AGRIVITA Journal of Agricultural Science. 2017. 39(3): 257-265

Development of *Simple-Sequence Repeats* Markers from Durian (*Durio zibethinus* Murr. cultv. Matahari) Genomic Library

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Received: November 30, 2016 /Accepted: April 18, 2017

ABSTRACT

Simple sequence repeats have been proved as powerful markers and widely used in molecular breeding to reduce cycles and cost efective. The availability of the marker is, however, very limited in durian. This research aimed to develop SSR markers from durian genomic library. Genomic DNA was isolated from durian shoot leaf, whilst SSR motifs were isolated using membrane-based oligonucleotide enrichment hybridization protocol. Annotation made on the library found 527 unique motifs from 354 durian libraries which form 425 loci. The SSR motifs obtained were generally short repeats which reached 89.6 %, whilst longer repeats were found consisted of compound motifs. Eleven loci were selected as representative for further test to prove their informativity. A number of unique allels were successfully amplified from 17 durian genomes. The analysis showed the polymorphic information content (PIC) values ranged from 0.000 to 0.662 with an average of 0.390. The SSR loci also showed their ability to be used for durian diversity analysis as the evident that the loci could be used as genetic markers for assisting further durian breeding program.

Keywords: durian (*Durio sp.*); genetic markers; informative loci; SSR

INTRODUCTION

Indonesia is occupied by abundant species and cultivars of durian (*Durio* sp.). At least 20 of 29 durian species in the world are native to this country. Durian in Indonesia is mainly grown in many traditional production areas which dominated by semi forest and arable land. The cultivars planted are mainly assorted local cultivars from open pollinated seed, whilst the fruits are for local consumption except for limited area near to the nation border. These conditions consequently affect to low fruit quality and product assurance. As the result, it decreases the image and price of Indonesian durian compare to the introduced cultivars.

Based on the above point of information, superior cultivar becomes an essential component to develop durian business in this country. The cultivar is needed to support the establishment of new plantation and upgrade the local assorted cultivars through top-working technique (Rebin et al., 2003). These efforts are believed to increase the quality of Indonesian durian. As a consequence, it will increase market acceptance and price (Rahman, 2000).

Superior durian cultivar criteria were determined based on the preferences of consumers and durian traders. Consumers choose durian based on the fruit quality attributes such as medium fruit weight, strong odor, yellowish aril, wrinkle seeds, thick aril, soft and firm aril texture, sweet tastes and nutty (Santoso, Novaril, Jawal, Wahyudi, & Hasyim, 2008). Traders want a long self-live and crack-persistent durian, while the growers require high productivity, disease resistant and early maturing durian. This kind of superior cultivar could be achieved through indigenous germplasm selection and artificial crossing (Somsri et al., 2008; Indriyani, Santoso, & Hermanto, 2012).

Durian breeding through artificial crossing requires enormous efforts and take a long period of time. Experiences of two neighboring countries Malaysia and Thailand showed that they took up to 30 years to obtain new cultivars from one breeding generation (Abidin, Mohammad, Shamsudin, Masdek, & Ghazali, 2000; Somsri et al., 2008). To shorten the period of selection and to make cost efective for durian breeding, it is therefore required molecular markers as tools for progeny selection. This method is commonly known as marker assisted selection (MAS) (Collard, Jahufer, Brouwer, & Pang, 2005).

Cite this as: Santoso, P. J., Pancoro, A., Suhandono, S., & Aryantha, I N. P. (2017). Development of simple-sequence repeats markers from durian (Durio zibethinus Murr. cultv. Matahari) genomic library. *AGRIVITA Journal of Agricultural Science*, *39*(3), 257–265. http://doi.org/10.17503/agrivita.v39i3.1171
Accredited: SK No. 60/E/KPT/2016

Application of molecular markers has been growing very rapidly and proven helpful for breeding activity in detecting expected alleles as early as possible. One molecular marker that is widely used for this purpose is *simple sequence repeats* (SSR) or often called as microsatellites DNA (Hancock, 1999; Liu, Cantrell, McCarty, & Stewart, 2000). The advantage of this markers are co-dominant character, high level of polymorphic, easily to be repeated and stable (Ritschel et al., 2004) and the analysis could be conducted automatically (Schuelke, 2000). High variability of these markers allows accurate calculation to detect paternal contribution to the progeny produced (Ottewell, Donnellan, Moran, & Paton, 2005). The technique has been widely used for estate crops such as oil palm (Billotte et al., 2005; Singh, Nagappan, Tan, Panandam, & Cheah, 2007).

