



Research Article

Molecular Identification of *Begomovirus* Infecting Angled Luffa

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ABSTRACT

Begomovirus was reported as one of the most aggressive and destructive viruses on several commercial crops, including cucurbits in Indonesia. Plants that infected with *Begomovirus* show the mosaic symptom on the leaves, change in leaf shape, stunts, change in color and shape of fruit. It was recently observed in cultivated angled luffa [*Luffa acutangula* (L.) Roxb] around Yogyakarta and Central Java. The aim of this research was to identify the virus by using *Polymerase chain reaction* (PCR). The result of *Begomovirus* amplification from the total DNA samples amplification using primer Krusty-Homer showed that DNA of *Begomovirus* from angled luffa was amplified at ~580bp. The DNA sequencing of angled luffa's leaf isolate GD1 had 97.8% homology with SCLV-China isolate MC1. However, amplification of DNA seed samples using the same primer showed negative result. It was concluded that *Begomovirus* was not a seed borne virus. This is the first molecular report on the occurrence of *Begomovirus* in angled luffa in Yogyakarta.

Keywords: *Begomovirus*; *Luffa acutangula*; molecular identification; not seed borne virus

INTRODUCTION

Cucurbits is an important commodity group that produces fruits and vegetables in various areas around Indonesia. Cucurbit producing centrals, such as East Java, Central Java, Jakarta, West Java, and Yogyakarta, contribute to approximately 80% of national production in Indonesia (BSCI, 2003 *cit.* Daryono & Natsuaki, 2009).

One of the challenges in cultivating cucurbit crops are plant diseases caused by viruses that result in mosaic symptoms, leaf distortions, stunting, discoloration and malformed fruit (Julijantono, 2006). Lovisololo (1980) discovered various diseases caused by 32 viruses or viroid that infect cucurbits. Many of those viruses are able to infect many plant species from different genera to families (Zitter *et al.*, 1996). Diseases caused by viruses from the *Geminiviridae* family is a major challenge in increasing production of stable and specialty crops. *Begomovirus* is one of the most important virus that majorly affects plant health and production (Fauquet & Stanley, 2003; King *et al.*, 2012).

Begomovirus owns circular singlestranded DNA (ssDNA) genome that consists of single molecules within the size of 2.7–2.8 kb (monopartite) and

mainly double molecules, DNA-A and DNA-B (bipartite) (Vanitharani *et al.*, 2005 *cit.* Wilisiani *et al.*, 2014). Identification the type of virus and determining the pathogen's characteristics is an essential step that determines the success of effective, safe, and efficient disease management (Wilisiani *et al.*, 2014). Identification using molecular techniques, such as *Polymerase Chain Reaction* (PCR), have been vastly used to identify viruses. PCR technique to identify *Begomovirus* on crops have widely used the Krusty Homer primer that is able to amplify this viruses' coat protein (Revill *et al.*, 2003).

Yellow-curly mosaic virus does not only cause decreases yield, but also affect seed quality. Seeds from infected plants reduce in size and weight. Defected seeds are recognized by the yellow discoloration (Nene, 1973; Varma *et al.*, 1992) and yellow lesions on newly sprouted leaves in nurseries (Nene, 1973).

There have been many reports regarding the characteristics of various plant species infected with *Begomovirus*. However, to our knowledge, there has not been any report on the molecular characteristics of *Begomovirus* on angled luffa cultivated in Yogyakarta and Central Java.

MATERIALS AND METHODS

Sample Collection and Disease Incidences

Disease incidence of yellow curly virus on angled luffa were observed on sample fields in Sleman, Yogyakarta and Magelang, Central Java. Leaf and seed samples were taken from angled luffa plants with yellow lesions, curly leaves, and stunting symptoms. Leaf samples were then placed in a plastic bag with silica gel and stored in -20°C for further processing.

