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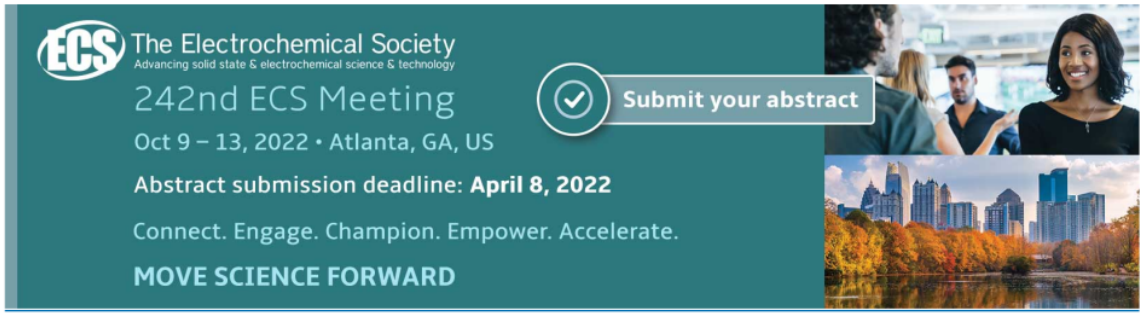

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Genetic diversity of Some Indonesian Local Rice Varieties based on Simple Sequence Repeat (SSR) marker related to Aromatic Genes

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Abstract. Rice plant (*Oryza sativa* L.) has various varieties. People generally prefers to rice with good taste and aroma. The aroma is part of the physicochemical properties of the rice plant. The aroma of rice arises because of the gene that controls the nature of the aroma. PCR (polymerase chain reaction) is an in vitro method for generating a large amount of specific DNA fragments with defined lengths and sequences in a small number of complex templates. The objective of this study was to analyze the genetic diversity of some Indonesian local rice varieties based on SSR marker related to aromatic genes. The selected Simple Sequence Repeat (SSR) primers associated with aromatic genes were: RM 484, RM 410, RM 251, RM 247, RM 223, and RM 9. Based on the DNA band pattern, 6 primers were derived, in which there were 14 loci related to the aromatic gene. The resulting dendrogram showed that two main groups of rice achieved a 57% similarity. Group A consisted of aromatic rice accessions, i.e. Rojolele, Rabeg, Rumbah, Cere Lintang, Manikam, Cao, Jawara Hawara, Jalawara, Pare Racik, Godok, Mayang, Caragol, Segubal, Konjal, Beuruem Batu, Tambleg, Parajaketra, Pondok Leger, Seungkeuhan, Waren, Pare Emas, Kapundung, and Cireh Hudang. Group B consisted of non-aromatic rice accessions, i.e. Ciherang, Care Wari, Care Beuruem, Cokrom, Kewal Bulu Hideung, Maninjau, Seren, Sidenuk, Tampai Beureum, Mira, and Pare Caok.

Keywords: rice, local varieties, aromatics, SSR

1. Introduction

Rice (*Oryza sativa* L.) has various varieties, among which some commonly used in Indonesia are: IR64, Ciherang, Pandan Wangi, and Inpari. In Banten province, there are also some local varieties, namely: Waler, Tambleg, Kewal Bulu Hideung, Gadog, Jaketra, Kewal Cere, Ketan Laleur, Jalawara Hawara, Ketan Solo, and Kewal Gudril [1][2]. As a primary food, rice can meet most of the nutrition needs of most Indonesian societies.

The amount of rice consumption of Indonesian people reached 114.6 kg/capita/year or 314 g per capita per day [3]. It is higher than that of other countries, which is 60-90 kg/capita/year. Because of such dependency, the government strongly needs to guarantee the annual rice-stock availability through even distribution and stable prices. Thus, the government should enhance the quantity and quality of rice production itself, so that the national rice needs will be adequately fulfilled.

The high rice production should be balanced with the enhancement of rice quality upon new advanced varieties, which has become one of the main objectives of the rice breeders. Palatability (good taste) is a trait of rice connected to quality, determined by the aroma, appearance, texture, and taste [4]. Indonesian people generally prefer rice with a delicious taste and aroma, and better tastes and aromas will increase the value of rice production. Aromatic rice is a distinguished variety of rice groups because of its excellent quality. The aroma itself emanates from its aroma-controller gene. [5] succeeded in identifying aromatic and non-aromatic rice according to its molecular markers based on the badh-2 gene, the encoder of rice fragrance.

