MOLECULAR CHARACTERIZATION OF Begomovirus INFECTING YARD LONG BEAN (*Vigna unguiculata* subsp. *sesquipedalis* L.) IN JAVA, INDONESIA

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ABSTRACT

Begomovirus was identified as one of the causal agents associated with yellow mosaic disease on yard long bean (*Vigna unguiculata* subsp. *sesquipedalis* L.) in Java. Previous study reported that *Begomovirus* has infected several Leguminosae in South Asia. Several *Begomoviruses* have been reported to infect important crops in Indonesia. Those *Begomoviruses* were characterized based on nucleotide sequences. This study was conducted to identify and characterize *Begomovirus* taken from yard long bean samples in Java based on specific genome property of common region. Three main activities were conducted: i) sample collection in yard long bean fields located in Central Java (Tegal, Magelang, and Klaten), Yogyakarta (Sleman), and West Java (Bogor and Subang) provinces; ii) virus detection using I-ELISA, PCR, and sequencing; iii) molecular characterization of *Begomovirus* using software BioEdit v.7.0.5 and MEGA 6.06. Yellow mosaic disease was found in almost all fields. Infection of *Potyvirus* and *Begomovirus* was detected using I-ELISA and PCR, respectively. Both viruses were detected as either single or mixed infection. Samples collected from Tegal, Klaten, Magelang, Subang and Bogor were positively infected by *Begomovirus* based on specific viral DNA amplification. Sequence analysis indicated that *Begomovirus* infecting yard long bean is Mungbean Yellow Mosaic India Virus (MYMIV) and it belongs to the same group with MYMIV from Bangladesh, India, Pakistan and Nepal. Further analysis showed the conserved region of *Begomovirus* around Common Region, i.e. "TATA box" sequence, hair pin loop structure, repetitive sequence and the conserved nonanucleotide sequence TAATATTAC were also determined. This is the first report of MYMIV infection in Indonesia.

Keywords: *Begomovirus,* common region, DNA sequencing, I-ELISA, MYMIV, PCR, yard long bean

INTRODUCTION

A yellow mosaic disease outbreak was reported in yard long bean (*Vigna unguiculata* subsp. *sesquipedalis* L.) growing area in Java since 2008 (Damayanti *et al.* 2009). The disease was associated with infection of Bean Common Mosaic Virus (BCMV), Cucumber Mosaic Virus (CMV) and *Begomovirus* (Damayanti *et al.* 2009; Hidayat 2011, *personal communication*; Tsai *et al*. 2013). At the same time, similar yellow mosaic disease caused by *Begomovirus* was first reported from Pakistan (Ilyas *et al.* 2010) and Nepal (Shahid *et al.* 2012).

Begomovirus is a member of Geminivirus group which is transmitted in nature by whitefly *Bemisia tabaci* Gen. (Hemiptera: Aleyrodidae). Molecular characteristics of its genome is very unique, having one or two circular single stranded DNA (2.6–2.7 kb) and represented as monopartite or bipartite, respectively (Hull 2002). Serious crop diseases caused by infection of *Begomovirus* have been reported in Indonesia. Pepper Yellow Leaf Curl Virus (PepYLCV), Tomato Yellow Leaf Curl Virus (TYLCV), and Tobacco Leaf Curl Virus (TLCV) are considered as the major factor causing yield loss on chilli pepper, tomato and tobacco, respectively (Aidawati *et al.* 2005; Hidayat *et al.* 2006; 2008;). Damage and potential yield loss in yard long bean caused by *Begomovirus* should be anticipated by establishing detection method and disease control strategy. To do so, basic information regarding molecular and biological characters of the virus is required.