Molecular Identification of Begomovirus

Plants that showed symptoms of *Begomovirus* infection, were sampled for further molecular identification using PCR and a universal *Begomovirus* primer, *Krusty-Homer* (Revill *et al.*, 2003). Sample of DNA were extracted from angled luffa leaves with yellow curly symptoms using a DNA Geneaid Minikit (Geneaid Biotech Ltd., Taiwan). As much as 0.05 g of leaf samples were placed on porcelain mortars, 400 µl GP1 buffer and 5 µl RNase A were added and leaf samples with mentioned solutions were macerated and put into a 1.5 mL tube. Solutions were homogenized. Incubation was done in a waterbath at 60°C for 10 minutes and every 5 minutes tubes' positions were moved to ensure equal temperature. After that, 100 µL GP2 buffer was added, homogenized, and incubated in ice for 3 minutes. Mixtures were moved into 2 mL tubes that were placed in a filter column and centrifuged for 1 minute at 1,000 G. Supernatant was taken and placed in 1.5 mL tubes and GP3 buffer was added by 1.5 times of the supernatant volume. This mixture was then homogenized for 5 seconds. Mixture was placed on GD column into the 2 mL tube, Elution Buffer was warmed at 60°C. The mixture inside the GD column was centrifuged at 10,000 G for 4 minutes. Supernatant was then disposed and 400 µL of W1 buffer was added and centrifuged at 10,000 G for 3 minutes. Supernatant was disposed and 600 µL of wash buffer was added, centrifuged at 10,000 G for 3 minutes, and repeated twice. Supernatant was disposed and recentrifuged at 10,000 G for 6 minutes until the column matrix was dry. GD columns were placed in 1.5 mL tubes and 100 µL of pre-heated elution buffer was added, left for 3 minutes and centrifuged at 10,000 G for 2 minutes. Extracted solutions were stored at -20°C. The DNA amplification used a primer specific *Begomovirus* Krusty (Forward): 5'CCNM

RDGGHTGTGARGGNCC'3; Homer (Reverse): 5'SVDGCRTGVGTRCANGCCAT'3) with a pre-denaturation stage of 95°C for 1 minute, denaturation 95°C for 14 seconds, annealing stage at 55°C for 15 seconds, elongation at 72°C for 10 seconds, repeated for 35 cycles, and ended with a final elongation stage at 72°C for 5 minutes. The DNA electrophoresis was visualized on 1% agarose gel at 50 V for 50 minutes. Targeted DNA band was 580 bp (Revill *et al.*, 2003).

Begomovirus Seed-borne Test

The possibility of *Begomovirus* to be contracted by seeds was tested using a growing on-test. Seeds were obtained from ripe angled luffa fruit that were cleaned. Seeds were placed in petri dishes with wet filter paper to maintain humidity. Seeds that have sprouted were identified using the similar molecular procedures used to test angled luffa leaves

Data Analysis

Sequencing results were analyzed through Basic Local Alignment Search Tool (BLAST) program of the National Center of Biotechnology Information (NCBI) for similarity search that at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> to obtain species with high homology. Corrections were done and sequences were multiple aligned using the ClustalOmega program, which was accessed through <http://www.ebi.ac.uk/tools>. Results from multiple alignments were used to create nucleotide homology percentage tables and phylogenetic tree using Molecular Evolutionary Genetical Analysis versi 7 (MEGA7) with a Maximum-Likelihood, 2 parameters Kimura, and 1,000 bootstraps (Kumar *et al.*, 2016).