The initial step of SSR application is to mine up the microsatellite loci and designing primers for amplification the DNA template. Microsatellite motifs could be obtained through *in silico* technique for crops that are already available their genomic data base, or through isolation from genomic library for those which their data base are not available yet (Sharma, Grover, & Kahl, 2007). The number of durian SSR loci listed in *GeneBank,* so far, is very limited. It is therefore necessary to isolate the loci from genomic library for providing molecular markers to assist durian breeding in the future.

This study was aimed to develop SSR loci from durian genomic library and to determine the potential of the loci as genetic markers to assist durian breeding.

MATERIALS AND METHODS

The research was conducted at Plant Genetics Laboratory, School of Life Science and Technology, Institut Teknologi Bandung from January 2012 to December 2013. Genomic DNA for SSR development were isolated from shoot leaf of durian cultivar 'matahari'. Locus informativity analysis were using 4 durian species consisted of 17 accessions.

SSR Motif Isolation

Genomic DNA isolations were folowing protocols of *Genomic DNA Isolation Kit (plant)* from *Geneaid*™ with modifications on the addition of PVP-40 and prolongation of the incubation period. SSR motifs isolation referred to the membrane-based oligonucleotide enrichment hybridization method (Edwards, Barker, Daly, Jones, & Karp, 1996). Transformant produced was cultured following the protocols developed by Xiang, Wang, Shiel, Berger, & Guerra (1994). Plasmid isolation was conducted based on *Geneaid*™ plasmid isolation kit. The DNA plasmids were then sequenced using the ABI 3730 XL machine in one direction using T7 primer conducted by Macrogen Inc., Korea.

Annotation of microsatellite motif of the DNA clone sequences was conducted using *Phobos* software program as plug-in to *Geneious Basic 5.5.6* software (Biomatters, New Zealand). Microsatellite loci were annotated based on the criteria such as containing repeating units of one to six nucleotides in length for at least 6 repetitions for mononucleotide, 3 repetitions for dinucleotide, and two repetitions for trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide. These motifs were then classified based on the perfection and length of repeat sequences. Sequences flanking the microsatellite region were then designed to generate primer pair using *Primer3* software.

Locus Informativity

Template DNAs of 17 durian accessions were amplified using 11 primer pairs. A total of 20 mL PCR mixture consisting of 10 uL KAPA 2G™ Fast 2x Ready Mix (KAPA BIOSYSTEMS), 0.3 μM forward primer, 1 μM reverse primer, 1 μM fluorescence label (6 - Fam or Hex), 0.6 ul DMSO, and 30 ng DNA template were put on 200 uL PCR tube. Amplification was conducted in *GenAmp Thermocycler* with the reactions of pre-denaturation at 94 °C for 3 minutes continued by initial 30 cycles of denaturation at 94 °C for 15 seconds, annealing at temperature of each primer (Tabel 2) for 15 seconds and elongation at 72 °C for 20 seconds. Reacrion were then followed by the label attachment for 8 cycles of denaturation at 94 °C for 30 seconds, annealing at 53 °C for 20 seconds, and elongation at 72 °C for 30 seconds, then continued by final elongation at 72 °C for 7 minutes. Labeled amplicon products were then sent for fragment analysis (gene - scanning) to Macrogen Inc., Korea. Fragments analysis results were received in the form of fluorescence peak graph represent the length of PCR product. This data was then read and analyzed using software *GeneMarker 2.4.0* (SoftGenetics). Analysis the level of heterozygosity (*He*), allele frequency distribution and Principle Coordinate Analysis (PCO) were conducted using *GeneAlEx ver.6* (Peakall & Smouse, 2006) software, whilst the *Polymorphic Information Content* (PIC) was analyzed using *Cervus ver.3.2.*(Field Genetics).