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Method for detection of *Begomovirus* is commonly based on polymerase chain reaction (PCR) technique using universal primers. Degenarate primers PAL1v1978/ PAR1c715 was first used by Rojas *et al.* (1993) as universal primer to detect several Geminiviruses from infected plants belong to Solanaceae, Leguminosae, Euphorbiaceae, and Malvaceae from South America. Since then, the primer pair has been used to identify and characterize various members of *Begomovirus*. Fragment of *Begomovirus* DNA amplified by this pair of primers covers part of AL1 region (replicase gene), common region and intergenic region, and part of AR1 region (coat protein gene). Molecular characters of *Begomovirus* can be determined by analyzing this DNA fragment sequences due to its conserved and diversed region. Common region for each *Begomovirus* is different, except loop region on hairpin structure, 5'-TAATATTAC-3' which is known as the conserved nonanucleotide region (Lazarowitz 1987). *Begomovirus* possessing >92% similarity on their hairpin loop structure can be grouped as one species (Fauquet *et al.* 2005).

This paper explained the attempts to identify and characterize *Begomovirus* from yard long bean samples in Java based on specific genome property of common region.

MATERIALS AND METHODS

Collection of Field Samples

Samples collection was conducted in West Java (Bogor and Subang), Central Java (Tegal, Klaten and Magelang) and Yogyakarta (Sleman). Leaf samples were collected from the plants age 6–9 WAP using purposive sampling method based on yellow mosaic symptom described by Damayanti *et al.* (2009) and Ilyas *et al.* (2010). Fresh tissue was directly subjected for virus detection and the remaining were stored as isolate collection in the laboratory at -80 °C.

Virus Detection by Indirect Enzyme-linked Immunosorbent Assay (I-ELISA)

Two major viruses on yard long bean i.e. *Potyvirus* and CMV were detected from leaf samples using I-ELISA commercial kit following manufacturer protocol (Leibniz-Institut DSMZ GmbH, Germany). Infected leaves was ground in coating buffer (1/10, v/v). Coating buffer (pH 9.6) containing 15 mM Na_2CO_3 (1.59 g), 2.38 mM $NaHCO_3$ (2.93 g), 3.08 mM NaN_3 (0.20 g), and H_2O was added to final volume of 1 L. Aliquots of each sample (100 µL) was dispensed to each microtitters' well, then incubated overnight at 4 °C. Microtitter plate was washed 8 times using PBS-Tween (0.137 M NaCl (8.0 g), 1.47 mM KH_2PO_4 (0.2 g), 8.1 mM Na_2HPO_4 (1.15 g), 2.68 mM KCl (0.2 g), 3.08 mM NaN_3 (0.2 g), add H_2O up to 1 L pH 7.4 and add 0.5 ml Tween 20/ L). Plates were dried up by tapping upside down on tissue paper. Blocking solution (2% skim milk diluted in PBS-Tween) was added 100 µL to each well then incubated at 37 °C for 30 minutes. Blocking solution was removed and plate was tapped dry. First antibody (IgG) was diluted in conjugate buffer (PBS-Tween containing 2% PVP (Serva PVP-15 polyvinyl pyrrolidon) and 0.2% egg albumin) according to manufacturer recommendation, then 100 µL conjugate buffer contains IgG was added to each well, incubated at 37 °C for 2-4 hours, then washed by PBS-Tween. Second antibody (IgG-AP) was diluted in conjugate buffer according to manufacturer recommendation, then 100 µL conjugate buffer containing IgG-AP was added to each well, incubated at 37 °C for 2 hours then washed using PBS-Tween. Substrate (10 mg p-nitrophenyl phosphate) was dissolved in substrate buffer (97 mL diethanolamine, 600 mL H_2O, 3.08 mM NaN_3 (0.2 g), adjust to pH 9.8 with HCl and make up to 1 L with H_2O) added 100 µL to each well. Plate was incubated in the room temperature under low light intensity and reaction was evaluated. Positive reaction was qualitatively indicated by appearance of yellow color and quantitatively determined by measuring absorbance value at 405 nm wave length, i.e. two times absorbance value of negative control (healthy plant), using ELISA reader (Biorad 550).