RESULTS AND DISCUSSION

Disease Incidence and Symptoms on Angled Luffa

Disease incidence observation at both location where *Begomovirus* infected angled luffa fields imply that geographical conditions of the field affects disease incidence. According to Febria (2015), different geographical conditions of fields significantly affected disease incidences. Disease incidence on angled luffa at Berbah, Sleman with altitude of 109 m asl was 80% while disease incidence at Dukun, Magelang was 100% (Table 1). Disease incidence of yellow curly on angled luffa was also affected by other factors, such as surrounding vegetation, existences of alternative host and this

Table 1. Sampling locations and disease incidences (Giovanni, 2017)

Season	GPS coordinates	Altitude	Region	Disease Incidences
Rainy	7°79'42.63"S 110°45'30.21"E	109 masl	Berbah, Sleman	80%
Rainy	7°53'92.28"S 110°36'66.28"E	766 masl	Dukun, Magelang	100%

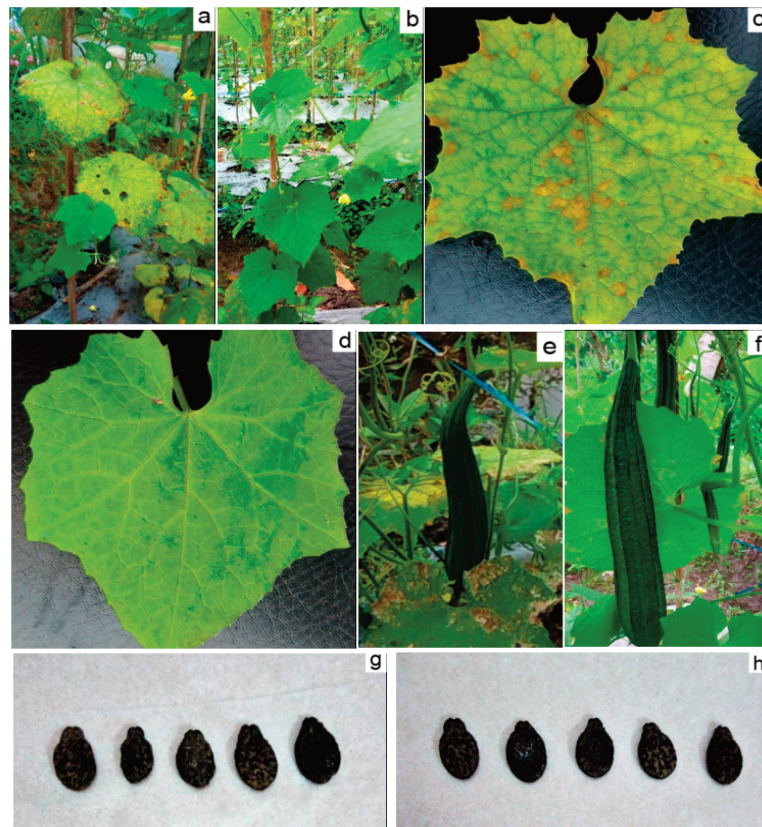


Figure 1. Morphological comparison between healthy and malformed angled luffa (*Luffa acutangula*) plants (a) angled luffa plant with almost all of its body turned yellow (b) healthy angled luffa plant (c) angled luffa leaf with yellow mosaic (d) healthy angled luffa leaf (e) curved angled luffa fruit (f) healthy angled luffa plant (g) shrunken and empty seeds (h) healthy angled luffa seeds (Giovanni, 2017)

disease's vector. The high disease incidence of yellow curly at Dukun, Magelang was caused by geographical condition of the location due to it being at a medium altitude land, which was an endemic area for *Begomovirus* (Sulandari *et al.*, 2006), surrounding vegetation of this field included other commodities that were host for *Begomovirus*, such as chili pepper, eggplants, tomato, various species of weeds and an existing population of its insect vector, *Bemisia tabaci* Genn. yang tinggi. The dispersion of *B. tabaci* is affected by abiotic factors, such as the change of average yearly air temperature (Kandito, 2017). Kandito (2017) stated that average yearly air temperature was 19.3°C–20.1°C between 2009–2015.

