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CROSSABILITY ELUCIDATION BETWEEN *Saccharum* spp. and *Erianthus* sp. ACCESSIONS USING SSR MARKER

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SUMMARY

Commercial sugarcane varieties, which are widely planted in Indonesia, originated from intraspecific and/or interspesific hybridization of *Saccharum* spp. However, hybridization in sugarcane is not always easy to do. The morphology of sugarcane flower and environment are two factors that influence the success of hybridization. The similarity of phenotypic appearance of progeny and parent is another limiting factor in indentifying the hybrid. The objective of this study was to identify the progeny from intra and interspecific hybridization as true hybrid, selfing, or off type using simple sequence repeat (SSR). This study was conducted in the Molecular Genetics laboratory of the Indonesian Sweetener and Fiber Crops Research Institute (ISFCRI) in Malang, Indonesia from August 2016 to July 2017. There were 91 genotypes consisting of four *Saccharum* spp. and one *Erianthus* sp. as parent and 86 F1 intra and interspecific progeny. Identification of putative hybrids was done by comparing the visualization band result from electrophoresis of male and female parent genetic marker in F1 hybrid. All primers could identify on average 62.7%, 52.44%, and 38.89% of the progenies as hybrid in crosses among sugarcane commercial varieties, between sugarcane commercial varieties and S. *spontaneum*, and between sugarcane commercial varieties and *Erianthus* sp. Further, primers also identified in average 8.02%, 30.21%, and 24.44% of the progenies as selfing from crosses among sugarcane commercial varieties, between sugarcane commercial varieties and S. *spontaneum*, and between sugarcane commercial varieties and *Erianthus* sp. Also, 29.42%, 17.46%, and 36.11% of the progenies could be identified as off type by primers from crosses among sugarcane commercial varieties, between sugarcane commercial varieties and between S. *spontaneum*, and crossing sugarcane commercial varieties and *Erianthus* sp.

Key words: Sugarcane, *Saccharum* spp., *Erianthus* sp, F1, intraspecific, interspecific, SSR

Key findings: DNA marker analysis was used to identify progeny as hybrid, selfing, or off type by the differences in its genetic make-up. This information enabled selection of the best parent combination with high heterogeneity and complementary traits to maximize heterosis and to assist in widening the germplasm base. The choice of male or female parent was equally important in sugarcane breeding programs.

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INTRODUCTION

Commercial sugarcane varieties that are widely planted in Indonesia and worldwide originated from intraspecific and/or interspecific hybridization of *Saccharum officinarum* L. (*noble cane*), *S. barberi* Jeswiet, *S. sinense* Roxb., and its wild relatives *S. spontaneum* L. and *S. robustum* Brandes, and Jeswiet ex Grassl (Price, 1963; Stevenson, 1965; D'Hont *et al*., 1996;; Singh *et al*., 2011). In addition, improvement of sugarcane through hybridization was also achieved by introgression of superior genes from existing varieties, hybrid clones, or another Saccharum complex genus (*Erianthus* sp.; *Mischantus* sp.) (Price, 1965: Glowacka *et al.*, 2016; Gao *et al.*, 2015; Jing *et al.*, 2009; Cai *et al.*, 2005; Aitken *et al*., 2007; Nair *et al*., 2006).

Compared to other crops, hybridization programme in sugarcane had lower chance of success. Sugarcane has a perfect flower (bisexual) and is very small in size, as doing emasculation cannot guarantee 100% elimination of the female's pollen viability. Furthermore, it also needed flowering induction in the sub-tropic region (Heinz and Tew, 1987). The other limitation is the possibility of both cross and self-pollination occurrence resulting into hybrid and selfing progenies (McIntyre and Jackson, 2001; Santos *et al.*, 2014). The environment is also a critical influencing factor in flowering and limiting the fruit set of hybridization either before, during, or after hybridization (Stevenson, 1965; Heinz and Tew, 1987; Moore and Berding, 2014).

During hybridization, the possibility of selfing varied from 0% to 8.69% under controlled environments (Nagarajan, *et al.*, 2001; McIntyre and Jackson, 2001; Tew and Pan, 2010; Santos, *et al.*, 2014) and between 0% to 98.5% under uncontrolled environment (inadequate temperature and humidity) (Melloni, *et al.,* 2014). Alongside with that, there were possibility of crossing between female parent and foreign pollen (off type). Tew and Pan (2010) and Santos *et al.* (2014) reported that there were 6.89% - 23.95% off type progenies.

Identification of true hybrid progeny using morphology or phenotypic markers was limited especially for characteristics that were influenced by environment. Another challenge was that the similarity between the progeny and parent especially with the female parent and sugarcane as a polyploid plant with high heterozygosity will generate various phenotypic diversity as a result of the high number of

chromosomal segregation in interspecific hybridization (Aitken *et al*., 2007; Wang *et al*., 2009). Identification of the progeny from hybridization is important to guarantee that selected genotypes are truly from crossing and not from selfing or fertilization by foreign pollens (Stevenson, 1965; Nagarajan, *et al.*, 2001).