RESULTS AND DISCUSSION

SSR Motifs and Primer Design

Microsatellite has become one of the molecular markers widely applied to assist breeding program of various crops. SSR marker-based research on durian, to our concern, however is very limited. Marker application on durian so far is still dominated by finger-printing activity such as RAPD (Nuchuchua et al., 2008), DAF-PCR (Somsri et al., 2008) and PCR-RFLP (Santoso & Saleh, 2009). More specifically associated with microsatellite markers there were only seven entries of sequence data in GeneBank, while isolation conducted by Kristianti (2005) has provided 38 loci. To conduct QTL mapping for molecular assisted breeding on durian, these loci number is still too little due to the considerable number of chromosomes of durian is n=28 (Soepadmo, 1979), where it could require hundreds to thousands loci.

Besides the advantages of their characteristics, the availability of protocols and advancement in molecular technology also make the isolation and development of SSR markers easier. One widely used method to mine microsatellite motifs was oligonucleotides-enrichment method (Edwards, Barker, Daly, Jones, & Karp, 1996). This method was simple and efficient in obtaining microsatellite loci and has been successfully applied in various crops (Edwards, Barker, Daly, Jones, & Karp, 1996; Cordeiro, Maguire, Edwards, & Henry, 1999; King et al., 2008; Mahmoodi et al., 2010).

To establish a set of SSr markers, a total of 767 libraries from durian genomic DNA which varied in the sequence length from 151 to 669 nucleotides. Of those, only 360 (46.9 %) clones were found as unique libraries which subsequently annotated to find microsatellite motif. The annotation made to 354 (98.3 %) inserts found a total of 1265 motifs or an average of 3.57 motifs per clone, whilst the remaining six inserts (1.7 %) contain no motif at all. Among the motifs annotated, there were 527 unique motifs that make up 425 loci. These motifs consisted of 72 mononucleotide, 76 dinucleotide, 210 trinucleotide, 115 tetranucleotide, 41 pentanucleotide, and 13 hexanucleotide. Ten motifs most commonly found in this study were $(GAA)_2$, $(C)_6$, $(CG)_3$, $(A)_6$, $(TGG)_2T$, $(G)_6$, $(TGC)_2$, $(GTT)_2G$, and $(C)_7$, as many as 52 , 43, 34, 23, 21, 21, 20, 20, 19, and 18 sequentially. If the motifs were grouped based on repeat perfection (Weber, 1990; Sharma, Grover, & Kahl, 2007), it was found 217 perfect, 121 imperfect, 25 perfect compound, and 71 imperfect compound motifs. In general, single motif found from durian genomic library has short repeat size (<10 nt) which reached 328 (89.6 %), whilst longer size varies from 11 to 89 nt were found only 38 (10.4 %) motifs. However, combination of more than one motif in a locus produced longer motifs. Table 1 displays microsatellite motifs that have a length >15 nucleotides.

A number of 67 primer pairs have been designed to flank the microsatellite loci of the selected libraries (data not shown). Locus selection was determined based on the length and type of microsatellite motif and the position of motif in the sequence which gave enough space on the left and right sides to the target sequence to make the product length between 100-300 nt. In general, primers which successfully designed in this study had sequence length ranged 17 to 25 nucleotides, and most primers were 20 nt. These primers were designed to amplify product ranged from 100 up to 280 nt. The primers had GC content ranged from 40 to 66.7 and TM values ranged from 57.01 to 60.88.

The success of designing the primer is depending on the length of the library. Primer is difficult to be designed from library less than 150 nucleotides, due to the products will be too short. Library with several motifs which separate in quite long gap could be used to design more primer pairs, for example, clone 2G11 produced two loci *i.e.* mDz2G111 and mDz2G112. Position of motif in the library is also determining the successful of designing the primers. Motif in the middle of library makes easy to design the primers, on the contrary motif on the edge makes it difficult to be designed because there is no conserve site, unless the motive itself is used as the primer. Another factor that led failure to design the primer is the target areas of primer does not enough GC or TM value.