Virus Detection by PCR

Total viral DNA was isolated from infected leaf following a procedure described by Doyle and Doyle (1987) with minor modification. Fresh tissue (0.1 g) was ground with liquid Nitrogen to powder, 500 µL of CTAB buffer (10% Cetyl-trimethyl-ammonium bromide (100 mL), 0.1 M Tris-HCl pH 8 (100 mL), 0.05 M EDTA (50 mL), 0.5 M NaCl (126 mL), 1% β-mercapto-ethanol

(10 mL and added H_2O up to 1 L) was added, and the sap was transferred to 1.5 mL clean tube. The sap was incubated in water bath at 65 °C for 1 hour, shaked every 10 minutes to separate lipid and protein. Five hundred microliter of chloroform/iso-amyl alcohol (24:1, v/v) was added to the liquid, then the tube was vortexed for 5 minutes and centrifuged at 14,000 rpm for 15 minutes. The supernatant was pipetted to 1.5 mL clean tube, 1/10 volume of 3 M ammonium acetate and 2/3 volume of isopropanol of was added, respectively. The liquid was mixed gently then incubated overnight at -20 °C or 4 hours at room temperature. After incubation, the liquid was centrifuged at 12,000 rpm for 10 minutes to precipitate DNA and then was discarded flow-through. The pellets were washed with 500 µL of 70% ethanol, centrifuged at 8,000 rpm for 5 minutes and dried in room temperature after discarding the flow through. The dried pellets containing total DNA were dissolved in 50 to 100 µL of nuclease free water or TE buffer, pH 8 and the DNA was ready for PCR.

Amplification of viral DNA was conducted following method described by Rojas *et al.* (1993) to confirm geminivirus infection. PCR reaction contained 10xPCR Buffer, 25mM MgCl, 2.5mM dNTPS, 10µM each of primer PAL1v1978 and PAR1c715, *Taq* polymerase (5U/µL), 1 µL of DNA, and the reaction was adjusted to 25 µL with nuclease free water. Amplifications was performed in GeneAmp PCR System 9700 machine with 5 minutes at 94 °C for pre-heating, followed by 30 cycles of denaturation (1 minute at 94 °C), annealing (1 minute at 50 °C) and extension (3 minutes at 72 °C). The last cycle was followed by 72 °C for 3 minutes and decreased at 4 °C. Agarose gel electrophoresis was used to visualize PCR products.

DNA Sequencing

Viral DNA fragments obtained from direct PCR amplification were sent to PT Genetika Science, Indonesia and Australian Genome Research Facility, Australia, respectively for DNA sequencing. Sequence data were compared with other sequences from GenBank (NCBI 2013) and analysed using software programs BioEdit V.7.0.5, CLC Sequence Viewer 7, and MEGA 6.06.

RESULTS AND DISCUSSION

Identification of Begomovirus from Field Samples

Incidence of yellow mosaic disease was very high i.e. 80% to 100% in most growing areas. Infected plants were easily recognized in the field based on visual symptoms. Three main symptoms of yellow mosaic disease in the field were observed i.e. (1) yellowing; (2) yellowing with green spot; and (3) mosaic vein banding (Fig. 1 and Table 1). The most common symptom found in every field was yellowing. Yellowing with green spot was thought as early symptom before it developed into yellowing. Further severe infection caused smaller pods and leaves. These types of symptom had also been reported as typical symptoms of yellow mosaic disease of yard long bean in South Asia (Ilyas *et al.* 2010). *Begomovirus* was detected in 11 infected plant samples (Fig. 2) showing yellowing and yellowing with green spot symptoms from all locations. However, *Begomovirus* fragment was not successfully amplified from samples showing mosaic vein banding from Subang Viral detection using I-ELISA revealed the infection of *Potyvirus*

Figure 1. Symptoms of yellow mosaic disease on yard long bean: (A) yellowing; (B) yellowing with green spot; (C) mosaic vein banding

Table 1. Detection of Cucumber Mosaic Virus, *Potyvirus* and *Begomovirus* from leaf samples collected in wet season 2012 from various locations using I-ELISA and PCR[a]