Observation of yellow curly symptoms on angled luffa that these plants were presumed to be infected by *Begomovirus* with early infection symptoms to be yellow lesions on leaves, thickening of leaf midribs, and curly leaves (Figure 1c) and stunting (Figure 1a). Other than the previous mentioned symptoms, *Begomovirus* infected plants have malformation on its leaves, fruits, and seeds. These malformations are usually shown by mosaic on leaves (Figure 1c), stunting (Figure 1a), small fruits followed by discoloration and misshaped fruits (Figure 1e), and shrunken and sometimes discolored seeds (Figure 1g). Comparison between healthy and *Begomovirus* infected plant are shown in Figure 1.

Virus infection may decrease photosynthetic efficiency by decreasing chlorophyll content in leaves, chlorophyll efficiency, leaf areas, and growth regulating substances (Mushtaq *et al.*, 2014), which will then hinder plant growth. Ariyanti (2012) stated that symptoms that appeared due to virus infection is caused by disruption in nutrition transportation from the source to sink. This later will disrupt plant growth in general and not produce fruit if plants are affected at early stages or during the vegetative stage. If plants are infected during the generative stage may result in smaller fruits with hard textures.

Molecular Identification for Virus Causing Yellow Curly Leaves on Angled Luffa and Seed-borne Test

Molecular identification from samples using the PCR Krusty-Homer Universal *Begomovirus* primer (Revill *et al.*, 2003) showed positive results from leaf samples while negative results from angled luffa seeds. Leaf samples positively infected by *Begomovirus* showed ± 580 bp bands (Figure 2). Krusty-Homer primer is able to amplify nucleotides that encode *Begomovirus* coat protein, which is a conserve gene from *Begomovirus* (Revil *et al.*, 2003; Reddy *et al.*, 2005). Thus, that primer can be used in early identification and surveillance method.

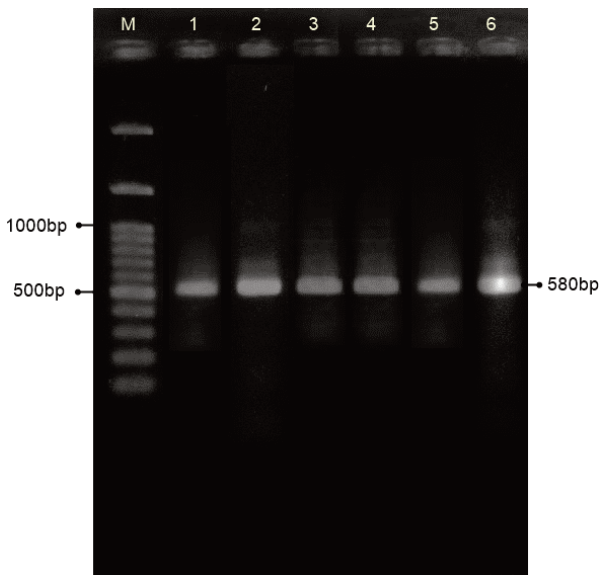


Figure 2. PCR product from *Begomovirus* DNA amplification results after electrophoresis on 1% agarose: (1) isolate GB1; (2) isolate GB2; (3) isolate GB3; (4) isolate GD1; (5) isolate GD2; (6) isolate GD3; (M) marker 100bp DNA ladder; ± 580 bp DNA band (Giovanni, 2017))