Until the early 1900s, there were only few information about the genome or genetic composition of sugarcane in the molecular level. The complexity of its genome or genetic composition, the long life cycle, and the absence of specific molecular markers were the limiting factors. (Singh *et al.*, 2008). However, in the onset of biotechnology, it gave rise in the utilization of molecular markers in sugarcane. One of the molecular markers that has been commonly used in sugarcane is the microsatellite or *simple sequence repeats* (SSR). SSR is a tandem repeat of DNA with one until seven base pairs, co-dominant inheritance, multiallelic, and is found in huge amount and spreaded all throughout the genome. One of the utilizations of SSR in sugarcane is to identify and authenticate progenies from hybridization programmes (Santos *et al*., 2014; Costa *et al.*, 2014; Gao *et al.*, 2015; Jing *et al*., 2009; Aitken *et al.*, 2007; Nair *et al.*, 2006; Cai *et al.*, 2005; McIntyre and Jackson, 2001). Identification and authentication of the progenies were done by identifying specific allele of male and female parent (Tew and Pan, 2010; Santos *et al.*, 2014; Melloni *et al*., 2014; Xavier *et al*., 2014). These specific alleles of parents will distinguish the progeny as a true hybrid, selfing, or off type (fertilized by foreign pollen).

There were many studies that has already been done prior to this research that routinely used molecular markers in some sugarcane breeding programs as a tool to analyze their results, especially for confirmimg the hybrids if either interspecific (Govindaraj *et al*., 2012; Aitken *et al.*, 2007; Gao *et al.*, 2015; Jing *et al*., 2009; Nair *et al.*, 2006; Cai *et al.*, 2005; Nair *et al.*, 2017; Fukuhara *et al.*, 2013) or intraspecific (Xavier *et al.*, 2005; Tew and Pan, 2010; Pan *et al.*, 2015) separately. Unlike in some previous works, in this study we tried to compare these different types of crosses together in producing hybrids using SSR markers.

The aim of this study was to determine the molecular efficiency of identification of the progeny from intra and interspecific hybridization as a true hybrid, selfing, or an off-type using simple sequence repeat (SSR).

MATERIALS AND METHODS

Eighty six (86) intra and interspecific F1 progenies which were derived from 12 different biparental crosses and five parents were used in this study. The parents were three sugarcane commercial varieties (VMC 7616, PS 881, and PSJT 941) as female and/or male parent and two wild relatives (*S. spontaneum* dan *Erianthus* sp.) as a male parent. The study was conducted in the Molecular Genetics laboratory of the Indonesian Sweetener and Fiber Crops Research Institute (ISFCRI) in Malang, Indonesia from August 2016 to July 2017. Crossing was conducted in Karangploso experimental garden of ISFCRI, from January to December 2014.

The crosses involved all the possible biparental combinations of

VMC 7616, PS 881, and PSJT 941 as female and/or male parent and *S. spontaneum* and *Erianthus* sp. as male parent. Crossing was done by the marcotting method (Heinz and Tew, 1987; Stevenson, 1965). Generally, five to ten nodes above the base of stalks secured with plastic sleeve, filled with moist soil so that three or four nodes were covered, and then were watered to ensure rooting. Stalks were selected from the field at a stage just prior to inflorescence emergence from the tip of panicle, which was marked by the appearance of the flag leaves. Then the rooted stalks were cut and moved to the crossing house after about four to five weeks when profuse rooting had been taken place. During crossing and seed setting, the stalks were placed in buckets of Hawaiian solution and were changed weekly. At crossing time, the male and female inflorescence were positioned together inside the lantern with the male placed above the female. Pollen test was conducted before crossing. The pollen grains were separately collected and observed microscopically after emasculation and staining with iodine solution and then placed in 70% alcohol for 5 minutes to eliminate pollen viability of the female parent. Furthermore, the progeny of each cross had been selected by their phenotypic appearance (as similar in appearance of both parents) for agronomic type only.

Three from 15 primers based on Pan's (2006) research were selected and which produced polymorphic bands across five parents. The three primers were mSSCIR43, mSSCIR66, and SMC119CG. DNA was extracted from the tissue of young leaf roll using GeneAll exgene Plant SV mini kit (General bio-system, Korea) following the instructions from the kit. Quality of the DNA was checked on 0.5% agarose gels in 70ml TBE 0.5x and electrophoresis at 100V for 30 min.

DNA amplification was done using PCR machine (Sensoquest Lab Cycler, Germany) and PCR Kit GoTaq® Green Master Mix (Promega Corporation, USA). PCR analysis was conducted in the final reaction volume of 25 µL containing 1 µL template DNA; 1 µL of each primer (forward and reverse); 9.5 µL dh_2O and 1 unit Master Mix. PCR reaction was conducted at 94°C for 5 min, followed by 35 cycles of denaturation (94°C for 1 min), annealing (2 min at 45.5°C for mSSCIR43, 41.3°C for mSSCIR66, and 63.5°C for SMC119CG), extension (1 min at 72°C) and final extension (72°C for 5 min).

Amplification products were separated by electrophoresis on 2% gel agarose (75% Metaphore and 25% agarose) in 70ml 1x TBE buffer and GelRed™ Nucleic Acid Stain Biotium with US Patents at 100V for 180 min. After electrophoresis, the SSR products were visualized under UV transilluminator and documented using Geldoc Wealtec KETA (Wealtec Corp).