Location	Code of Isolates	Symptoms description	I-ELISA[b]		PCR
			CMV	*Potyvirus*	*Begomovirus*
Tegal	Tegal 1	Yellowing	-	+	+
	Tegal 2	Yellowing mosaic	-	-	+
Klaten	Klaten 1	Yellowing	-	-	+
	Klaten 2	Yellowing mosaic	-	-	-
Sleman	Sleman 1	Yellowing	-	+	+
	Sleman2	Yellowing mosaic	-	+	+
Magelang	Magelang 1	Yellowing mosaic	-	-	+
	Magelang 2	Yellowing	-	+	+
	Magelang 3	Yellowing mosaic	-	-	+
Subang	Subang 1	Mosaic vein banding	-	+	-
	Subang 2	Mosaic vein banding	-	+	-
	Subang 3	Mosaic vein banding	-	-	-
	Subang 4	Yellowing	-	-	+
Bogor	Bogor 1	Yellowing	-	-	+
	Bogor 2	Yellowing	-	-	+

[a] Most plants were in generative stage when samples was collected
[b] - = not detected; + = detected

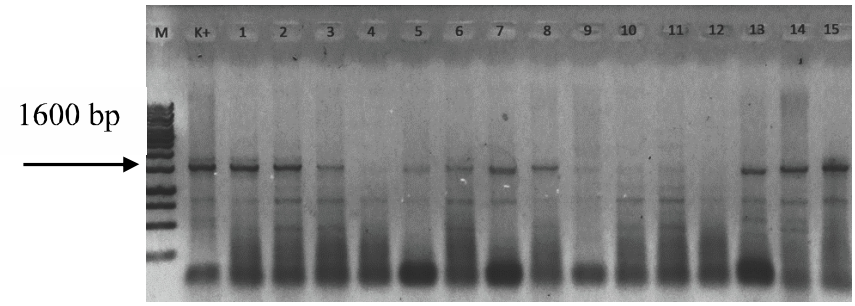

Figure 2. Visualization of *Begomovirus* amplification from leaf samples using universal primers PAL1v1978/ PAR1c715 on 1 % agarose gel. M, 1 kb marker DNA (Thermo Scientific, US); K+, DNA of PepYLCIV; 1-15 leaf samples from fields (1, Tegal 1; 2, Tegal 2; 3, Klaten 1; 4, Klaten 2; 5, Sleman 1; 6, Sleman 2; 7, Magelang 1; 8, Magelang 2; 9, Magelang 3; 10, Subang 1; 11, Subang 2; 12, Subang 3; 13, Subang 4; 14, Bogor 1; 15, Bogor 2)

but not of Cucumber Mosaic Virus (CMV). Mosaic vein banding symptom caused by *Potyvirus* infection had been described previously by Damayanti *et al.* (2009). This result indicated the association of *Begomovirus* with yellow mosaic disease of yard long bean in Java.

Analysis of Sequence Identity of Begomovirus Infecting Yard Long Bean

Nucleotide sequences were obtained for *Begomovirus* isolates from Tegal, Klaten, Magelang, Subang and Bogor. The sequence of isolate from Sleman was not good due to unreadable chromatogram, therefore it was not included in the further sequence analysis. Analysis of their identity by comparing to sequences on the GeneBank showed their highest homology with Mungbean Yellow Mosaic India Virus (MYMIV) from Brebes and Purwakarta, i.e. >92%, followed by MYMIV from Bangladesh, Nepal, Pakistan, and India, i.e. >87% (Table 2). Their homology to MYMV, another virus causing yellow mosaic disease in South Asia, was only 71-76% and to other *Begomovirus* reported from Indonesia was even lower i.e. 51- 54%. Further dendogram analysis to study their relationship showed that all

Table 2. Nucleotide sequence homology (%) of *Begomovirus* infecting yard long bean in Java with other *Begomoviruses* reported earlier in GeneBank

Begomovirus infecting yard long bean	*Begomovirus* isolates from GeneBank[a])											
	1	2	3	4	5	6	7	8	9	10	11	12
pTgl1	99.7	97.9	92.5	91.1	92.2	92.1	76.0	55.8	53.8	51.4	51.5	16.6
Tegal 2	95.7	94.7	89.5	88.3	89.4	89.3	73.1	54.6	53.9	52.5	52.0	17.4
Klaten	96.0	93.2	88.0	86.8	87.9	87.9	71.9	54.1	54.2	52.9	51.9	17.2
Magelang	95.9	92.8	87.7	86.4	87.5	87.5	71.7	53.5	53.8	52.1	51.6	16.8
Magelang 2	95.3	92.8	87.7	86.4	87.5	87.5	71.7	53.5	53.8	52.1	51.6	16.8
Subang	95.5	94.7	89.5	88.3	89.4	89.3	73.0	54.6	54.0	52.5	52.0	17.4
Bogor 1	95.4	94.8	89.6	88.4	89.5	89.4	73.0	54.7	54.1	52.5	52.2	17.4
Bogor 2	95.1	93.8	88.6	87.4	88.5	88.4	72.6	53.7	54.1	52.7	52.1	17.5

