Screening and characterization of molecular markers for sugarcane (*Saccharum officinarum* L.) resistance to shoot borer pest

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**Abstract.** Sugarcane (*Saccharum officinarum* L.) is one of the main crops in Indonesia and is known as a sugar-producing plant. Sugar belongs to one of the 9 essential culinary ingredients in Indonesia. With the high demand for sugar the government in 2017 imported 109,147 tons of sugar or equivalent to IDR 823.8 billion to fulfill the demand. Efforts to increase sugarcane productivity are often constrained by complex pests that attack sugarcane shoots. One way to overcome it is to breed plant varieties that are resistant and of good quality. The purposes of the present study were to screen sugarcane shoot borer resistance gene using the molecular marker *SacMPI*-Like 3, to determine the phylogenetic relationship between *MPI* in sugarcane with other species in the family Andropogoneae, and to determine the most effective molecular markers for detecting *MPI*. The primer was designed by using Primer3 Plus. DNA samples of 22 sugarcane varieties derived from the Genetics and Breeding Laboratory, Biology Faculty of Gadjah Mada University were PCR-amplified using 3 selected primer designs. Results showed that all of the 22 samples of sugarcane DNA contained *SacMPI*-Like 3 ranged from 500 to 600 bp. Some of the samples were sequenced to obtain the arrangement of nucleotide bases for comparison with samples from the NCBI GeneBank. The phylogenetic tree analysis showed that 3 large clusters were formed depended on the presence of *MPI*. The first cluster consisted of *SacMPI1_PS_58*, *SacMPI1_PS_951*, *SacMPI2_PS_384*, *SacMPI2_PS_951*, *SacMPI3_PS_851*, *CA282462.1_SHC* (*Saccharum officinarum* L.), *XR002447315.1_SH*, *XR002447314.1_SH* (*Sorghum bicolor*), *XR003227569.1_PH*, *XR003227568.1_PH* (*Panicum hallii*) *EU972719.1_ZM*, *NM001158424.2_ZM*, *EU960955.1_ZM*, and *EU951995.1_ZM* (*Zea mays*). The second cluster consisted of *XM024458314.1_BD* (*Brachypodium distachyon*), *CT827984.1_OZ*, and *XM026021872.1_OZ* (*Oryza sativa*).
1. Introduction

Sugarcane (*Saccharum officinarum* L.) is a large monocotyledonous plant and is often cultivated in tropical and subtropical regions worldwide. It is known as a plant that effectively stores large amount of biomass of fiber, sucrose or sugar in its trunk. The latest statistical data from the Directorate General of Plantations reported that the total sugarcane plantation area throughout Indonesia was 447,350 Ha with a total production of 2,363,042 tons [1, 2]. The high consumption of sugar in Indonesia led the government to import 109,148 tons of sugar worth IDR 823.8 billion in 2017.

Due to a number of improper agronomic techniques and the presence of complex pests that attack sugarcane shoots, sugar cane production in Indonesia continues to face such constraints as productivity is decreasing. The pests remain to be a production inhibiting factor since the amount of loss can reach 20% [3]. Sugarcane shoot-borer insect pests may include *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae) [4, 5] *Scirpophaga nivella* [6], *Scirpophaga excerptalis*, [3] and *Chilo infuscatus* [7].

Among the sugarcane shoot borers are the moth larvae of *Chilo infuscatus*. Sugarcane shoot-borer larvae make vertical holes in the stem and shoot which become the main pathways for microorganisms such as fungi and bacteria to enter. These microbes take sucrose for their growth and reduce sugarcane sucrose contents [8]. The decay-causing fungi *Colleotrichum falcatum* and *Fusarium verticillioides* are often found in holes made by these insect pests [4]. These pests attacks on young sugarcane could cause death, while attacks on 5 mo old or older sugarcane could cause the formation of lateral shoots, hence cause a decrease in sugarcane production [9].

The purposes of this study were to determine the results of screening and characterization of the molecular markers for *SacMPI*-Like 3, which was a shoot borer resistance gene in the 22 analyzed sugarcane varieties and to determine the character of *SacMPI*-Like 3 which encode resistance to sugarcane shoot borers.

2. Methods

2.1. Sampling

A total of 22 sugarcane genomes from 22 varieties were obtained from the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, consisted of PS 41, PS 58, PS 384, PS 851, PS 862, PS 864, PS 865, PS 881, PS 891, PS 921, PS 951, PS 80.1649PSCO 902, PSBM 901, Bulu Lawang (BL), Kidang Kencana (KK), Kentung, VMC 76-16, PSDK 923, TLH 2 and BZ 132.