The polymorphic band assessment was based on the visualization of band size differences of the sample and the genetic marker used as male and female parent of the progeny. The progeny of each cross were then classified as a true hybrid if there were male or both of the parent specific bands were present while a selfing progeny only have a female parent specific band present, and it is an off type if none of the parent's band was present.

RESULTS

Figure 1 showed that each primer could produce a variety of polymorphic bands with different location in each parent. The marker mSSCIR43 was able to recognize one to three bands with a band size between 181 – 275 bps (basepairs), while mSSCIR66 was able to recognize two to four bands with a band size between 137 – 2500 bps, and the SMC119CG could generated two to eight bands measuring 115 – 3000 bps. Additionally, the primer SMC119CG produced more polymorphic bands compared to the two others primers (mSSCIR43 and mSSCIR66).

In general, all primers had different sensitivity to detect progenies as true hybrid, selfing, or off type (Table 1). SMC119CG could detect selfing rate (0 - 100%) better than mSSCIR43 and mSSCIR66, albeit less sensitive to detect off type. The two primers (mSSCIR66 and SMC119CG) were consistent together (>54%) in detecting six true crosses (50%) from the 12 crosses that had been done. mSSCIR66 and SMC119CG could detect eight desirable crosses (66.67%) in the amount of 54.4% - 100%, however, mSSCIR43 could only detect six desirable crosses (50%) in the amount of 50% - 100%. The eight desirable crosses that had been detected by mSSCIR66 were B, C2, D2, E1, E2, F2, G1, and H; while SMC119CG detected the crosses A, B, C2, D1, E2, F2, G1, and H, and the six desirable crosses that had been detected by mSSCIR43 were A, C2, D1, F1, F2, and H.

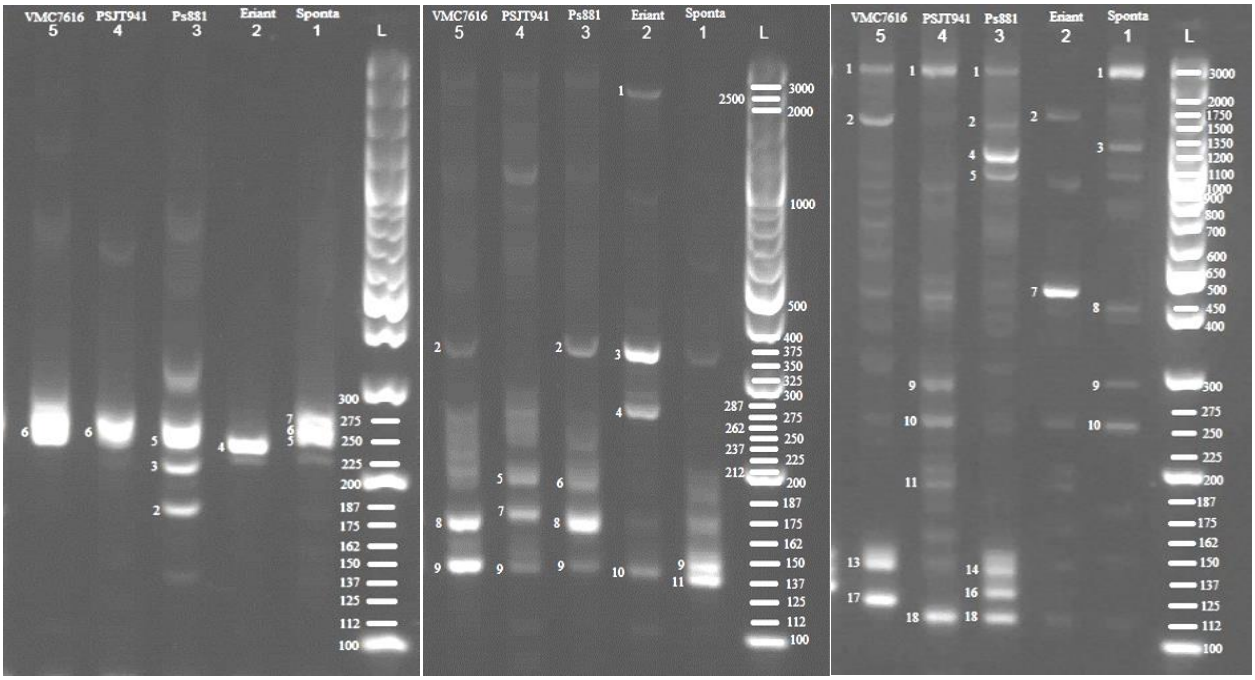

Figure 1. The polymorphism level of three primers in five parents. Left: Primer mSSCIR43; Center: Primer mSSCIR66; Right Primer: SMC119CG.

The effectivity of the primers in detecting progenies as true-hybrid, selfing, and off type from the combination of crosses between three sugarcane commercial varieties with their wild relatives as male parent

(*Erianthus* sp. and S. *spontaneum*) were shown in Table 1. Primer mSSCIR43 could detect 100% of the F2 crossing progenies as true-hybrid. On the other hand, 100 % of E1 crossing progenies were detected as off-types (Figure 2).

