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Identification of RNA Viruses Causing Sugarcane (*Saccharum officinarum* L.) Mosaic Disease by Simultaneously Multiplex-RT-PCR

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This study aims to determine the type of RNA viruses that cause mosaic disease in four sugarcane varieties in Indonesia based on simultaneous detection using multiplex-RT-PCR as well as relationship between types of RNA viruses that cause mosaic disease in four varieties of sugarcane in East Java, Indonesia from the analysis of cp gene sequence. The results showed that there were two types of RNA viruses, SCMV and SCSMV, which caused mosaic disease in four sugarcane varieties at four locations in East Java, Indonesia based on RT-PCR molecular detection either singleplex-RT-PCR or multiplex-RT-PCR. Molecular detection using the multiplex-RT-PCR method is considered superior to singleplex-RT-PCR because it is able to diagnose several types of RNA mosaic sugarcane at once, so it is more practical and time saving. SCMV that attacked sugarcane plantations in East Java, Indonesia on two sugarcane varieties namely Ps 864 (Magetan district) and Ps 881 (Madiun district) is a SCMV cluster closely related to the SCMV isolate (EU196453.1) from Argentina. SCSMV that attacked sugarcane plantations in East Java, Indonesia on four sugarcane varieties namely Ps 864 (Magetan district), Ps 881 (Madiun district), Ps 862 (Situbondo district), and NXI 1-3 (Jember district) is a SCSMV cluster closely related to the SCSMV isolate (AB563503.1) from Indonesia. RNA viruses that cause sugarcane mosaic disease in Indonesia, SCMV and SCSMV, can be detected simultaneously by multiplex-RT-PCR method

Keywords: SCMV, SCSMV, mosaic disease, sugarcane, RT-PCR.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is one of the major commercial crops grown widely in the tropics and subtropics of the world. Sugarcane is also one of the important industrial crops in Indonesia with an area of about 400,000 ha with an average production of 60-70 tons of sugarcane per ha (Putra et al. 2013). There are many factors responsible for the production decline, one of which is the presence of pests and diseases.

A number of pathogens capable to attacking sugarcane include viruses, fungi, bacteria, and nematodes, and among these pathogens, the virus, is one of the leading causes of endemic diseases to consistently and significantly lead to a decrease in world sugar production (Li et al. 2008, Parameswari et al. 2013). Sugarcane mosaic disease is one of the endemic diseases caused by the virus first identified in 1892 and it was apparently caused by Potyvirus (Artschwager and Brandes, 1958). In a subsequent report, it has been identified that the mosaic disease of sugar

cane in natural conditions caused by the genus Potyvirus, the Sugarcane Mosaic Virus (SCMV), and the genus Poacevirus, the Sugarcane Streak Mosaic Virus (SCSMV) (Grisham et al. 2000; Hall et al. 1998; Hema et al. 1999; Pirone and Anzalone, 1966; Shukla et al. 1992).

In previous studies, it has been reported that the diversity of Potyviridae is interconnected in the variation of its coat protein (cp) gene (Adams et al. 2005). The molecular variation in the cp gene suggests that SCMV and SCSMV may vary genetically based on differences in geographic distribution (Alegria et al. 2003). The cp gene encodes capsid-forming proteins in viruses and proteins associated with vector transmission, assisting the transfer of host cells to other host cells via plasmodesmata, and regulation of specific RNA amplification in each strain of the virus (Bedoya and Rojas, 2012).

Recently, both viruses capable of causing the mosaic of sugarcane disease have been reported to be widely distributed in many superior and commercial sugarcane in Indonesia (Asnawi, 2009; Damayanti and Putra, 2010; Putra et al. 2013). These viruses cause mosaic disease of sugarcane with symptoms of yellow chlorosis lines along the typical leaf (Grisham et al. 2000; Hema et al. 1999). The spread of mosaic disease in sugarcane crops can occur due to the presence of aphid vectors and the use of planting materials infected with the virus. The spread of the virus more quickly if the population of high vectors, varieties cannot stand the disease, and the presence of plants that have been infected. These viruses are capable of causing mosaic disease in sugarcane independently or simultaneously with other types (Viswanathan et al. 2007).In this study, multiplex-RT-PCR is used because it has the potential advantage to detect many types of viruses in a single reaction (Viswanathan et al. 2010). In addition, this detection is based on the precision of the primary pair of specific cp gene for cp gene amplification followed by sequence analysis of cp gene DNA thus optimizing the multiplex-RT-PCR condition to be able to detect the presence of RNA viruses causing mosaic disease from samples of sugarcane leaf affected by mosaic disease. Nucleotide sequence analysis is compared with data already in the database at the National Center for Biotechnology Information (NCBI), so that it will give information on the relationship of viruses that cause sugarcane mosaic disease. This study aims to determine the type of RNA viruses that cause mosaic disease in four sugarcane varieties in Indonesia based on simultaneous detection using multiplex-RT-PCR as well as relationship between types of RNA viruses that cause mosaic disease in four varieties of sugarcane in East Java, Indonesia from the analysis of cp gene sequence.