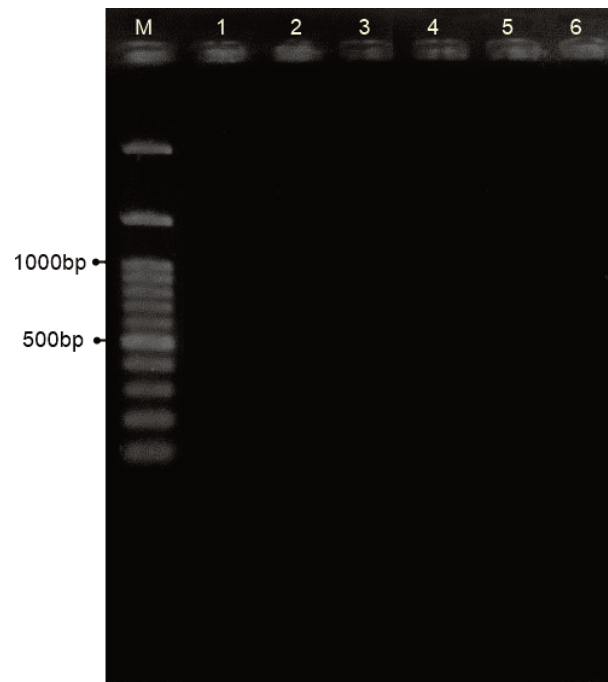


Figure 3. PCR product from DNA amplification from root and seed sample after electrophoresis using 1% agarose gel: (1) isolate GB1; (2) isolate GB2; (3) isolate GB3; (4) isolate GD1; (5) isolate GD2; (6) isolate GD3; (M) marker 100bp DNA ladder; negative results, no bands formed (Giovanni, 2017)

Seed samples did not result in these bands; therefore, it implies that seeds did not contain *Begomovirus* (Figure 3).

Begomovirus that cause yellow curly leaves in Indonesia are not seed-borne, are not able to be transmitted mechanically and can only be transmitted through its vector *B. tabaci* (Rusli *et al.*, 2000; Sulandari *et al.*, 2006; Sulandari *et al.*, 2001). However, Kil *et al.* (2012) proved that *Tomato yellow leaf curl virus* (ToYLCV) was seed-borne and had significant importance of its survival through planting seasons and geographical dispersion. This implies that although there are species of *Begomovirus* that are seed-borne, the species found on angled luffa in Berbah, Sleman and Dukun, Magelang were not one of them. *Begomovirus* may not become seed-borne due to seeds that go into dormant stages (Akin, 2006) or viruses not reaching seed embryo causing them to not survive (Matthews, 1970).

Sequence Analysis

The BLAST analysis from the NCBI implied that sequences from GD1 sample (angled luffa infected

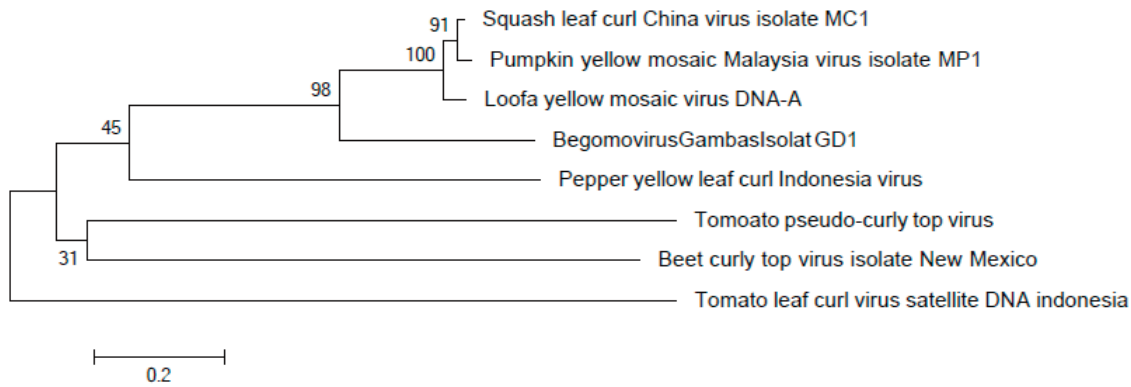


Figure 4. Phylogenetic tree of angled luffa *Begomovirus* form nucleotide sequences of samples collected at Dukun, Magelang (GD1) compared to other *Begomovirus* isolates from the GenBank; each node has the bootstrap values (Giovanni, 2017)

Table 2. Nucleotide sequence's homology percentage between samples (Giovanni, 2017)