[a])1= MYMIV Brebes (JN368436); 2= MYMIV Purwakarta (JN368434); 3= MYMIV India (KC852204); 4= MYMIV Bangladesh (AF314145); 5= MYMIV Nepal (AY271895); 6= MYMIV Pakistan (AM992618); 7= MYMV India (KC911271); 8= PepYLCIV (AB246170); 9= TLCIV (AB241671), 10= ToLCNDV (data unpublished); 11= ToLCJV (189848); 12= BCMV Bogor (FJ653916)

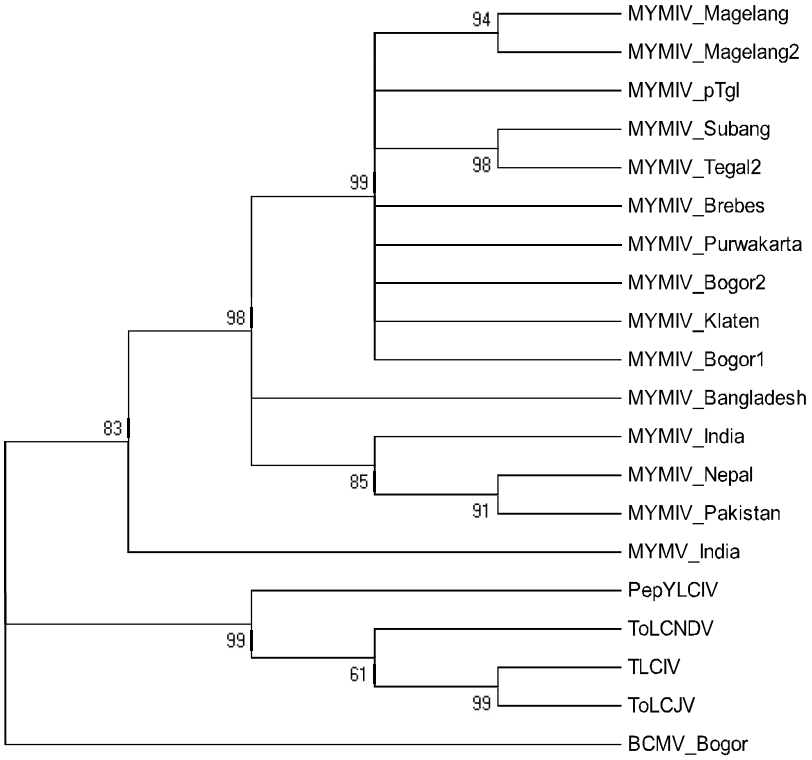

Figure 3. Phylogenetic analysis of Mungbean Yellow Mosaic India Virus based on alignment of partial nucleotide sequences of the DNA-A of *Begomoviruses* using Mega 6.06 (Algorithm Neighbor Joining with 1,000 bootstraps replicates)

Begomovirus infecting mungbean (MYMIV and MYMV) belonged to similar cluster and they were separated from *Begomoviruses* infecting other crops (PepYLCIV, TLCIV, TYLCNDV, ToLCJV) (Fig. 3). It indicated that MYMIV from Java had closer genetic relationship to MYM(I)V from South Asia than other *Begomoviruses* from Indonesia.