MATERIALS AND METHODS

Sampling of sugarcane plants with symptoms of mosaic disease

In this study, the exploration and collection of four varieties of sugarcane cultivation symptom of mosaic was conducted in sugarcane plantations of PT. Perkebunan Nusantara XI, East Java, Indonesia. Sugarcane samples used in this study consisted of four varieties of sugarcane, namely Ps 864 (Magetan district), Ps 881 (Madiun district), Ps 862 (Situbondo district), and NXI 1-3 (Jember district). Negative control used for this study is SUT variety which is free from symptoms of mosaic disease and obtained by in vitro through apical bud tissue culture technique.

Isolation of RNA samples

As many as 100 mg of prepared sugarcane young leaves samples were crushed with liquid nitrogen. The RNA isolation was carried out according to the guidelines that contained in the *TianGen RNAprep Pure Plant Kit*. The obtained RNA samples are stored at -80°C and then used as RT-PCR material.

RT-PCR

The cDNA synthesis stage is performed according to the *Roche Transcriptor First cDNA Synthesis Transcript Kit*. Synthesis of cDNA is done in two stages. They are RNA denaturation and reverse transcription (RT). In the RNA denaturation stage, 5 µL RNA samples were mixed in PCR microtube with 1 µL anchored-oligo $(dT)_{18}$ Primer (50 pmol/µL) and H_2O-PCR grade to a total volume of 13 µL. Then incubated the mixture at 65^oC for 10 minutes in water bath. At the RT stage, the mixture was added with 4 µL transcriptor RT reaction buffer, 0.5 µL protector RNAse, 2 µL deoxynucleotide mix, and 0.5 µL transcriptor reverse transcriptase. Then incubated the mixture at 55^oC in water bath for 45 minutes. Transcriptor reverse transcriptase must be disabled by incubation at 85°C for 5 minutes. The obtained cDNA templates are stored at -20^oC.

The PCR stage uses a *Bio-Rad* thermal cycler tool. Two µL cDNA template (250ng/µL) is mixed with 12.5 µL *Promega Go Taq Green Master Mix*, each 1 µL Forward Primer and

Table 1. Primers and their sequences

	Primer	Sequence (5' – 3')	Fragmen Size (bp)
First Primer	SCMV-F	GTTTTYCACCAAGCTGGAACAGTC	±900
	SCMV-R	AGCTGTGTGTCTCTCTGTATTCTC	
Second Primer	SCSMV-F	GTGGGTTCAGTTCTCGGTTC	±572
	SCSMV-R	CCTCCTCACGGGGCAGGTTGATTG	

Note: Y = C/T

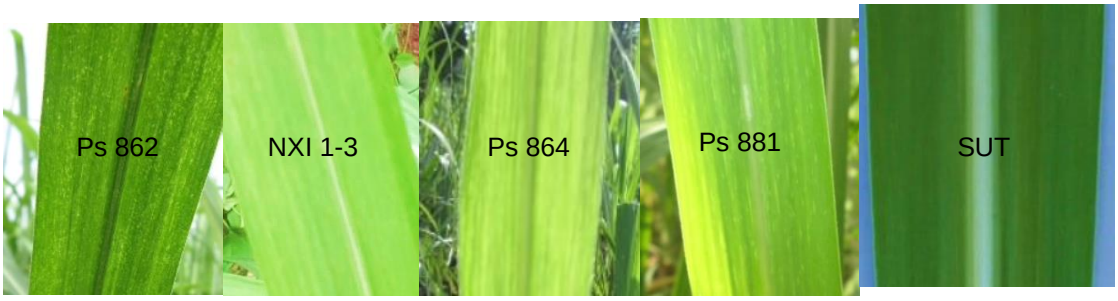

Figure 1: The leaves of sugarcane plant used as study samples. The symptoms of sugarcane mosaic disease from varieties of sugarcane: Ps 862, NXI 1-3, 862, Ps 881, and SUT not symptomatic (healthy) for negative control.