Table 1. Percentage (%) of crossing, selfing, and off type of 12 inter and intraspecific hybridization based on band visualization using three selected SSR primers.

Crossing	ΣF1	mSSCIR43			mSSCIR66			SMC119CG		
		1	2	3	1	2	3	1	2	3
Cross A	11	72.7	0.0	28.3	36.4	27.2	36.4	54.5	36.4	9.1
Cross B	11	18.2	9.1	72.2	63.6	0.0	36.4	54.5	27.3	18.3
Cross C2	4	50.0	25.0	25.0	25.0	50.0	25.0	0.0	100.0	0.0
Cross C1	4	25.0	0.0	75.0	100.0	0.0	0.0	100.0	0.0	0.0
Cross D2	7	42.9	0.0	57.1	28.6	0.0	71.4	71.4	28.6	0.0
Cross D1	2	50.0	0.0	50.0	100.0	0.0	0.0	0.0	50.0	50.0
Cross E1	1	0.0	0.0	100.0	100.0	0.0	0.0	0.0	100.0	0.0
Cross E2	8	25.0	0.0	75.0	62.5	25.0	12.5	100.0	0.0	0.0
Cross F1	4	75.0	25.0	0.0	0.0	25.0	75.0	25.0	20.0	50.0
Cross F2	3	100.0	0.0	0.0	66.7	33.3	0.0	66.7	0.0	33.3
Cross G1	6	16.7	16.7	67.7	100.0	0.0	0.0	83.3	0.0	16.7
Cross H	25	70.7	17.2	12.1	78.7	9.1	12.2	85.7	11.4	2.9

1. Crossing; 2. Selfing; 3. Off type; A (VMC7616 × *S. spontaneum*); B (PSJT941 × PS881); C1 (PS881 × *S .spontaneum*); C2 (VMC7616 × PSJT941); D2 (PSJT941 × VMC7616); D1 (VMC7616 × *Erianthus* sp); E1 (PS881 × *Erianthus* sp.); E2 (VMC7616 × PS881); F1 (PSJT941 × *Erianthus* sp.); F2 (PSJT941 × *S. spontaneum*); G1 (PS881 × PSJT941) and H (PS881 × VMC7616).

Primer mSSCIR66 could detect 100% of progenies from the combination of the two crosses as true-hybrid, i.e. crossing between *Erianthus* sp. (as male parent) with female parent VMC7616 (D1, Figure 2) and PS 881 (E1). mSSCIR66 detected 0% progeny of crosses of PSJT and *Erianthus* sp. (F1) as true-hybrid. Whereas, SMC119CG detected 0% progeny from the 3 crosses as true-hybrid, i.e. between VMC7616 with *Erianthus* sp. (D1) and between PS881 as female parent with male parent *Erianthus* sp. (E1) and S. *spontaneum* (C2, Figure 2). This result indicated that in crosses between sugarcane commercial varieties with their wild relatives (*Erianthus* sp. and S. *spontaneum*), the primers mSSCIR66 and mSSCIR43 had the ability to detect the fidelity of crosses better than SMC119CG.

Table 1 also showed the ability of the primers to identify progenies as true hybrid, selfing, and off types from the crosses among sugarcane commercial varieties. Only mSSCIR43 had the ability to detect progenies as hybrid <43% except in crosses H. In crosses H, mSSCIR43 had the ability to detect 70.7% progeny as true hybrid, while 17.2% and 12.2% were selfing and off types, respectively. Conversely, mSSCIR66 and SMC119CG had the ability to detect >60% progeny as hybrid except in crosses D2 (mSSCIR66) and crosses B (SMC119CG). In crosses D2, mSSCIR66 only had the ability to