Analysis of Common Region Sequences of Begomovirus Infecting Yard Long Bean

Molecular characters of *Begomovirus* could be determined by analyzing the top region of its genome covering common region and intergenic region representing the conserved and diversed region, respectively. Common region for each *Begomovirus* was different, except loop region on

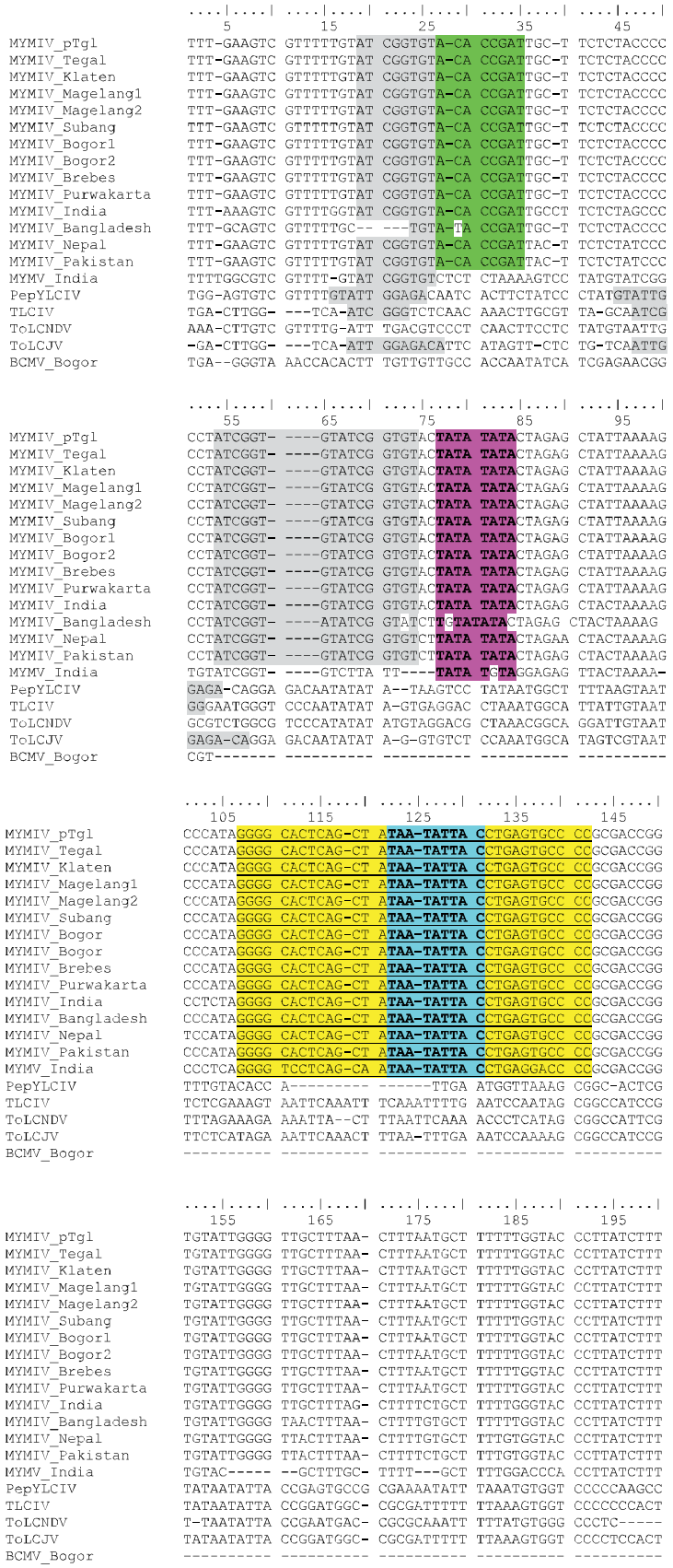

Figure 4. Alignment of nucleotide sequences of the CR of Mungbean Yellow Mosaic India Virus (MVMIV) isolates from Java with other reported MYMIV in Genebank. The alignment showing repetitive sequences (grey shadow), invert repeat (green shadow), TATA sequences (bold letter with purple shadow), and the hairpin loop region (underlined letter with yellow shadow) with nonanucleotide sequence (bold and underlined letter with turquoise shadow)

