Genetic Diversity of Cowpea Mild Mottle Irus on Soybean in Several Region in Indonesia

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Abstract: Soybean is one of the most important food commodities in Indonesia. Virus infection on soybean has been reported worldwide as factors affecting yield loss. This study was aimed to detect *Cowpea mild mottle virus* (CPMMV) from several soybean cultivation areas in Java, Sumatra and Southeast Sulawesi; and further characterize their genetic variation based on nucleotide sequences of their coat protein. Several virus infection was detected using double antibody sandwhich enzyme-linked immunosorbent assay (DAS-ELISA),including CPMMV, *Cucumber mosaic virus* (CMV), and *Soybean mosaic virus* (SMV). Generally, the symptoms caused by CPMMV, CMV, and SMV are similar, involving mottle, rugose, and vein banding. Coat protein gene of 5 CPMMV isolates (Bantul, Musi Banyuasin, Cirebon, Kendari, Cianjur) was successfully amplified and cloned. Sequence of this 5 clones of CPMMV showed high similarity, ranging from 88.2 to 99.8%; whereas their sequence homology to those of Taiwan and China ranging from 88.2 to 98.6%. Phylogenetic analysis showed different clusters of CPMMV Indonesian isolates: isolates from Bantul,Cirebon, Musi Banyuasin (Palembang) is clustered with Taiwan isolate (JX020701); isolate from Cianjur is clustered with China isolate (KX534092); isolate from Kendari is clustered with Puerto Rico (GU191840),Brazil (KC884247), and USA (KC774020) isolates.

1 INTRODUCTION

Several types of viruses reported to infect soybean plants are Alfalfa mosaic virus (AlMV), Bean common mosaic virus (BCMV), Bean yellow mosaic virus (BYMV), Blackeye cowpea mosaic virus (BlCMV), Cucumber mosaic virus (CMV), Pea enation mosaic virus (PEMV), Peanut mottle virus (PeMoV), Soybean mosaic virus (SMV), Tobacco mosaic virus (TMV), Tobacco ringspot virus (TRSV), Tobacco streak virus (TSV), Tomato ringspot virus (ToRSV), and Spotted Tomato wilt virus (TSWV) (Golnaraghi *et al*. 2004). In Indonesia, several viruses have been reported in soybean plants: Cowpea mild mottle virus (CPMMV) (Iwaki *et al*. 1986), SMV (Andayanie 2012), CMV soybean strain (CMV-S) and Pepper yellow leafcurl virus (PYLCV) (Rahim *et al*. 2015). CPMMV infection in soybean has caused endemic diseases in Java and Sumatra (Jumanto *et al*. 1999). CPMMV infection in soybeans in Lampung caused a decrease in dry weight with soybean crop weight between 15.5-53.4% and a decrease in soybean seed weight between 11.5-51.6% and a decrease in seed quality cause an abnormal seed shape of 7.6-54.35% (Akin 2003).

CPMMV is a member of the Genus Carlavirus, Family Betaflexiviridae (Martelli *et al*. 2007). Losses due to CPMMV infection were also reported in several other countries, including in Argentina and Iran CPMMV reportedly caused severe damage to soybeans (Laguna *et al*. 2006; Tavassoli *et al*. 2009). In addition to infecting soybean CPMMV was also reported in yard long bean (Brito *et al*. 2012), tomatoes, beans, Bambara peanuts (Almeida et al. (2005). Offei and Albrechtsen (2005) reported CPMMV infection in bambara peanuts (*Vigna subterranea* L.) causes the leaf area index to be reduced by 70%, and decreases the number of pods and seeds and the weight of the seeds CPMMV infection in peanuts causes dwarf plants, reduction in length of internodes and size of leaves, stripes on leaves, chlorosis and leaf rolling CPMMV infection has been reported to be a serious disease in peanuts

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in Sudan with a disease incidence of up to 50% even in some regions up to 100% (El-Hassan *et al*. 1997). Surveys on long bean plants in Venezuela show the incidence of diseases ranging from CPMMV infections. 15-40% (Brito *et al*. 2012). In India, CPMMV infection in soybean causes systemic, mosaic, and leaf deformation with 25.1-71 disease incidence. % (Yadav *et al*. 2013).

Expansion of soybean cultivation areas in Indonesia must anticipate the emergence of diseases that have the potential to cause loss of results. The current status of the area spread CPMMV on soybeans in Indonesia needs to be known. Recently on 2015, we conducted a field survey to collected soybean's leaves with typical symptom of virus-like infection from several cultivation areas in Java, Sumatra, and Sulawesi. In the earlier reports, the the yield impact of CPMMV infection were studied, but none of these were reported about genetic diversity of CPMMV in Indonesia. Here, we reported the genetic diversity of CPMMV from several soybean cultivation areas in Indonesia.