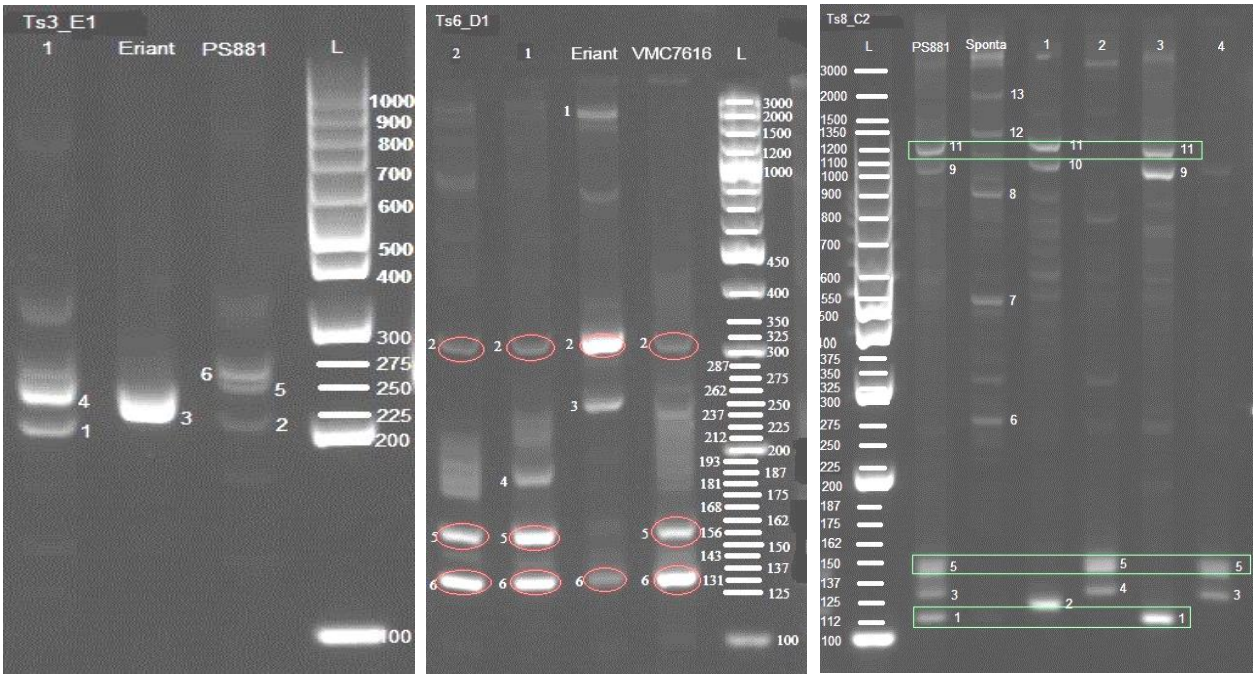

Figure 2. SSR profiles of F1 and their respective parent crossing between sugarcane commercial varieties and their wild type (S. *spontaneum* and *Erianthus* sp.) generated by polymorphic primers. Left: mSSCIR43 (Ts3) in Crosses E1; Center: mSSCIR66 (Ts6) in Crosses D1; Right: SMC119CG (Ts8) in Crosses C2; No.1-4: F1 of each crosses; L: marker lane.

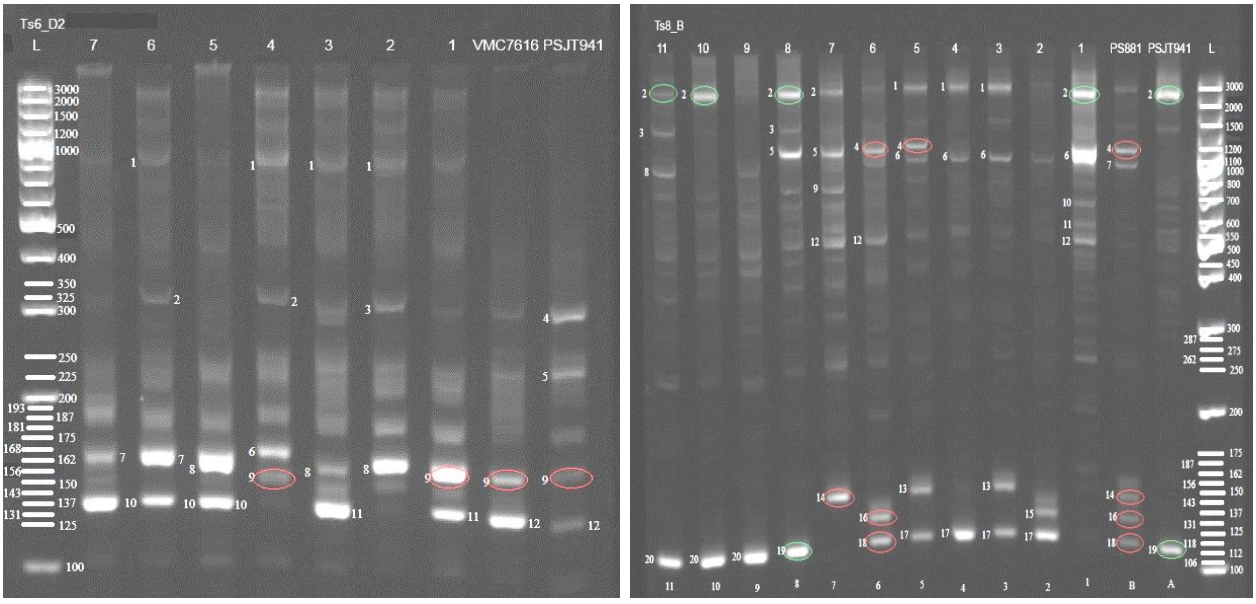

Figure 3. SSR profiles of F1 and their respective parent from crossing among sugarcane commercial varieties generated by polymorphic primers. Left: mSSCIR66 (Ts6) in Crosses D2; Right: SMC119CG (Ts8) in Crosses B; No.1-7 and No.1-11: F1 of each crosses; L: marker lane.

detect 28.6% progeny as hybrid, whereas 0% and 71.4% were detected as selfing and off types, respectively (Figure 3). In crosses B, SMC119CG only had the ability to detect 54.5% progeny as hybrid, whereas 27.3% and 18.3% were detected as selfing and off types, respectively (Figure 3). This result indicated that in crosses among sugarcane commercial varieties, primers mSSCIR66 and SMC119CG had better ability than mSSCIR43 to detect the fidelity crosses.