Molecular markers are a strong means of understanding the genetics of several models associated with agricultural science. According to [6], these markers are used widely by breeders because they provide beneficial genetic information. Such markers become one of the methods used in biotechnology, which is the development of molecular biology in modern plant breeding and plays a pivotal role in the maintenance of plant characters. Likewise, to produce excellent rice variety needs biotechnology. The intervention of DNA recombination and molecular breeding will enhance the excellent properties of rice, which can give it more value. To seek such excellent properties, DNA isolation needs to be conducted as the first stage of any technology-assisted DNA analysis.

PCR (*polymerase chain reaction*) is an in-vitro method to produce a large number of DNA fragments, specifically with its lengths and sequences that have been determined from small amounts of complex template. PCR is really strong and sensitive, which is applicable in several fields, such as molecular biology, diagnostic, population genetics, and forensic analysis. Commonly used PCR-group molecular markers are those that use a pair of primers and a single primer, Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), SNP, Sequence Tagged Sites (STS), and microsatellite or SSR (Simple Sequence Repeat) [7], [8], SSR molecular marker does have a connection with the specific gene and has some advantages, such as: easy and inexpensive; and it has fast detection methods and only requires a small number of tissue samples. DNA fingerprint or fingerprinting is a method to identify the peculiarities of individual DNA patterns. The fingerprints will show the identity of rice varieties indispensable in the development of plant breeding to create superior rice varieties and support the increase of national rice production.

Many studies had been reported on the use of SSR markers to identify a character in rice plants. [2] conducted a study on the genetic diversity of red rice varieties originating from West Java and Banten based on SSR markers related to palatability. [9] reported on SSR-based genetic diversity of pigmented and aromatic rice genotypes of the western Himalayan region (India), as well as [10] reported some Egyptian rice genotypes. According to [11], SSR markers can be used to identify potential parent in future aromatic rice breeding programs. Herein, the objective of this study was to analyze the genetic diversity of some Indonesian local rice varieties based on SSR marker related to aromatic genes.

2. Materials and Method

This research was conducted from March to April, 2017, at Greenhouse of Certification Center of Horticulture Plant Seed, Serang, Banten, and Laboratory of Biotechnology, Faculty of Agriculture, University of Sultan Ageng Tirtayasa, as well as Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) Bogor, West Java.

This research used the descriptive qualitative method. It was a method that includes collecting data, analyzing data, interpreting data, and ending with a conclusion that refers to the results of data analysis. The procedure started from the rice seed seedling for up to 21 days after planting, and then the DNA was isolated from the rice leaves. The DNA isolation used the CTAB method employing 6 aromatic rice primers. After that, a PCR process with SSR markers was performed, whose results were

processed by electrophoresis using an agarose gel. The finished running was stained with a staining solution in EtBr and washed with ddH2O, then documented with Chemidoc Transilluminator EQ Biorad. The DNA bands were then scanned for the presence of bands and DNA profiles embedded in binary data for subsequent clustering.

2.1. Rice Seedling

Rice seedling was performed on 34 accessions of rice. The seeds were seeded on a bucket, composed of medium soil mixed with compost (3:1) and 1 g of NPK fertilizer. 5 seeds were planted in each bucket under one variety type.

2.2. DNA Isolation

In this study, the rice's young leaves were taken at age 21 days after planting, and the DNA was isolated. For DNA isolation, the solution was made by using the sampling method, taking 0.25 – 0.5 gram leaves crushed in porcelain with the help of liquid nitrogen. To avoid oxidation at the scouring process, ± 0.02 gram of PVPP was added.

The quantitative DNA/RNA assay with the Nano-drop of spectrophotometry was measured with an absorbance ratio of 260/280. The quantitative DNA test used 2% agarose. The gel was visualized inside Gel Doc. The DNA stock solution was stored at 20°C until PCR analysis was later performed.

2.3. DNA Amplification with PCR

Diluted DNA was then processed by PCR reaction performed at 10 μl of volume, containing the PCR mixture, and poured into the PCR tube. DNA amplification with PCR was done for 30 cycles. The procedure comprised of denaturation for 2 minutes at 94ºC, denaturation for 30 seconds at 94ºC, primary attachment process for 30 seconds at 63ºC (for all primers), and 30 seconds at 72ºC for primary elongation. The last primary elongation was carried out for 7 minutes at 72ºC.