2.2. Primer designs produced by the Primer3 Plus

The *SacMPI*-Like 3 used as the reference for primer design was obtained from the National Center for Biotechnology Information (NCBI) with the accession number CA282462.1 [4]. The DNA sequence of *SacMPI*-Like 3 was downloaded from the NCBI website at http://www.ncbi.nlm.nih.gov and subsequently copied and saved in notepad. The DNA sequences were then inputted to the Primer 3 Plus page for primer design.

2.3. DNA selection and amplification based on molecular markers

DNA was amplified using the Applied Biosystems Veriti Thermal Cycler PCR machine. The premix of each reaction consisted of 12.5 μL of Bioline My Taq HS DNA Polymerase PCR kit per reaction, 8.5 μL of sterile ddH2O, 1 μL of forward and reverse primer at 25 ng/μL, and 2 μL of DNA samples at 20 ng/μL.

The PCR amplification protocol included stages of predenaturation at 94 °C for 60 sec, denaturation at 94 °C for 45 sec, annealing at 65 °C for 75 sec, elongation at 72 °C for 45 sec, post-elongation at 72 °C for 70 sec. Denaturation, annealing and elongation stages were repeated 39 times.
The PCR amplification products were analyzed using 2% agarose gel electrophoresis. Electrophoresis results were observed using a gel doc system to visualize DNA bands from electrophoresis and compared with 100-bp Promega marker to determine the length of DNA.

2.4. Sequencing of PCR products
DNA bands were subsequently sequenced to determine the sequence of nitrogen bases. DNA was sequenced by DNA sequencing service provider, the 1st Base Sequencing Division of PT. Genetika Science. Samples sent for sequencing were PS 58 and PS 951 samples that have been amplified using the MP1 primer, hereinafter referred to as SacMPI1_PS_58 and SacMPI1_PS_901. The PS 384 and PS 951 samples were subsequently amplified using the MP2 primer, hereinafter referred to as SacMPI2_PS_384 and SacMPI2_PS_901. The PS 851 and PS 951 samples that have been amplified using the MP3 primer are hereinafter referred to as SacMPI3_PS_851 and SacMPI3_PS_901.

2.5. Analysis of phylogenetic relationship based on SacMPI-Like 3 sequence
Results of DNA sequencing in the form of nucleotide base sequences were further processed using the Gene Studio, Mesquite, and MEGA7 applications.

3. Results and discussion

3.1. Results of Primer Selection
The present study required primers specific to the genes to be detected, so that the primers were designed manually according to the research requirements.

| Primer | Size | Start | Length | Tm (°C) | GC% | any | 3’ | Sequences                  |
|--------|------|-------|--------|---------|-----|-----|----|---------------------------|
| MP1 (F)| 458  | 1     | 22     | 58.79   | 50.00| 4.00| 1.00| CAAGCAGTACGTTAGGGTCATC    |
| MP1 (R)| 458  | 21    | 21     | 58.66   | 47.62| 6.00| 3.00| GGATCCGTACAGAAAAGATGC     |
| MP2 (F)| 455  | 4     | 22     | 58.79   | 50.00| 4.00| 1.00| GCAGTACGTTAGGGTCATCAAG    |
| MP2 (R)| 458  | 21    | 21     | 58.66   | 47.62| 6.00| 4.00| GGATCCGTACAGAAAAGATGC     |
| MP3 (F)| 450  | 9     | 21     | 58.46   | 47.62| 0.00| 0.00| ACGTATGGGTCATCAAGGAT      |
| MP3 (R)| 458  | 22    | 22     | 58.47   | 45.45| 0.00| 0.00| GGATCCGTACAGAAAAGATGCA    |

Each primer had several characters (Table 1). ‘Start’ was the initial position of the primer and showed the location of the first base at the 5’ end of the primer. ‘Length’ or oligo length was the primer length which was the sum of all nucleotide bases of the primer. The manually designed primer length ranged from 21 to 22 nucleotide bases [10]. The ‘Tm’ or melting temperature was the primer melting temperature which served as the primer size for the annealing process during the PCR amplification. The percentage of nucleotide bases of guanine and cytosine found in the primer was denoted as GC%. The character ‘any’ was self-complementary which was a measure of possible attachment of a primer region to another primer region in one primer to form a secondary structure. The character ‘3’ was a measure of possible attachment of the 3’ region of a primer to the 3’ region of the primer itself so as to form a dimer primer. The character ‘size’ or product size was the size of the area of the template to be PCR-amplified. The size of the target genes amplified by the primers MP1, MP2 and MP3 is 458, 455 and 450 bp, respectively.
3.2. 

SacMPI-Like 3 amplification products

SacMPI-Like 3 was amplified from DNA samples of 22 sugarcane varieties at 20 ng/µL. The PCR amplification products were observed using the electrophoresis method. The 100-bp Promega was used as the marker to determine the length of the gene. A band of specific length on the marker was used as size reference for the electrophoresed genes.