Reverse Primer, and Nuclease free water up to a total volume of 25 µL. The cDNA was amplified in a PCR machine with 40 cycles reactions including predenaturation for 2 minutes at 95^{o}C for 1 cycle followed by 40 cycles for denaturation 95^{o}C for 30 seconds, annealing 56^{o}C for 20 seconds, extension 72^{o}C for 1 minute, and final extension 72^{o}C for 5 minutes. The application of multiplex-RT-PCR is directly using two pairs of detection primers, for SCMV and SCSMV, in a single reaction (Table 1).

Visualization of RT-PCR products

One percen of agarose gel electrophoresis was performed to determine the PCR products visually. The 5 µL PCR products and 5 µL DNA marker were inserted into each wells prepared on agarose gel. Electrophoresis is carried out for 25 minutes with a voltage of 100 Volts. The electrophoretic DNA was then visualized with a UV transilluminator.

Analysis of genetic relationship between types of mosaic viruses

A total of 40 µL DNA of RT-PCR products was sequenced by *DNA 1st Base Laboratories*, Malaysia, using the *ABI PRISM 3730 XL* sequencer type. Analysis of genetic relationship between types of RNA viruses causing sugarcane mosaic diseases sequenced results based on sequences that have been deposited on *GenBank* using Basic Local Alignment Search Tools (BLAST) at the website address https://blast.ncbi.nlm.nih.gov/. Furthermore, phylogenetic tree is described by Blast Tree program through Neighbor Joining method with Rectangle Cladogram layout to know the existing data group and its genetic relationship distance.

RESULTS AND DISSCUSSION

Symptoms of sugarcane mosaic disease

Characteristics of symptoms of sugarcane mosaic disease are characterized by the development of green and yellow patterns along the leaves caused by chlorosis. The apparent symptom of consistency of infection is linear spot chlorosis distributed in the middle or basal along the leaf margins (Gonçalves et al. 2007). The expression of symptoms of this disease may vary depending on the sugarcane cultivar, growing conditions, leaf age, infection age, and striking viral strains (Grisham, 2000). In Figure 1, they are sugarcane leaves that were sampled in this study because they have the characteristics of symptoms that are similar to the symptoms of sugarcane mosaic disease. From the observation of the symptoms is known that the sugarcane plants that have leaves similar to the phenomenon of mosaic is mostly found in the young leaves or leaves are still rolled and gradually thinning along

with the increasing age of the leaf. Mosaic symptoms that appear from the observation is still unclear. Wijayani and Indradewa (2004) also explained that the mosaic patterns present in sugarcane leaves can also be caused by the lack of nitrogen or phosphorus. While Nayudu (2008) explains that mosaic symptoms in sugarcane plants are caused by viruses when symptoms can be transmitted to healthy plants. From the test of Wahyudi (2016), one of several varieties of sugarcane showing mosaic symptoms to healthy plants has indicated that healthy plants have been infected by mosaic disease. Viruses are known as obligate parasites that must use host cells to survive. Death to the host cell also means death to the virus itself so before the host cell dies the virus will soon infect other host cells as a form of virus survival. Therefore, this study was conducted to ascertain the cause of symptoms of mosaic disease seen in some varieties of sugar cane which is considered superior and commercial by some sugar factories from several cities in East Java, Indonesia, as well as to know the type of virus that causes the mosaic disease through RT- PCR.

Molecular detection of mosaic viruses through RT-PCR

To determine whether the cause of symptoms of mosaic disease seen in some varieties of sugarcane is caused by mosaic virus or not then molecular virus molecular detection through RT-PCR method. As has been reported by some Indonesian researchers (Asnawi, 2009; Damayanti and Putra, 2010; Putra et al. 2013), there are several types of viruses that are capable of causing mosaic sugarcane disease namely SCMV and SCSMV.

