sumatra have different structures. Repetitive sequences for most isolates of begomovirus infecting pepper in West Sumatra (GGAGACA) were similar with those of ToLCIV (Sukamto *et al.* 2005) and PYLCIV-Bgr (Hidayat *et al.* 2006), but different from those of ToLCJAV (Sukamto *et al.* 2005). Differences were also observed with geminiviruses reported from other countries, for instance *bean golden mosaic virus-Guatemala* (Ikegami *et al.* 1988), *tomato leaf curl Joydebpur virus-Mild* (ToLCJV-Mild), and *tomato leaf curl New Delhi virus-severe* from Jessore (ToLCNDV-Svr[Jes])(Maruthi *et al.* 2005), each with repetitive sequence of TCGGAGTGTCTCCAA, GGTGT, GGAGT, respectively. Variability in hairpin-loop structure and iteron sequences indicates the possibility of genetic diversity among begomoviruses from West Sumatra. Therefore, further analysis of genome sequences is necessary.

Phylogenetic analysis based on the common-region sequences revealed that the West Sumatra isolates of begomovirus infecting pepper can be differentiated into three groups. Sequence similarity ranged from 63 to 91% with selected begomoviruses from Asia (Table 3). Padidam *et al.* (1995) proposed that virus isolates displaying more than 90% sequence identity should be considered as isolates or strains rather than different viruses. In this study, strain grouping did not represent different altitude for sample location, where the group 1 comes from locations sampled at low, medium and high altitudes, i.e. PepPSS1-1, PepSo3-5, PepAg1-4, and PepTD1-3, respectively. We could not make any conclusion

yet regarding the identity of begomovirus from West Sumatra based on analysis of the common-region sequences. Availability of the full genome sequence will provide comprehensive and complete analysis enable such a conclusion. However, there is an indication that genetic variability of begomovirus isolates infecting pepper occurred in West Sumatra and that the viruses are related to begomovirus infecting pepper in Java. This fact should be considered in developing strategies to control pepper yellow leaf curl disease, especially in the development of resistant varieties.

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