Figure 1 was shown the presence of SacMPI-Like 3 gene between 500–600 bp in all sugarcane samples. The 1463 bp and 232 bp DNA were detected using the MP1 primer, while 1232 bp DNA was detected using the MP3 primer. However, the MP2 primer only detected the SacMPI-Like 3 gene at 583 bp, indicated by the emergence of only a single band in all samples. It showed that MP1 and MP3 primers were not specific to detect only SacMPI-Like 3 gene. In their study, Tamayo et al. subjected corn leaves to physical wounding treatment in order to demonstrate MPI responses [11]. The responses were marked by the appearance of mRNA after the physical wounding treatment of the leaves. Prior to the physical wounding treatment, no MPI mRNA was detected on the corn leaves. However, the MPI mRNA was detected after 2 hr of treatment. At 6 and 12 hr of treatment the MPI mRNA levels were increased. This study demonstrated that all plants had MPI, included the sugarcane samples used in the present study. Figure 1 showed the presence of the gene after amplification using the primers MP1, MP2, and MP3. However, the levels of expression depended on the duration of wounding on the plants.

![Figure 1](image-url)

**Figure 1.** Amplified resistance genes of all sugarcane samples using primers; (a) MP1; (b) MP2; (c) MP3 M: 100-bp. Notes: Promega marker; 1) PS 41; 2) PS 58; 3) PS 384; 4) PS 851; 5) PS 862; 6) PS 864; 7) PS 865; 8) PS 881; 9) PS 891; 10) PS 921; 11) PS 951; 12) PS 80910; 13) PS 801649; 14) PSCO 902; 15) PSBM 901; 16) Bulu Lawang (BL); 17) Kentung; 18) Kidang Kencana (KK); 19) BZ 132; 20) TLH 2; 21) PSDK 923; 22) VMC 76-16.
**SacMPI-**Like 3 was a resistance gene in sugarcane which was analogous to *Maize Proteinase Inhibitor* (MPI) found in corn plants. Madeiros et al. stated that *SacMPI*-Like 3 also had similarities with the potato type 1 inhibitor family. Potato type 1 proteinase inhibitor family was one of the several members of the Protease Inhibitor (PI) in plant tissues [4]. The members of PI were Soybean trypsin inhibitor family, Bowman-Birk inhibitor family, Barley trypsin inhibitor family, Potato inhibitor I family, Potato inhibitor II family, Squash inhibitor family, Maize Bifunctional inhibitor family, Carboxypeptidase A and B inhibitor family, Cysteine Proteinase inhibitor family, and aspartyl proteinase inhibitor family [12]. *SacMPI*-Like 3 generally respond to both physical damage and insect pest attacks [4]. It is similar to *MPI* that encodes proteins whose mRNAs accumulate responses to physical attacks. *MPI* expression play a role in the inhibition of larval growth rate of several pest species [11].

### 3.3. DNA sequencing results of several samples

Several sugarcane samples found to have *SacMPI*-Like 3 were sequenced to determine the sequence of nucleotide bases of the gene in the representative samples. DNA was sequenced by DNA sequencing services. The results in the Table 2 corresponded to those of electrophoregram of samples amplified with the primers MP1, MP2, and MP3 to determine the presence of *SacMPI*-Like 3. The length of sequenced DNAs did not differ to a great extent from the estimated length of DNAs in the electrophoregram, ranged from 500 to 600 bp.

| No. | Samples       | Contig | Initial length | Final length | Ambiguities |
|-----|--------------|--------|----------------|--------------|-------------|
| 1   | *SacMPI1* _PS 58_ | 1      | 556            | 509          | 43          |
| 2   | *SacMPI1* _PS 951_ | 1      | 554            | 554          | 25          |
| 3   | *SacMPI2* _PS 384_ | 1      | 550            | 509          | 63          |
| 4   | *SacMPI2* _PS 951_ | 1      | 545            | 509          | 19          |
| 5   | *SacMPI3* _PS 851_ | 1      | 550            | 515          | 24          |
| 6   | *SacMPI3* _PS 951_ | 1      | 540            | 530          | 22          |

A good sequencing result could form one contig, in which during the sequencing the forward and reverse primers could meet and cut certain gene strands. Ambiguities were the results of imperfect nucleotide base readings, lead to the ‘N’ notation on the sequenced nucleotide bases, rather than nucleotide bases. As a consequence, it was not known whether these ambiguous bases were guanine, adenine, cytosine, or thymine bases. These ambiguous bases should be removed or replaced by looking at its complementary bases. Subsequently, some BLAST results were copied and compared with the DNA sequence of tested sugarcane samples to determine the phylogenetic relationship.