Viswanathan (2010) states that serology is the most common detection method used to detect the presence of viruses because it is fast, cheap and suitable for specific viruses, but the enzyme linked immunosorbent assay (ELISA) method is limited by the availability of antisera, especially for new viruses identified in the sugarcane plant such as SCSMV, and in some cases the specificity of the antisera is considered less when the resulting antisera contains two or more viruses, for example an antiserum is produced against SCMV but also detected by SCSMV (Viswanathan et al. 2007). In contrast to serological techniques, RT-PCR can be rapidly performed in the laboratory independently after basic protocols and primary sequences are made available (Peiman and Xie, 2006). However, the singleplex-RT-PCR test requires separate amplification of each of the desired viruses that potentially more cost, therefore the multiplex-RT-PCR method is used to detect some types of mosaic virus in a single reaction. This study shows that SCMV and SCSMV cp genes are viral genome areas that can be relied upon for detection by using multiplex-RT-PCR. This study demonstrates the advantages of multiplex-RT-PCR to diagnose two types of mosaic RNA virus simultaneously.

Visualization of RT-PCR products were performed using electrophoresis method with 1% agarose gel. Figure 2 A-B shows the results of the singleplex-RT-PCR test with each of SCMV and SCSMV primers. Figure 2A shows fluorescent band on ±900 bp area for sugarcane samples Ps 864 and Ps 881 as symptomatic of mosaic disease. The fluorescent band is suspected to be a nucleotide of the SCMV virus, so that the symptoms of mosaic disease that attack sugarcane Ps 864 and Ps 881 are suspected to come from SCMV. Figure 2B shows fluorescent band on ±500 bp area for sugarcane samples Ps 862, NXI 1-3, Ps 864 and Ps 881 as symptomatic of mosaic disease. The fluorescent band is suspected to be a nucleotide of the SCSMV virus, so that the symptoms of mosaic disease that attack sugarcane cane Ps 862, NXI 1-3, Ps 864 and Ps 881 are suspected to come from the SCSMV. In Figure 2 A-B, Sugarcane SUT did not show the fluorescent band because SUT was an asymptomatic sugarcane and used as a negative control in this study.

Figure 2C shows the results of a multiplex-RT-PCR test that directly mixes two pairs of SCMV and SCSMV virus primers in a single reaction. From the results of multiplex-RT-PCR assay is suspected all samples of sugarcane mosaic symptom has contained RNA viruses causing mosaic disease cane mosaic. The electrophoretic visual results show that sugarcane Ps 864 and Ps 881 contain two fluorescent bands each on ± 900bp and ± 500bp, whereas sugarcanes Ps 862 and NXI 1-3 there is only one fluorescent band in an area of ± 500bp. This is in accordance with the results of the singleplex-RT-PCR test (Figure 2 A-B). The results of this study are in line with Viswanathan's (2010) study which shows that the multiplex-RT-PCR method is superior to diagnose more than one type of mosaic RNA mosaic virus in one reaction compared to the singleplex-RT-PCR method because the multiplex-RT-PCR method is more practical and time saving.