Furthermore, crosses between sugarcane commercial varieties (VMC 7616, PS 881, and PSJT 941) and sugarcane commercial varieties with S*. spontaneum* provided more occurrence of desirable crosses than undesirable ones. On the other hand, the crosses between sugarcane commercial varieties and *Erianthus* sp. produced more selfed or off types. The wide genetic distance between the two genera was probably caused more occurrences of undesirable crosses.

DISCUSSION

The choice of proper primer is the key in carrying out genetic analyzes based on molecular markers. One of the criteria to choose the proper primer is the ability of the primer to generate polymorphic locus which can be seen from the visualization of electrophoretic images. Polymorphic primer will show the various bands in different base pairs among different samples. The more variability among the band location that could be produced, the more polymorphic and reproducible is the primer. Both polymorphism and reproducibility are the main criteria that a primer must have to be efficient in molecular studies (Powell *et al*., 1995). The success of the primer in identifying the polymorphic locus will provide an overview of the primary ability in the accuracy of genetic analysis between genotypes.

Despite the sugarcane was incorporated as an open pollinated crop, the chance of self pollination still remains high even pollen control was strictly done. In this regards, SSR marker could serve as an effective tool to distinguish hybrids from selfing progenies because the selection based on morphological trait of promising hybrids among crosses is often unreliable (Heinz and Tew, 1987). SSR markers are valuable in dealing with the complexity created by the interspecific hybrids. Additionally, sugarcane and wild species have similar phenotypic properties. Hence, it is difficult to morphologically identify the crossed hybrids (Wang *et al.*, 2009).

Parera *et al*. (2012) reported that of 57 morphological traits based on UPOV (2005), only eight traits exhibited stability in different locations and seasons. In addition, aside from morphological markers, Parera *et al*. (2012) also used AFLPs and SSRs as DNA markers. The results showed that the use of DNA markers was better in identifying genetic diversity, genetic similarity, coefficient of parentage (f), and determining the results of crosses through segregation patterns. The use of SSR markers was better than AFLP. In terms of monitoring genetic diversity and identification of germplasm, Silva *et al.* (2012) also reported the ability of SSR primers to discriminate and determine unique genetic profiles. Thus, this unique genetic profiles could be used in DNA fingerprinting for the protection of new

varieties developed by the breeding program.

Unlike in previous research, in this study the comparison of three different types of crosses in producing hybrids using SSR markers was done. The SSR marker was effective in distinguishing three types of progenies in each type of crosses. SSR primers were also able to produce polymorphic bands in *Erianthus* sp. even with a much smaller amount of polymorphism. The polymorphic band in *Erianthus* sp. could also be identified in F1 so that it could be used as a molecular marker to distinguish and ensure that an F1 is a true hybrid.

The results indicated that mSSCIR66 was the primer that could be used to detect the success of crosses among commercial sugarcane varieties and between commercial sugarcane varieties, and their wild relatives (S. *spontaneum* and *Erianthus* sp.), while mSSCIR43 and SMC119CG were only sensitive in detecting the success of crosses on one of the three categories of crosses. The primer mSSCIR43 was more sensitive in detecting the success of crosses between commercial sugarcane varieties and their wild relatives, in contrast to SMC119CG which was more sensitive in detecting the success of crosses among commercial sugarcane varieties.

In previous studies, the use of molecular markers for confirming the hybridity of crosses from either interspecific or intraspecific sugarcane hybridization was done separately i.e. crosses among sugarcane commercial variety, between sugarcane commercial variety and *Saccharum spontaneum*, or between sugarcane commercial variety and *Erianthus* sp. Costa, *et al*. (2014) successfully detected 71.7% - 97.6% of self-pollination occurrences in a population of five cultivars of *Saccharum* spp. with SSR markers. Non-significantly different results were also reported by Tew and Pan (2010) that microsatellite markers were able to recognize the presence of male parent alleles in 79% - 99% of clones from polycross of *Saccharum* spp. Fukuhara, *et al*. (2003) had successfully identified intergeneric hybrid of *Saccharum* spp. hybrid and E. *arundinaceus* (Restz.) Jeswit using 5S rDNA marker. The percentage of successful intergeneric hybrids obtained was 2.9% (five hybrids from a total of 173 progenies).

Wang, *et al*. (2009) reported that by using the SCAR molecular marker, they were successful in identifying hybrids from *Saccharum* spp/E*. fulvus* of about 38.5% - 95.5%. Further, Wang, *et al*. (2009) explained that the chromosome transmission 2n + n or n + n will not always be in equal numbers as transmitted by both parents. The elimination of male chromosome, the new variant DNA sequence (recombination) in hybrids by crossing-over event at meiosis are some of the factors that some markers could not amplify and identify the parent allele in the progeny. The same result had been reported by Nair, *et al*. (2006). In that study, it was reported that RAPD markers failed to identify the presence of specific alleles of *Erianthus* spp. in the progeny, probably because of the lost *Erianthus* spp. chromosome (eliminated) although the primer was able to identify 107 specific alleles of the parent (*Erianthus* spp.)