2 MATERIAL AND METHODS

Sampling activities for collection of CPMMV isolates were carried out during the 2014-2015 planting season in Cirebon, Cianjur, Bogor (West Java Province); Bantul, (Yogyakarta); Ngawi (East Java Province); Musi Banyuasin (South Sumatra Province), Sungai Hitam (Bengkulu Province), Kota Baru (Jambi Province); and Kendari (Southeast Sulawesi Province). Virus detection and cloning were carried out at the Plant Virology Laboratory, Department of Plant Protection, Faculty of Agriculture, IPB.

2.1 Samples Collection

Samples of soybean leaves taken from the field are young leaves that show symptoms of viral infection including mottle, vein clearing, chlorosis, dwarf, leaves malformation. Leaf samples are put into a plastic bag and stored in a box to be brought to the laboratory. Symptoms of leafy leaves were then separated into two groups, namely leaves with symptomatic stripes, chlorosis, distortion of leaves and dwarfs and a group of yellowing leaves. Each sample is weighed 0.1 g each, labeled and stored at -80 ˚C until it is used for the next stage.

2.2 Serological Detection

Serological detection of viruses was carried out with DAS-ELISA following the Clark and Adams (1977) protocol using 3 types of antiserum separately, namely CMV, SMV, and CPMMV antiserum (DSMZ, Germany).

2.3 Reverse Transcription-Polimerase Chain Reaction (RT-PCR)

Total RNA extraction. Total RNA extraction was carried out using RNeasy Plant Mini Kits (Qiagen, Hilder, Germany) according to the Qiagen protocol (Qiagen 2003). RNA extraction results were synthesized into cDNA using reverse transcription (RT) method. CDNA amplification was performed using a specific primary pair for CP CPMMV gene. The primary forward used was CPF (5'-ATTAAGGATCCGAGTTGATTTAAATAAGT-3') and the reverse CPR primer (5'-ATTAAGAATTCCTTGTGATTGAAATTGCG-3') with an expected amplification product measuring 958 bp. The composition of the PCR reactant consisted of 12.5 µl Dream taq PCR master mix (Thermo Scientific), 1 µl primer forward 10 µM, and 1 µl reverse primer 10 µM, 1 µl cDNA, and 9.51 µl H2O. Amplification of cDNA with 94 ° C stages for 5 minutes, then followed by 35 cycles consisting of denaturation of 94 ° C for 1 minute, annealing 45 ° C for 1 minute, and DNA synthesis (extension) 72 ° C for 2 minutes, then extension 72 ° C for 10 minutes and the cycle ends at 4 ° C. Amplification of agarose gel follows the method described previously. CPMMV isolates which showed a clear and thick DNA band of ± 958 bp then continued to cloning stage.

2.4 DNA Cloning and Sequences Analysis

PCR products were cloned into pTZ57R/T aesy vector system based on the protocol provided by Thermo Scientific, USA. Plasmid DNA recombinant was sequenced and analyzed. The nucleotide sequences of the gene were aligned with those corresponding virus sequences deposited in GenBank database by using software Clustal-W (www.ebi.ac.uk). Phylogenetic tree CPMMV was constructed using the ClustalX Bio Edit version 7.05 program and the MEGA 5.0 program with the neighbor-joining algorithm and 1000 repetition bootstrap methods.

3 RESULT AND DISCUSSION

3.1 Results

3.1.1 Symptoms on Infected Soybean Leaves

Symptoms of viral infection in soybean in the field varied, namely systemic chlorosis and leaf distortion, systemic leaf and curly leaf surface, systemic stripes with green patches, striped with lamina blisters, stripes and leaf distortion, chlorosis in leaf lamina and vein banding, and some mixed symptoms in one plant (figure 1). The symptom variation was depending on the soybean cultivar and climate on each location.

Figure 1: Symptoms of viral infection in soybean: systemic chlorosis and leaf distortion (A), systemic stripes and curly leaf surface (B), systemic stripes with green spots (C), striped with lamina of blister (D) leaves, stripes and leaf distortion (E), and chlorosis in the leaf lamina and vein banding (F).

3.1.2 Serological Test

Based on serological test using three antisera, there are three virures detetcted on samples with various frequences and showed multiple infection occurred naturally (data not shown). CPMMV detected on samples in 8 regions from 9 regions of origin of soybean leaf samples. Besides CPMMV also detected several other types of viruses, namely CMV and SMV. CMV infection is detected in 4 regions, namely Cirebon, Cianjur, Bantul, Kendari; while SMV is only detected in Kendari and Cirebon. CPMMV was consider as endemic disease on soybean in Java, further characterization of biological, physical and nucleic acid characters are necessary to identify to confirm its existence in Indonesia.

3.1.3 Amplification and Nucleotide Sequences Analysis

CPMMV specific DNA was successfully amplified from 5 CPMMV isolates from Bantul (B), Banyuasin Musi (MB), Cirebon (CB), Kendari (KN), and Cianjur (CR) (figure 2). Furthermore, the PCR product is used for cloning stages.