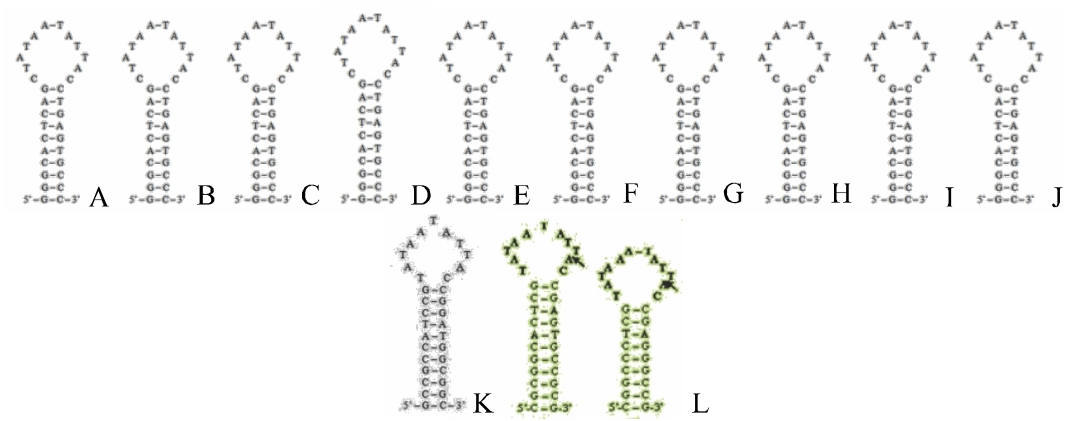

Figure 5. Hairpin loop structures of several *Begomovirus* isolates. A. MYMIV Tegal; B. MYMIV Klaten; C. MYMIV Magelang; D. MYMIV Subang; E. MYMIV Purwakarta; F. MYMIV Bogor; G. MYMIV Bangladesh; H. MYMIV Pakistan; I. MYMIV India; J. MYMIV Nepal; K. TLCIV Indonesia; L. PepYLCIV Indonesia

hairpin structure, 5'-TAATATTAC-3' which is known as the conserved nonanucleotide region (Lazarowitz 1987).

Comparison of iteron and protein products variations have been used to determine of MYMV and MYMIV from several regionals in India (Varma & Malathi 2003; Usharani *et al*. 2004) and TYLCV isolates from Israel, Sardinia, and Thailand (Argüelo-Astorga *et al*. 1994). However, exception was reported on several cases, for example Potato Yellow Mosaic Virus with TYLCV-Sardinia and BGMV-Brazil with TYLCV-Israel have identical iterons although they have a rather far distance relationship (Argüelo-Astorga *et al.* 1994).

Our analysis showed that common region of *Begomovirus* isolates infecting yard long bean in Java consists of repetitive sequences (ATCGGTGT), TATA box, and hair pin loop structure (Fig 4). Three direct repeats (two of them are tandem repeat sequences) can be found before TATA box in all MYMIV and MYMV isolates (Fig 4). Iteron and TATA box sequence always present together in CR of each Begomovirus species (Lazarowitz 1987). The function of iteron and TATA box was described by Argüelo-Astorga *et al.* (1994) as initiator for rolling circle replication which was known as RAP-specific binding sites. Similar characteristics were reported for Eastern Hemisphere geminivirus except ACMV and ICMV, Western Hemisphere geminivirus, and SqLCV-E and –R (Argüelo-Astorga *et al.* 1994), and MYMIV on soybean (Usharani *et al*. 2004).

Hair pin loop structure of each isolates was identical to MYMIV from Bangladesh, Pakistan, India, Nepal, and one isolate of PepYLCIV, but they were different from TLCIV and PepYLCIV (Fig. 5). *Begomovirus* possessing >92% similarity on their hairpin loop structure could be grouped as one species (Fauquet *et al.* 2005; Hidayat *et al*. 2008). Therefore, all *Begomovirus* isolates from this study was the same species with MYMIV from Bangladesh, India, Pakistan, and Nepal.

CONCLUSIONS

Molecular detection and characterization confirmed the association of MYMIV in yellow mosaic disease of yard long bean in Java. Virus isolates from Java had the highest similarity (>85%) with MYMIV isolates from Bangladesh, Pakistan, India, and Nepal. Further study on characters of the virus, such as host range, insect transmission and disease spread, are very important to understand disease development and control.

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