2.4. Data Analysis and DNA Fingerprint Profile Creation

The resulting DNA band patterns from the amplification of each primer were analyzed based on the presence or absence of an amplification band, indicating that the sample rice DNA had an aromatic-linked gene. Gel Analyzer software program and Power Marker were employed to get the value of PIC (Polymorphic Information Content). Furthermore, based on the molecular markers used, the dominant marker produces "existing" (positive) and "none" (negative) DNA bands.

The resulting band patterns were converted into binary values, with 1 indicating "positive" on the presence of a DNA band, while 0 indicating "negative". The results obtained from the data analysis were then made into a unique DNA fingerprint profile of each accession based on the results of primary amplification in the binary values sequentially from left to right in the order of 6 primers used so that the digital value system could be displayed. The data were also analyzed by using the NT-SYS software program for "cluster tree analysis" to reveal the genetic relationship and proximity among all the genotypes studied.

3. Results and Discussion

The primers used in this study yielded 2 to 3 loci. Primers RM 484, RM 223, and RM 9 yielded 3 loci, while Primers RM 410, RM 251, and RM 247 yielded 2 loci. Based on the results of [12] research using 10 SSR markers (RM 9, RM 247, RM 251, RM 335, RM 410. RM 411, RM 433, RM 484 RM 444, and RM 535) a polymorphic band pattern was produced.

The SSR-based aromatic gene primers used can be seen in Table 1. The PCR results using the primers were then translated into digital values (Table 2). Details of the PIC of the primers used can be seen in Table 3.

Table 1. The SSR primers based on aromatic gene

Loci name	Chromosome	Bases sequence	TM (°C)
RM 484	10	F: TCTCCCTCCTCACCATTGTC R: TGCTGCCCTCTCTCTCTCTC	63
RM 410	9	F: GCTCAACGTTTCGTTCCTG R: GAAGATGCGTAAAGTGAACGG	63
RM 251	3	F: GAATGGCAATGGCGCTAG R: ATGCGGTTCAAGATTCGATC	63
RM 247	12	F: TAGTGCCGATCGATGTAACG R: CATATGGTTTTGACAAAGCG	63
RM 223	8	F: GAGTGAGCTTGGGCTGAAAC R: GAAGGCAAGTCTTGGCACTG	63
RM 9	1	F: GGTGCCATTGTCGTCCTC R: ACGGCCCTCATCACCTTC	63

Source : [12]

Table 2. Digital values of PCR amplification results on 6 SSR marker primers

No	Accession Name	Digital Value
1	Rabeg	001.010.000.100.000
2	Rumbah	010.010.000.100.000
3	Cere Lintang	100.010.000.100.000
4	Pare Emas	100.000.000.100.000
5	Pare Racik	100.100.000.110.000
6	Manikam	100.100.000.100.000
7	Kapundung	010.000.000.100.000
8	Caok	010.100.000.100.000
9	Carogol	010.100.000.001.010
10	Care Wari	010.000.001.001.010
11	Beureum Batu	010.100.000.001.000
12	Care Beureum	010.000.001.001.100
13	Cokrom	001.100.001.001.100
14	Seungkeuhan	001.100.100.001.100
15	Waren	001.100.100.001.100
16	Jalawara Hawara	001.100.000.001.100
17	Pare Caok	010.000.001.000.000
18	Godok	001.000.000.000.000
19	Mayang	100.100.000.000.000
20	Jalawara	100.100.000.100.000
21	Pare Jaketra	010.100.000.100.010
22	Tambleg	010.100.000.100.010
23	Seren	010.100.001.100.001
24	Cireh Hideuang	001.000.000.100.011
25	Konjal	001.100.000.100.010
26	Pondok Leger	001.100.000.001.010
27	Segubal	001.100.000.001.010
28	Tampai Beureum	001.100.010.001.000
29	Kewal Bulu Hideung	001.100.010.001.001
30	Maninjau	001.100.010.001.001
31	Sidenuk	001.010.010.001.001
32	Ciherang	001.010.010.001.001

33	Mira	001.100.010.100.000
34	Rojolele	001.101.000.100.000

Fragrance genes and badh2 genes were fragrance-encoding genes found in aromatic rice. Badh2 gene was present on chromosome number 8 which is responsible for exuding the aroma. The genes were not only present in aromatic rice but also non-aromatic ones. The most important component contributing to the aroma of aromatic rice is 2-*AP* [13]. Aromatic rice has a volatile or 2-*AP* compound which gives an aroma to the rice. The 2-*AP* can be found in all parts of aromatic rice plants, except at its roots. The 2-*AP* content is relatively higher in rice crops than in milled rice grains [14].