Loci Informativity

Eleven loci have been selected and analyzed for their informativity on 17 durian accessions (Table 2). From the experiments, a total of 187 amplification products were produced. Distribution and frequency of allel are shown in Table 3. A total 40 alleles were found with an average value of 3.635. Loci which had the highest number of six alleles were mDz03A31 and mDz6F06, whilst that had the lowest number of one allele was mDz3G72.

Table 1. SSR motifs from durian genomic library which have length >15 nt

No	Motif	Length	Remarks
1	$(CAR)_{33}$	99	CS
2	$(CGS)_5gc(CG)_3ggg(SG)_4(TAT)_2a(CT)_3$	46	CS and SSR-IC
3	$(YG)_{19}$	38	CS
4	$(G)_{25}$	25	SSR-P
5	$(T)_{25}$	25	SSR-P
6	$(G)_{15}(C)_4(TCC)_2$	25	SSR-PC
7	$(GGC)_2G_7tgT_{10}$	25	SSR-IC
8	$(CAT)_2gtg(CA)_3C_6$	24	SSR-IC
9	$G_6T_{11}g(CA)_3$	24	SSR-IC
10	$(TAAA)_2$ $a(AAG)_2$ ttt $(TAA)_2$	24	SSR-IC
11	$(CCG)_3$ gcG_7cG_5	24	SSR-IC
12	$(TGG)_5a(GTTG)_2$	24	SSR-IC
13	$(AG)_4(CA)_3(CTA)_3$	23	SSR-PC
14	$(GGGGGC)_2ggcG_8$	23	SSR-IC
15	$(TTTC)_2T_8(GT)_3$	22	SSR-PC
16	$(TTGG)_2gc(CCT)_2c(TG)_3$	22	SSR-IC
17	$(CGT)_2t(TCC)_2(GCTC)_2g$	22	SSR-IC
18	$(CAT)_2gtg(CA)_3C_6$	21	SSR-IC
19	$(CA)_3tcg(TG)_2(CA)_3$	21	SSR-IC
20	$(CAT)_2gtg(CA)_3C_6$	21	SSR-IC
21	$C_6g(GCC)_2atG_6$	21	SSR-IC
22	$(GGA)_2ccc(TCTA)_2tc$	19	SSR-IC
23	$(TTGGA)_2$ $(AAY)_3$	19	SSR-P and CS
24	$(TGG)_2aat(TGG)_3$	18	SSR-IC
25	$(ACCCC)_2(GATT)_2$	18	SSR-PC
26	$(CCGG)_3G_6$	18	SSR-PC
27	$(TGG)_6$	18	SSR-P
28	$A_7tc(ATT)_3$	18	SSR-IC
29	$A_7gaa(GA)_4$	18	SSR-IC
30	$C_7tgg(CCTG)_2$	18	SSR-IC
31	$G_5cG_6(TG)_3$	18	SSR-IC
32	$G_8(TG)_2(CA)_3$	18	SSR-PC
33	G_9cgcG_6	18	SSR-IC
34	$(CGGCT)_2cg(TTC)_2$	18	SSR-IC
35	$(ACA)_3gcg(AGAA)_2$	17	SSR-IC
36	$(AAAAG)_2a(GAA)_2$	17	SSR-IC
37	$(ACT)_3ct(AAG)_2$	17	SSR-IC
38	$TT(ATT)_2ctc(TG)_3$	17	SSR-IC
39	$(CAT)_2gtg(CA)_3C$	16	SSR-IC
40	$(ACCC)_2(GATT)_2$	16	SSR-PC
41	$(CAGA)_2(GTGA)_2$	16	SSR-PC
42	$(GGA)_2(GAAAA)_2$	16	SSR-PC
43	T_{16}	16	SSR-P
44	$(GAA)_2ta(ATTG)_2$	16	SSR-IC
45	$(GAG)_2G(TAK)_3$	16	SSR and CS
46	A_{15}	15	SSR-P
47	G_{15}	15	SSR-P
48	$(ACC)_3(TCA)_2$	15	SSR-PC