The primer that had been used in this study was designed from two sugarcane commercial varieties R570 (French) and Q124 (Australia) (Pan, 2006), with the probability that it

doesn't have any pedigree of E*. arundinaceus,* resulting to the primer wasn't able to work with *Erianthus* sp and resulted in low polymorphism. The same result had been reported by Govindaraj, *et al*. (2012), that the primer could identify the hybrids from hybridization of E*. arundinaceus*/S*. spontaneum*, but with only few alleles in E*. arundinaceus* than in S*. spontaneum*, probably because the primer was designed without any pedigree from E*. arundinaceus*.

Furthermore, Pan (2006) stated that there were three forms of SSR utilization. The first was the registration and verification of the true identity of the variety. Second was the examination of genetic identity of vegetative propagation of sugarcane originating from different locations. Third was to find out the genetic purity of a genotype in population mapping.

Pan *et al.* (2006) demonstrated that the molecular approach of fingerprinting the progeny to confirm parentage prior to field planting even with only one microsatellite marker might substantially increase selection efficiency. It also has opened the way for identifying large number of seedlings in the early stage of selection with limited number of primers thus saving resources and time. Accordingly, future studies could be targeted to understand the sequence feature and its functional significance associated with these unique cultivars. Additionally, this unique feature could also be used as specific genetic profile, and will enable the establishment of criteria for variety protection and to identify the duplication of germplasm.

The results also showed that there were differences in producing hybrids among the three types of crosses. It also showed that there were differences in the compatibility of parent combination of crosses in producing hybrids. When compared with the results of previous studies in producing selfing and off types, higher percentages were produced in this study.

Generally, crosses among sugarcane commercial varieties could produce more hybrids (average of 62.6%) than crosses between sugarcane commercial varieties and their wild relatives (average of 45.67%). Conversely, crosses between sugarcane commercial varieties and their wild relatives produced more selfing and off type progenies (average of 27.33% and 26.78%, respectively) than crosses among sugarcane commercial varieties (8.02% and 29.42%). In crosses among sugarcane commercial varieties, all crosses could produce hybrid (>50%) higher than self (<11%) or off types (<36%).

Specifically, crossing between VMC7616 as female parent and its wild relatives (S. *spontaneum*, crosses A and *Erianthus* sp., crosses D1) as male parent produced more hybrids (52.27%) than selfing or off type (18.93% and 28.97%, respectively). The same results were found in crosses between PSJT941 as female parent and their wild relatives (S. *spontaneum*, crosses F2 and *Erianthus* sp., crosses F1) as male parent. Compared with both previous results, more selfing progenies were produced (45.83%) in crosses between PS881 and S. *spontaneum* (crosses C2) or *Erianthus* sp. (crosses E1) than progenies as hybrid (29.17%) or off type (25%). Generally, crosses between PS881 and S. *spontaneum* or *Erianthus* sp. has less compatibility than crosses between PSJT941 and S.

spontaneum or *Erianthus* sp. or crosses between VMC7616 and S. *spontaneum* or *Erianthus* sp. in producing hybrids.

In S. *spontaneum*, although it had a few genetic markers and not much different from *Erianthus* sp., there were more hybrids identified when it was crossed with sugarcane commercial varieties. These results were not surprising. S. *spontaneum* was one of the ancestors of modern sugarcane which now had been developed and used either for production or genetic material in crosses. Intensive use of the same genetic material as parent caused the genetic similarity between the parent and the progeny that gave advantages such as removing obstacles or incompatibilities in crosses and also by decreasing the genetic variability in sugarcane. Previous research reported that the decrease of genetic diversity resulted from the intensive use of the same genetic material and probably the one factor in causing slow breeding progress in sugarcane (Stevenson, 1965; Zhang, *et al*., 2001; Perera *et al.*, 2012; Filho *et al*., 2010; Pan, *et al*., 2003; Hapsoro, *et al*., 2015; Chen, *et al*., 2017). Hogarth (1976) reported that less than one variety could be released with less than 1% yield rate increase in a year (Tew and Pan, 2010).

This study also showed that in crosses among sugarcane commercial varieties, VMC7616 and PS881 was the best parent combination to generate hybrid because in this cross (crosses E2) and its reciprocal (crosses H) could be detected with progenies as hybrid and with equally high percentage. Furthermore, VMC7616 could become the best female parent (crosses C1, crosses E2) whereas, PSJT941 could become the best male parent (crosses C1, crosses G) and PS881 could become the best parent combiner (as male, crosses B and crosses E2 or female, crosses G and crosses H) to produce more hybrid in intraspecific sugarcane hybridization program.

Selection of the best parent and the best parent combination were the two main factors that determine the success of a sugarcane breeding program and these factors might probably alleviate the difficulty of obtaining genotype in F1 seedling population. Additionally, gene linkages that controls desirable and undesirable character will further minimize the chances of obtaining ideal genotype. Polyploidy with complex genome arrangement and inter- and intraspecific hybridization with irregular chromosome transmission pattern were the other limiting factors in sugarcane crosses (Bremer, 1962; D'Hont *et al*., 1996; Grivet *et al*., 2001; Filho *et al*., 2010; Piperedis *et al*., 2010; Sigh *et al*., 2011; Budhisantosa, 2012; Perera *et al*., 2012; Huang *et al*., 2015; Nair *et al.*, 2017). Warner (1953) with the simple assumption illustrated that only one ideal genotype could result from three million seedlings (Budhisantosa, 2012).