### 3.4. Results of phylogenetic tree construction

The phylogenetic tree was constructed using the MEGA7 application and the Neighbor Joining (NJ) method using Kimura-2-Parameter with 1000X bootstrap. Figure 2 was shown the phylogenetic relationship of 18 accession numbers. The formation of clusters was related to the genes belong to each accession number. In general, 3 large clusters were formed, where the first cluster were consisted of *SacMPI1* _PS_58, *SacMPI1* _PS_951, *SacMPI2* _PS_384, *SacMPI2* _PS_951, *SacMPI3* _PS_851, *SacMPI3* _PS_851, *SacMPI3* _PS_951, and CA282462.1_SHC (*Saccharum officinarum* L.). They were found to have *SacMPI*-Like 3, namely *Maize Proteinase Inhibitor* (MPI) found in *S. officinarum* L. Genomic clones and cDNA of MPI have a high homology with members of potato I proteinase inhibitor family [11]. Furthermore, the other members were XR002447315.1_SB and XR002447314.1_SB (*Sorghum bicolor*). Based on information from the NCBI, XR002447315.1_SB and XR002447314.1_SB had an uncategorized gene at the locus LOC8067309 on chromosome
number 9 of *Sorghum bicolor*. The next members were XR003227569.1 PH and XR003227568.1_PH (*Panicum hallii*). Based on the NCBI, XR003227569.1 PH and XR003227568.1_PH had an uncategorized gene at the locus LOC112887497 on chromosome number 3 of *Panicum hallii*.

The only members of the second cluster were EU972719.1_ZM, NM001158424.2_ZM, EU960955.1_ZM, and EU951995.1_ZM (*Zea mays*). Based on information from the NCBI, EU972719.1_ZM, NM001158424.2_ZM, EU960955.1_ZM and EU951995.1_ZM had an uncategorized gene at the locus LOC100285533 of *Zea mays*, described as subtilisin-chymotrypsin inhibitor CI-B1.

The 3rd cluster consisted of XM024458314.1_BD (*Brachypodium distachyon*). Based on the information from the NCBI, XM024458314.1_BD had a gene at the locus LOC112270584, described as subtilisin-chymotrypsin inhibitor 2A-like on chromosome number 2 of *Brachypodium distachyon*. Furthermore, the next members were CT827984.1_OZ and XM026021872.1_OZ (*Oryza sativa*). Based on the information from the NCBI, both accession numbers had a gene at the locus LOC112937351, described as subtilisin-chymotrypsin inhibitor CI-B1.

The number next to the branching was the percentage repetition of the branching pattern (Figure 2). The higher the number the more replications were performed by bootstrap to form the branching pattern. If the bootstrap percentage number was below 60%, the branching pattern formed was not strong since 1000 bootstrap replications only <600 times the branching pattern appear and vice versa.

**Figure 2.** Phylogenetic tree analysis of all samples using BLAST.

The phylogenetic tree showed that the primers MP1, MP2, and MP3 indicated the presence of SacMPI-Like 3 in sugarcane (Figure 2). With regard to the closeness of branching of samples to the control *Saccharum hybrid cultivar* (CA282462.1), the PS 58 samples amplified with the primer MP1 showed a branching closest to the control relative to other samples amplified with the primers MP2 and MP3. This showed that the primer MP1 was most effective to amplify the SacMPI-Like 3 in those samples relative to other primers.

4. **Conclusions**

There are 3 molecular markers produced by primer designs, namely MP1, MP2, and MP3. They are used in the PCR amplification for further visualization by the electrophoresis method. Those primers are successfully amplified the target gene in 22 sugarcane varieties. The character of the 3 designed
molecular markers qualifies as good molecular markers for the PCR process. The character of shoot borer resistance gene (SacMPI-Like 3) in all varieties is of 500–600 bp in length. MP2 primer shows specific result to detect SacMPI-Like 3 gene than other primers. The constructed phylogenetic tree shows that the formation of 3 large clusters based on the presence of the resistance genes belong to several accession numbers analyzed. The first cluster consists of SacMPI1_PS_58, SacMPI1_PS_951, SacMPI2_PS_384, SacMPI2_PS_951, SacMPI3_PS_851, SacMPI3_PS_951, CA282462.1_SHC (Saccharum officinarum L.), XR002447315.1_SB, XR002447314.1_SB (Sorghum bicolor), XR003227569.1_PH, and XR003227568.1_PH (Panicum hallii). The second cluster consist of EU972719.1_ZM, NM001158424.2_ZM, and EU960955.1_ZM, and EU951995.1_ZM (Zea mays). The third cluster consists of XM024458314.1_BD (Brachypodium distachyon), CT827984.1_OZ and XM026021872.1_OZ (Oryza sativa).

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