Remarks: SSR= simple sequence repeat; CS= cryptic simplicity; P= perfect; I= imperfect; C= compound; R= G/A; S= G/C; Y= T/C; K= T/G; W= A/T

Table 2. List of 11 SSR loci, motif and primer to be used to informativity analysis

No	Locus	Motif	Forward & reverse primer	Annealing Temp. (°C)
1.	mDz3B71	$(TGG)_{4,}$ $(TGG)_6$	f-GATGGTGGAATTGGTGGTGG r-ATCGGCTCCAACCCTTAACT	58
2.	mDz03F10	$(GAA)_{3,}$ $A(GA)_4$	f-GGACTAGACAACCAAGCAGAG r-GCGTGGACTACTTCAAACCC	57
3.	mDz3B72	$C_{12,}$ C_8	f-TGAACGTTCTCCACCCCTC r-GAAGTTGGTTCCTTGCGGTT	58
4.	mDz1C12	$(CGCT)_{3,}$ $(GGGT)_2$	f-CGTTGTTGCCTGTCGGAT r-CACAACCATAGCACCACTCA	57
5.	mDz03A31	$(ATT)_{3,}$ $(GA)_4$	f-TGTGGAGTCTTGTTCGGGAA r-AGCAACAAACAGAACCACCG	60
6.	mDz3G72	$(CCCCG)_{2,}$ $(CCCCT)_{2,}$ $(CCCCT)_{2,}$ $(CCCA)_{2,}$ $(CA)_4$	f-AGTTAAGGGTTGGAGCCGAT r-TACGTGTGAGGTCAAGCTGT	60
7.	mDz4A6	$A_{12,}$ $(CAAAA)_2$	f-AGAGAAGTTCGTTTGGAGCCA r-ATCAACACCTGGCTTGATCC	57
8.	mDz03H9	$(ACA)_3gcg(AGAA)_2$	f-AGCCTCCGTATCTTTACATGT r-CATTCGATGCTACCACACCG	57
9.	mDz6F06	$(TAAA)_{2,}$ $(AGG)_3$	f-GGTTACAACTTG CCC CAC TG r-GACCACCAACACAAACGGAA	57
10.	mDz03A1	$(CAR)_{33}$	f-CGTGGACTACTTTTATTGCAGAGG r-CAAGTCCATTCGTATTGCCATTTAG	57
11.	mDz6A11	$(ACCC)_2(GATT)_{2,}(GCCAC)_2$	f-GCACAACCATAGCACCACTC r-TGTTATTCTCGTGCCAAGCG	57

Remarks: coma (,) in between two motifs shows the gaps more than 3 bases

Table 3. Allele frequency of each SSR locus on durian population

Locus	Allele	Frequency	Locus	Allele	Frequency
mDz3B71	97	0.941	mDz4A6	97	0.500
	162	0.059		165	0.118
mDz03F10	82	0.706		167	0.382
	105	0.176	mDz03H9	82	0.147
	201	0.118		188	0.853
mDz3B72	97	0.676	mDz6F06	97	0.353
	102	0.029		147	0.029
	107	0.235		157	0.500
	182	0.059		178	0.059
mDz1C12	82	0.353		193	0.029
	105	0.324		223	0.029
	165	0.206	mDz03A1	82	0.529
	169	0.118		91	0.029
mDz03A31	97	0.618		105	0.235
	120	0.029		165	0.059
	162	0.118		225	0.147
	206	0.029	mDz6A11	82	0.118
	214	0.176		98	0.029
	220	0.029		162	0.029
mDz3G72	82	0.883		167	0.824
	N	0.117			

In general, microsatellite alleles of the 11 loci were found in all durian accessions, except for locus mDz3G72 which indicated there were null allele in two accessions namely 'Namlung Petaling' and 'Kerantungan'. These data fact showed the potential of the loci to produce the variation among the accessions.