No.	Identity	1	2	3	4	5	6	7	8
1	Squash_leaf_curl_China_virus_isolate_MC1	ID							
2	Squash_leaf_curl_China_virus_isolate_Pumpkin-Nakhon	97.3	ID						
3	Squash_leaf_curl_China_virus_isolate_Hanoi	97.1	99	ID					
4	Begomovirus_wax_gourd/Suphan_Buri2008/Feb2008	96.5	97	96.9	ID				
5	Pumpkin_yellow_mosaic_Malaysia_virus	96.5	96	95.9	95	ID			
6	Squash_leaf_curl_China_virus_Thailand	96.5	99	98.2	97	95	ID		
7	Squash_leaf_curl_China_virus_isolate_GZ01	95.8	97	96.5	97	95	96.5	ID	
8	Angled_luffa_Dukun_Magelang_Indonesia	97.8	96	96.1	96	96	95.9	95.4	ID

with *Begomovirus* from Dukun, Magelang) were similar with nucleotide sequences from previous studies. Sequence alignment of GD1 were similar with *Begomovirus* from other viruses known to cause yellow curly leaf symptoms, such as *Beet curly top virus* isolate New Mexico (EF501977), *Loofa yellow mosaic virus* (AF509739), *Pepper yellow leaf curl Indonesia virus* (NC_008284), *Pumpkin yellow mosaic Malaysia virus isolate MP1* (EF197941), *Squash leaf curl China virus isolate MC1* (EF197940), *Tomato pseudo curly top virus* (NC_003825) and *Tomato leaf curl virus satellite DNA isolate Indonesia* (AB113651), and compared using a phylogenetic tree (Figure 4).

The phylogenetic tree showed 2 main clusters. Several *Begomovirus* isolates and other genera were chosen to compare relationship with angled luffa *Begomovirus* gambas isolat GD1. Isolate GD1 was in the same cluster as SCLV-China isolate MC1 access code EF197940.1 and *Pumpkin yellow mosaic virus Malaysia* (PYMV-M) isolate MP1 access code EF197941.1. *Beet curly top virus* New Mexico (BCTV-NM) (access code EF501977.1) had distinct

relationship due to it being in different cluster as. In addition, *Tomato leaf curl virus* satelite Indonesia (access code AB113651.1) was an outgroup species. Angled luffa *Begomovirus* isolate GD1 also had high relations with LYMV (access code AF509739.1) due being in the same cluster. Meanwhile *Tomato leaf curl virus* satelite Indonesia (access code AB113651.1) had relations with BCTV-NM (access code EF501977.1) due being in the same cluster. Different cluster can be implied that isolates between them have distinct relations.

The BLAST analysis showed that GD1 had similarities to *Squash leaf curl virus* from China isolate MC1 (GenBank EF197940) based on it phylogenetics. Homology percentage between both isolates were taken from Genbank (Table 2).

The multiple alignment and homology analysis showed that GD1 were almost identical to the *Begomovirus* spesies SLCV China isolate MC1. Alignment resulted in 95% homology and identical percentage of 98% (Table 2). Molecular identification of yellow curly leaf causing viruses on angled luffa showed high relations with SCLV China (EF197940)

based on the analysis of nucleotide sequence and *Begomovirus* coat protein coding amino acid. This study firstly reports the molecular characteristic of *Begomovirus* on angled luffa from Indonesia. Other viruses reported to infect angled luffa are *Zucchini yellow mosaic virus* (ZYMV) (Milne & Grogan, 1969 *cit.* Ohtsu *et al.*, 1985), *Watermelon mosaic virus 2* (WMV-2) (Webb, 1965), *Cucumber green mottle mosaic virus* (CGMMV) and *Kyuri green mottle mosaic virus* (KGMMV) (Daryono & Natsuaki, 2009).

CONCLUSION

All samples tested using PCR were positively infected by *Begomovirus*. Analysis of sequence results of GD1 *Begomovirus* from angled luffa isolates had homologies closed to SLCV MC1 isolates from China with 97.8% similarity and close relationships with the same isolate. Root samples from angled luffa seedlings detected using PCR showed negative results implying that this virus is not seed-borne.

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