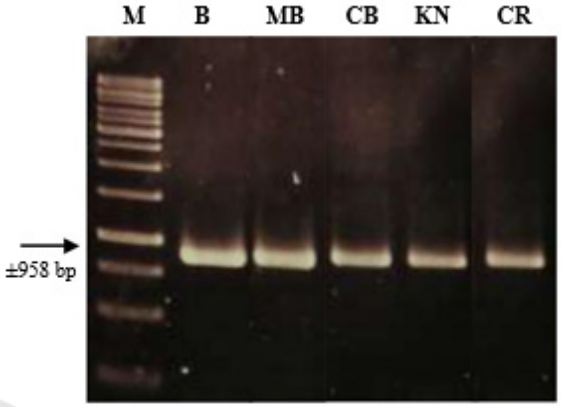

Figure 2: DNA amplification visualization of CP CPMMV gene from Bantul (B), Banyuasin Musi (MB), Cirebon (CB), Kendari (KN), and Cianjur (CR), M, 1 kb DNA (Thermo Scientific) marker.

Isolates from 5 regions in Indonesia have homology between isolates as much as 88.2-99.8% (Table 1). Similarity of nucleotide sequences between isolates in Indonesia and Taiwan and China isolates with a range of 88.2-98.6% higher than similarities with isolates from America; while the lowest homology is with Ghana isolates. CPMMV isolates in Indonesia showed more than 72% homology with all isolates compared to GenBank.

Table 1: The nucleotide sequences of CPMMV isolates in Indonesia with sequences of isolates from several other countries in GenBank.

Sequence comparison	CP gene (%)
Among Indonesian isolates	88.2-99.8
Between Indonesian isolates with Taiwan and China	8.2-98.6
Between Indonesian isolates with Amerika	6.9-88.8
Between Indonesian isolates with Ghana	6.9-79.2

3.1.4 Phylogenetic Analysis

Phylogenetic analysis showed that Bantul, Cirebon, Musi Banyu asin (Palembang) isolates formed a group with Taiwan isolates (accession number JX020701); whereas Cianjur isolates were separated from other isolates and closer to Chinese isolates (accession number KX534092). Kendari isolates are closer to Puerto Rico isolates (accession number GU191840, Brazil (accession number KC884247), and USA (accession number KC774020) (Figure 3).

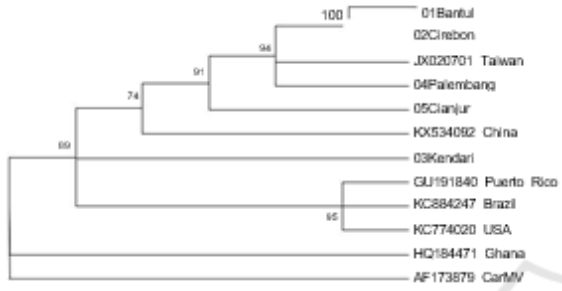

Figure 3. Phylogeny tree isolates CPMMV from Bantul, Cirebon, Musi Banyuasin, Cianjur, and Kendari based on the CP CPMMV gene sequence from Asian, American and African groups and CarMV as outgroup

3.2 Discussion

Jumanto *et al*. (1999) reported mottled disease in soybeans associated with CPMMV infection in Java and Sumatra. However, after the report, there is no current information regarding the distribution of CPMMV in Indonesia, even though the area of soybean cultivation in Indonesia is increasingly widespread. In this study it was found that CPMMV was detected on soybeans in Bengkulu, Musi Banyuasin (South Sumatra); Cianjur, Bogor and Cirebon in West Java; Bantul (Yogyakarta); Ngawi (East Java); and Kendari (Southeast Sulawesi).

The results of this study indicate that the diversity of CPMMV not only occurs in virulence and the type of symptoms caused but also in diversity its molecular level. Based on nucleotide homology analysis, CPMMV isolates obtained from Sumatra, Java, and Kendari in this study, had a relationship with isolates from Asia (China, Taiwan), and America (USA, Brazil, Puerto Rico). Close relationship with Asian and American isolates indicates the possibility of CPMMV Indonesia coming from that country. Nevertheless, CPMMV from various regions turned out to show genetic variation at the level of its nucleotide sequence. Genetic variation in viruses can occur through two events, namely mutation and recombination. The high rate of mutation in the RNA virus indicates an evolutionary strategy. According to Agrios (2005) the evolution of viruses occurs as a form of adaptation to environmental suitability, such as host plants, strains of viruses, insect vectors. Different environmental conditions between regions in Indonesia, and climate differences between Indonesia and other countries may cause environmental stresses that cause genetic changes in the virus.

4 CONCLUSIONS

The distribution of CPMMV on soybeans covering several areas of soybean production field in Java, Sumatra, and Sulawesi. CPMMV is the dominant virus found in 8 soybean cultivation locations from 9 sampling locations. Besides CPMMV, CMV, and SMV were also found. Based on phylogenetic analysis CPMMV Bantul, Cirebon, and Musi Banyuasin isolates formed a group with Taiwan isolates; whereas Cianjur isolates are closer to Chinese isolates; and Kendari isolates are closer to isolates from Puerto Rico, Brazil, and USA.

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If any, should be placed before the references section without numbering.

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