Table 4 shows the value of *Ho*, *He* and PIC of the loci analyzed. Value of *Ho* is an average of 0.422, with the variation from the lowest of 0.00 (mDz3G72) to the highest of 0.941 (mDz1C12). Value of *He* is an average of 0.431 which is higher than the *Ho* with variation from 0.00 (mDz3G72) to 0.715 (mDz1C12). Value of the *PIC* is an average of 0.390, ranging from the lowest of 0.000 to the highest 0.662. Based on the PIC it could be drawn that five loci are categorized as high informative with PIC value higher than 0.500 (mDz1C12, mDz03A31, mDz4A6, mDz6F06, mDz03A1); three loci are medium informative with PIC value between 0.250-0.500 (mDz03F10, mDz3B72, mDz6A11); and three loci are low informative with PIC value <0.250 (mDz3B71, mDz3G72, and mDz03H9).

Genetic distances among durian accessions based on allele variation of 11 SSR loci shows high disparity of genetic distances among accessions (Table 5). The shortest genetic distance was occurred between durian 'Pelangi' and 'Sibaluik' with the value of 2, whilst the furthest were among 'Lai Mas' and two other accessions 'Namlung Petaling' and 'Kerantungan' with the value of 22. PCO analysis based on the genetic distance matrices shows 13 of 14 durian accessions (*Durio zibethinus*) are closer one to another and forming a distinct group, whilst an accession 'Kamlung Petaling' separated from the *D. zibethinus* group and forms a distinct group with 'Kerantungan' (*D. oxleyanus*). Two other accessions, 'Lai Mahakam' (*D. kutejensis*) and 'Lai Mas' (*Durio sp.*) separate from the two groups and show distinct position each other (Fig. 1).

This research had succesfully developed a set of SSR markers of durian by using oligonucleotides-enrichment method (Edwards, Barker, Daly, Jones, & Karp, 1996). In general, the SSR motifs found in durian genomic libraries in this experiment formed short motifs which doubtly to be not potential genetic marker. However, many libraries found consisted of compound motifs which form longer motifs >15 nt which consider as potential marker. The potential of the loci developed in this research as genetic markers are indicated through durian diversity analysis. Amplification made up to 11 SSR loci onto17 durian accessions showed the PIC values were an average of 0.390 which indicating the loci used have high informatifity (Hildebrand, Torney, & Wagner, 1992). More evident for the potential of the loci as genetic markers is that the loci able to divided the 17 durian accession into four groups based on the species as the botanists have done (Fig. 1).

Table 4. Number of population, allele, value of Ho, He, and PIC of 11 SSR loci

Locus	N	Na	Ho	He	PIC	F
mDz3B71	17	2	0.118	0.111	0.105	-0.063
mDz03F10	17	3	0.588	0.457	0.411	-0.288
mDz3B72	17	4	0.412	0.483	0.428	0.147
mDz1C12	17	4	0.941	0.715	0.662	-0.317
mDz03A31	17	6	0.471	0.571	0.534	0,176
mDz3G72	15	1	0.000	0.000	0.000	#N/A
mDz4A6	17	3	0.529	0.590	0.506	0.103
mDz03H9	17	2	0.059	0.251	0.219	0.766
mDz6F06	17	6	0.588	0.619	0.553	0.050
mDz03A1	17	5	0.765	0.638	0.590	-0.198
mDz6A11	17	4	0.176	0.306	0.285	0.424
Average		3.636	0.422	0.431	0.390	0.080

Remarks: N= population number; Na = allele number; Ho = *Observed Heterozygosity*; He = *Expected Heterozygosity*; PIC = *Polymorphic Information Content*; F = *Fixation Index*; Percentage of polimorphic loci: 90.91 %

Table 5. Genetic distance matices of 17 durian accessions based on polimorphism of 11 SSR loci