The percentage of selfing and off type progenies in this study was different from previous studies. In this study, there were 4.77% - 45.83% and 19.22% - 35.22% of progenies as selfing and off type, higher than previous studies. Also, in previous studies around <10% of progenies were identified as selfing in the controlled environment (Nagarajan *et al.*, 2001; McIntyre and Jackson, 2001; Tew and Pan, 2010) and >50% in the uncontrolled environment (Melloni *et al.,* 2014), although <25%

of progenies carry foreign pollens (Tew and Pan, 2010; Santos *et al.*, 2014). The off-type progenies were also found in selfing breeding programmes with the percentage <30% (Costa *et al*., 2014).

The high level of undesirable crossing (selfing and off type) was possibly due to the characteristic of the sugarcane flower. Sugarcane inflorescence is very small so the emasculation cannot guarantee 100% elimination of the pollens from the female parent. Sugarcane flowers are also perfect flower and hermaphrodite where both stamen and pistil are present in the same flower. The pistil is mature and receptive before the stamens mature (protogyni) so both selfing and fertilization by foreign pollen could happen before the crossing itself (Heinz and Tew, 1987; James, 2004 *in* OECD, 2013; McIntyre and Jackson, 2001; Santos *et al.*, 2014).

On the other hand, the seeds that had been harvested and germinated were derived from those that fell from the lantern or were still attached to the panicle. This indicated that the seed might be mixed with other seeds from selfing or foreign pollen (off type). Flower anthesis occurs at different time, but starting from the tip of inflorescence and the tip of rachillae (branches) with the period of receptivity longer than the period of anthesis (Moore and Berding, 2014) so the probability of an undesirable crossing is still high despite pollen testing and emasculation before the crosses are made.

The flowering of sugarcane is also strongly influenced by the environment. Temperature, rainfall, and day length could accelerate, delay, or defeat the transformation from vegetative stage to reproductive stage in sugarcane (Stevenson, 1965; Gosnell, 1973; Manhaly *et al*., 1984; Srivastava *et al*., 2006; Shanmugavadivu and Rao, 2009; Caraballoso *et al*., 2012; Cordoza and Sentelhas, 2013; LaBorde *et al*., 2014; Moore and Berding, 2014). The environmental influence is highest during flowering induction and initiation process and is the main factor that causes variability among flowering time in sugarcane, some cultivars are only slightly affected by the environment to induce flowering (early flowering sugarcane) but some other cultivars are strongly influenced by the environment to induce flowering (Glassop *et al*., 2014).

During 2014 when the crosses was made, based on Indonesian Agency for Meteorology, Climatology, and Geophysics data, the research location has an average rainfall of 139 mm/month with a rain frequency of 12 days, an average minimum and maximum relative humidity of 41% and 96%, respectively.As well as an average minimum and maximum temperatures of 20ºC and 28.9ºC, respectively. In fact, the research location has temperature (minimum and maximum) and relative humidity (minimumand maximum) relatively appropriate for sugarcane flowering. Flower formation is expected to increase by 4.2% with increased temperature of 1ºC above 31.9ºC (vegetative stage) and decrease by 4.4% with increased temperature of 1ºC above 32.1ºC (pre-initiation stage with constant 12 hr and 30 min day length) and decrease by 4.7% with increased temperature of 1ºC above 33.1ºC (booting stage) (LaBorde *et al*., 2014).

Temperature (minimum and maximum), minimum relative

humidity, cloudiness, the frequency of rainy days, the fertility of pollen, and wind speed were the factors that have correlation with altitude and influence the flowering ability of sugarcane, although varieties and flowering intensity has a negative impact to flowering initiation of sugarcane (Caraballoso *et al*., 2012). Maximum temperature with range of 29.4ºC - 31.6ºC and minimum temperature with range of 19.5ºC - 21.4ºC with frequency of days within the range of 18ºC - 31ºC had around 85%, higher rainfall with the precipitation of 276.7 mm during flowering induction and initiation increased the average flowering intensity by 28% (Shanmugavadivu and Rao, 2009).

CONCLUSION

Hybrid identification in sugarcane is the important step to do after crossing to guarantee the selected genotype is truly a hybrid and not selfing or off type. SSR as one of the molecular markers can be the best tool to assist in the identification of hybrids from the breeding programme. All primers (mSSCIR43, mSSCIR66, and SMC119CG) could identify in average 62.7%; 8.02%, and 29.42% of progenies as hybrid, selfing, and off type in the crosses among sugarcane commercial varieties. While 52.44% hybrid, 30.21% selfing, and 17.46% off type progenies could be identified in crosses between sugarcane commercial varieties and S. *spontaneum* and 38.89% hybrids, 24.44% selfing, and 36.11% off type progenies were identified in crosses between sugarcane commercial varieties and *Erianthus* sp., respectively. Based on this study, the three SSR primers were quite effective as genetic markers to confirm the true identity of the progenies.

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