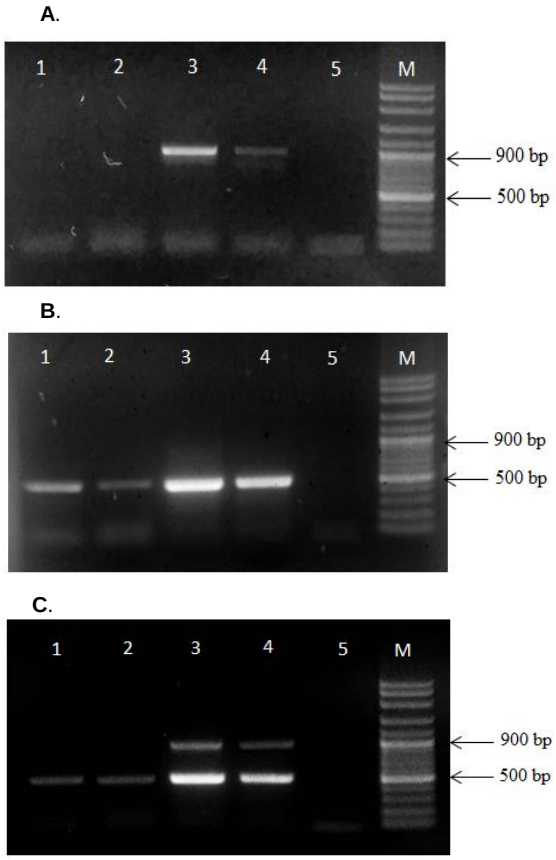

Figure 2: Detection of (A) SCMV and (B) SCSMV by singleplex-RT-PCR and by simultaneously (C) multiplex-RT-PCR. Line 1, Ps 862; line 2, NXI 1-3; line 3, Ps 864; line 4, Ps 881; line 5, SUT (healthy); line M, DNA marker.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Sugarcane mosaic virus isolate TUC-7B polyprotein gene, partial cds	1225	1225	100%	0.0	91%	EU196453.1
Sugarcane mosaic virus isolate TUC-7A polyprotein gene, partial cds	1214	1214	100%	0.0	91%	EU196452.1
Sugarcane mosaic virus isolate TUC-2D polyprotein gene, partial cds	1197	1197	100%	0.0	91%	EU196440.1
Sugarcane mosaic virus isolate TUC-1A polyprotein gene, partial cds	1192	1192	100%	0.0	91%	EU196421.1
Sugarcane mosaic virus isolate TUC-1C polyprotein gene, partial cds	1186	1186	100%	0.0	91%	EU196423.1
Sugarcane mosaic virus isolate TUC-1B polyprotein gene, partial cds	1181	1181	100%	0.0	90%	EU196422.1
Sugarcane mosaic virus isolate TUC-2A polyprotein gene, partial cds	1175	1175	100%	0.0	90%	EU196436.1
Sugarcane mosaic virus isolate TUC-1G polyprotein gene, partial cds	1175	1175	100%	0.0	90%	EU196427.1
Sugarcane mosaic virus isolate TUC-2C polyprotein gene, partial cds	1170	1170	100%	0.0	90%	EU196439.1
Sugarcane mosaic virus isolate TUC-2B polyprotein gene, partial cds	1170	1170	100%	0.0	90%	EU196437.1
Sugarcane mosaic virus isolate ARG-345 polyprotein gene, partial cds	1131	1131	100%	0.0	89%	JX237865.1
Sugarcane mosaic virus isolate TUC-5A polyprotein gene, partial cds	1131	1131	100%	0.0	89%	EU196449.1
Sugarcane mosaic virus isolate SAL-9A polyprotein gene, partial cds	1131	1131	100%	0.0	89%	EU196438.1
Sugarcane mosaic virus isolate SAL-1N polyprotein gene, partial cds	1125	1125	100%	0.0	89%	EU196434.1
Sugarcane mosaic virus isolate SAL-1O polyprotein gene, partial cds	1120	1120	100%	0.0	89%	EU196435.1
Sugarcane mosaic virus isolate TUC-1I polyprotein gene, partial cds	1120	1120	100%	0.0	89%	EU196429.1
Sugarcane mosaic virus isolate TUC-1H polyprotein gene, partial cds	1120	1120	100%	0.0	89%	EU196428.1
Sugarcane mosaic virus isolate TUC-3B polyprotein gene, partial cds	1114	1114	100%	0.0	89%	EU196443.1
Sugarcane mosaic virus isolate SAL-2E polyprotein gene, partial cds	1114	1114	100%	0.0	89%	EU196441.1
Sugarcane mosaic virus isolate TUC-1J polyprotein gene, partial cds	1114	1114	100%	0.0	89%	EU196430.1

Figure 3: The DNA sequence analysis of samples suspected to contain SCMV with the 20 most identical strains already deposited *GenBank* using BLAST.