Accession	Katiman	Matahari	Namlung	ParakKopi	Pelangi	Salisun	Saripan	S. Gading	Sibaluik	Sigundul	Sijapang	Siradio	T. Medaye	Violeta	Mahakam	Lai Mas	Kerantungan
Katiman	0																
Matahari	10	0															
Namlung Ptlg	18	18	0														
Parak Kopi	7	9	10	0													
Pelangi	12	13	7	7	0												
Salisun	14	10	11	9	6	0											
Saripan	15	13	7	8	5	7	0										
Saweri Gading	15	14	14	9	10	7	11	0									
Sibaluik	12	12	7	6	2	6	4	10	0								
Sigundul	10	9	8	6	4	4	5	8	3	0							
Sijapang	6	11	17	6	13	14	13	14	10	10	0						
Siradio	9	7	14	7	9	8	10	11	7	7	9	0					
Tong Medaye	6	10	18	6	14	14	14	15	11	10	3	6	0				
Violeta	11	10	13	10	8	9	9	10	7	7	9	10	13	0			
Mahakam	11	15	17	8	12	14	15	14	12	10	12	13	13	16	0		
Lai Mas	16	15	22	13	18	17	17	20	17	14	16	16	17	18	8	0	
Kerantungan	16	16	7	13	9	11	11	16	9	10	17	13	17	12	15	22	0

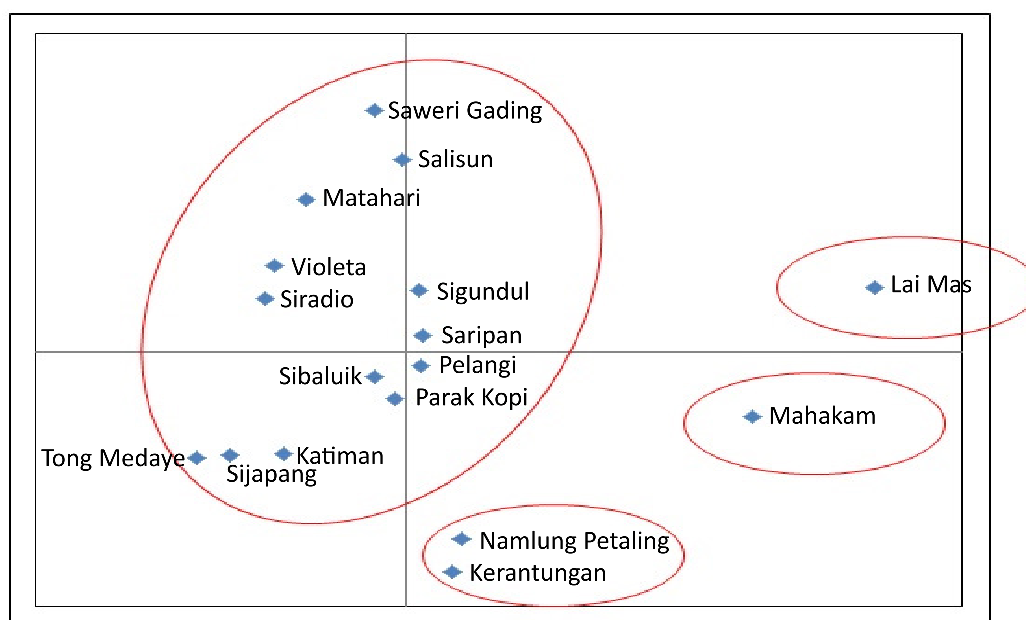

Fig. 1. PCO diagram of 17 durian accessions based on allele distribution of 11 SSR loci

CONCLUSION

Annotation made on the library found 527 unique motifs from 354 durian libraries which form 425 loci. The SSR motifs obtained were generally short repeats which reached 89.6 %, whilst longer repeats were found consisted of compound motifs. Eleven loci were selected as representative for further test to prove their informativity. A number of unique allels were successfully amplified from 17 durian genomes. The analysis showed the polymorphic information content (PIC) values ranged from 0.000 to 0.662 with an average of 0.390. The SSR loci also showed their ability to be used for durian diversity analysis as the evident that the loci could be used as genetic markers for assisting further durian breeding program.

ACKNOWLEDGEMENT

The authors thank to Dr. Tati Kristianty for lab assisstance, and Prof. Rosichon Abdillah for review the first draft of the manuscript. This research was funded by KKP3N Program of IAARD years 2012-2013.

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