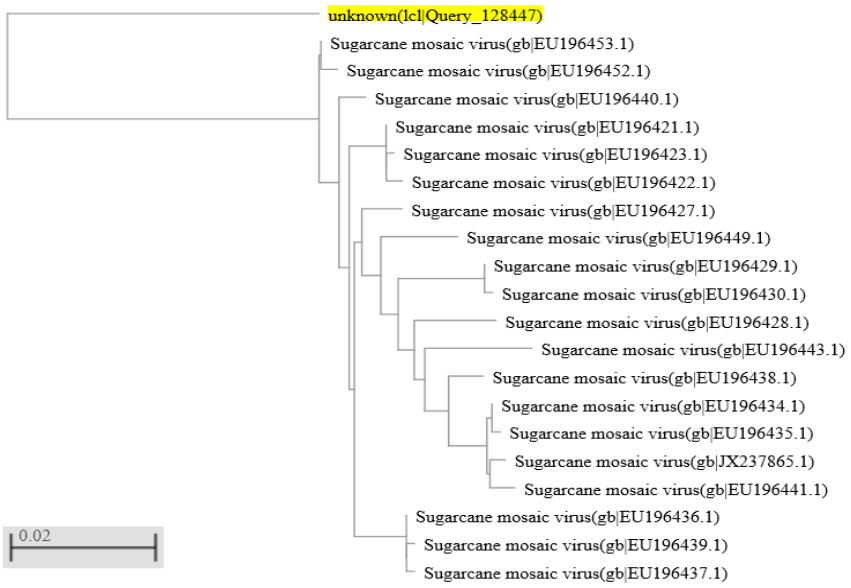

Figure 4: The phylogenetic tree is described by the Blast Tree program through the Neighbor Joining method with the Rectangle Cladogram layout. Unknown, DNA sequence analysis results from samples suspected to contain SCMV.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Sugarcane streak mosaic virus CP gene for coat protein, partial cds	859	859	100%	0.0	98%	AB563503.1
Sugarcane Streak mosaic virus partial mRNA for coat protein (Nlb gene), isolate Co 94012-3	813	813	99%	0.0	96%	AM749393.1
Sugarcane streak mosaic virus coat protein mRNA, partial cds	813	813	99%	0.0	96%	U75456.1
Sugarcane streak mosaic virus isolate PAK, complete genome	808	808	99%	0.0	96%	GQ388116.1
Sugarcane streak mosaic virus isolate IND671, complete sequence	785	785	99%	0.0	95%	JN941985.1
Sugarcane streak mosaic virus coat protein-like mRNA, partial sequence	767	767	100%	0.0	94%	AY887086.1
Sugarcane streak mosaic virus isolate M117 clone NIACP-CL4 polyprotein gene, partial cds	741	741	90%	0.0	96%	KT257289.1
Sugarcane streak mosaic virus isolate M117 clone NIACP-CL1 polyprotein gene, partial cds	741	741	90%	0.0	96%	KT257288.1
Sugarcane streak mosaic virus isolate M117 clone NIACP-CL2 polyprotein gene, partial cds	741	741	90%	0.0	96%	KT257287.1
Sugarcane streak mosaic virus isolate scsmv_tn polyprotein mRNA, partial cds	715	715	100%	0.0	93%	AY193784.1
Sugarcane streak mosaic virus polyprotein mRNA, partial cds	710	710	100%	0.0	92%	AY193783.1
Sugarcane streak mosaic virus isolate ta coat protein mRNA, partial cds	710	710	100%	0.0	92%	AY189681.1
Sugarcane streak mosaic virus isolate Anantapur coat protein gene, partial cds	708	708	100%	0.0	92%	JN626910.1
Sugarcane streak mosaic virus isolate ID, complete genome	708	708	100%	0.0	92%	JF488066.1
Sugarcane streak mosaic virus isolate GX coat protein gene, partial cds	702	702	84%	0.0	97%	JX467699.1
Sugarcane streak mosaic virus isolate TN1 polyprotein gene, partial cds	702	702	99%	0.0	92%	GQ386845.1
Sugarcane streak mosaic virus isolate KA1 polyprotein gene, partial cds	702	702	99%	0.0	92%	GQ386844.1
Sugarcane streak mosaic virus partial mRNA for polyprotein, isolate Andhra Pradesh	699	699	100%	0.0	92%	Y17738.2
Sugarcane streak mosaic virus isolate JP2, complete genome	697	697	100%	0.0	92%	JF488065.1
Sugarcane streak mosaic virus isolate JP1, complete genome	693	693	99%	0.0	92%	JF488064.1

Figure 5: The DNA sequence analysis of samples suspected to contain SCSMV with the 20 most identical strains already deposited *GenBank* using BLAST.

Figure 6: The phylogenetic tree is described by the Blast Tree program through the Neighbor Joining method with the Rectangle Cladogram layout. Unknown, DNA sequence analysis results from samples suspected to contain SCSMV.

Results of genetic relationship analysis between types of mosaic virus

Based on Figure 3, there were 5 highest sequences with the highest identical value of 91% of the sequence of DNA samples suspected to contain SCMV which is identical with SCMV sequence with *GenBank* code EU196453.1. Supported by Figure 4, these results explain that the symptoms of mosaic disease in true sugarcane samples are caused by SCMV with an identical value of 91% for SCMV EU196453.1. This means that SCMV that attacked sugarcane plantations in East Java, Indonesia, on two varieties of sugarcane namely Ps 864 (Magetan district), Ps 881 (Madiun district) is a SCMV cluster that is closely related to SCMV EU196453.1 isolate from Argentina.

Based on Figure 5, there were 1 highest sequence with the highest identical value of 98% of the sequence of DNA samples suspected to contain SCSMV which is identical with SCSMV sequence with *GenBank* code AB563503.1. Supported by Figure 6, these results explain that the symptoms of mosaic disease in true sugarcane samples are caused by SCSMV with an identical value of 98% for SCSMV AB563503.1. This means that SCSMV that attacked sugarcane plantations in East Java, Indonesia, on four varieties of sugarcane namely Ps 864 (Magetan district), Ps 881 (Madiun district), Ps 862 (Situbondo district), and NXI 1-3 (Jember district) is a SCSMV cluster that is closely related to SCSMV AB563503.1 isolate from Indonesia.

The results of this BLAST analysis are the same as those reported by Frankel et al. (1989) which states that the equivalence of nucleotide sequences of the Potyvirus group ranges between 83-99% for strains in the same viruses and 39-53% for strains in different viruses. If a virus has a nucleotide similarity level <79.4%, the virus enters another group (Tsai et al., 2008).

The similarity of virus isolates either SCMV or SCSMV obtained from the results of this study with isolates from other countries allegedly occurred when there was exchange of varieties from sugar producing countries in the world. This is very possible considering almost all the superior varieties circulating in Indonesia is the result of research from one research institute center that is P3GI Pasuruan, East Java, Indonesia. P3GI is the oldest sugar research institution in the world. Some varieties of breeding P3GI, for example POJ 2878 has been sowing on the northwest side of Columbia (Thamrin, 2013).

Genetic differences in viruses can be attributed to viral evolution in host populations in their home countries along with their many vector species, as well as differences in environmental conditions that predominate in the tropics and

subtropics. According to Agrios (2005), viral evolution leads to environmental compatibility, such as host plants, viral strains, and vector insects. Mutations in plant virus often occur during genetic recombination, especially when replicating inside the host cell. Recombination between RNA genomes from a species of virus is one of the causes of biological variation in plant virus (Matthews, 2002). In addition recombination may occur if two identical strains of the virus infect the same plant, resulting in a new strain (recombinant) that has properties different from those of the original strains (Agrios, 2005).

CONCLUSION

There were two types of RNA viruses, SCMV and SCSMV, which caused mosaic disease in four sugarcane varieties at four locations in East Java, Indonesia based on RT-PCR molecular detection either singleplex-RT-PCR or multiplex-RT-PCR. Molecular detection using the multiplex-RT-PCR method is considered superior to singleplex-RT-PCR because it is able to diagnose several types of RNA mosaic sugarcane at once, so it is more practical and time saving. SCMV that attacked sugarcane plantations in East Java, Indonesia on two sugarcane varieties namely Ps 864 (Magetan district), Ps 881 (Madiun district) is a SCMV cluster closely related to the SCMV isolate (EU196453.1) from Argentina. SCSMV that attacked sugarcane plantations in East Java, Indonesia on four sugarcane varieties namely Ps 864 (Magetan district), Ps 881 (Madiun district), Ps 862 (Situbondo district), and NXI 1-3 (Jember district) is a SCSMV cluster closely related to the SCSMV isolate (AB563503.1) from Indonesia. RNA viruses that cause sugarcane mosaic disease in Indonesia, SCMV and SCSMV, can be detected simultaneously by multiplex-RT-PCR method.

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CONFLICT OF INTEREST

The present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

ND designed the experiments. RKA performed the experiments, data analysis and also wrote the manuscript. YSWM and SPAW reviewed the manuscript. All authors read and approved the final version.

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