The results of the identification of the aromatic genes displayed in Table 2 show that the mean of genetic diversity was 0.86, with the highest genetic diversity of 0.92 on primer RM 9, while the lowest was 0.67 on RM 251. The heterozygosity and genetic diversity levels were quite high, showing that the SSR locus used could distinguish the clones being analyzed. RM223 was the only primer that had the second-highest diversity value of heterozygosity of 0.90.

From the 6 loci, only one locus had a heterozygosity value, namely RM 223 (0.03). Heterozygosity is related to the probability that 2 alleles took randomly from a population can be distinguished by using a marker [15]. One of the advantages of microsatellite markers or SSRs as well as other codominant markers is to detect heterozygosity. A heterozygous focus will produce more than one band of each primer, where the limit of the number of alleles produced depends on the number of individual ploidies being analyzed. Meanwhile, homozygous loci will only produce one band or allele of each primer [16].

The resulting polymorphic alleles were analyzed in the form of percentages by calculating the polymorphic alleles to see how many percent of the polymorphic alleles was obtained in each primer used. The level of informative primers was determined by the calculation of Polymorphic Information Content (PIC). PIC value provides an estimate of the distinguishing power of a marker by computing not only the number of alleles in one locus but also the relative frequency of the alleles of an identified population. PIC value becomes the standard for evaluating the genetic markers based on PCR amplified DNA bands [17]. Therefore, PIC value is divided into 3 classes: PIC>0.5 = highly informative; 0.25> PIC> 0.5 = moderately informative; and PIC <0.25 = slightly informative [18].

All markers showed highly informative of PIC value with the average 0.84. Primer RM 484 obtained a PIC value of 0.87, both RM 410 and RM 223 gained 0.89, and RM 247 gained 0.88. The highest and the lowest were RM 9 and RM 251 with PIC value of 0.91 and 0.59, respectively. Besides, all primers had polymorphic DNA band properties.

Table 3. Number of alleles, Gene diversity, Heterozigosity, and PIC Values of 34 rice accessions with 6 SSR markers in terms of aromatic gene

Marker	First Allele Frequency	Number of Alleles	Gene Diversity (He)	Heterozygosity (Ho)	PIC
RM 484	0.22	13	0.88	0.00	0.87
RM 410	0.21	14	0.89	0.00	0.89
RM 251	0.33	3	0.67	0.00	0.59
RM 247	0.17	10	0.89	0.00	0.88
RM 223	0.19	13	0.90	0.03	0.89
RM 9	0.15	14	0.92	0.00	0.91
Mean	**0.21**	**11**	**0.86**	**0.00**	**0.84**

Notes: 1. General allele: Allele Frequency ≥0.75; Moderate allele: Allele Frequency 0.75 <P≤0.25; Rare allele: Allele Frequency 0.25 <P≤0.01; Specific Allele: Allele Frequency <0.01.
2. PIC> 0.5 = Highly informative; 0.25> PIC> 0.5 = moderately informative; PIC <0.25 = slightly informative

The primers with larger PIC values are the best primers that can be used as molecular markers [18]. The selected samples of primer RM 9 were: #9 (Carogol), #10 (Cere Wari), #12 (Care Beureum), #13 (Cokrom), #14 (Seungkeuhan), #15 (Waren), #16 (Jalawara Hawara), #21 (Pare Jaketra), #22 (Tambleg), #23 (Seren), #24 (Cireh Hideung), #25 (Konjal), #26 (Pondok Leger), #27 (Segubal), #29 (Kewal Bulu Hideung), #30 (Maninjau), #31 (Sidenuk) and #32 (Ciherang). These primers had a locus range of 70-1850 bp (136 bp). The samples corresponding to the target were #23 (Seren) and #24 (Cireh Hideung). These genotypes can be recommended as the elders for rice crossing to rice assembly. Sample #32 (Ciherang), which was non-aromatic rice, had a locus size of below 100 bp, or approximately 70 bp.

The use of primer RM 223 was able to amplify aromatic and non-aromatic rice with variations in the length of DNA fragment between 120 bp and 160 bp [19]. DNA amplification using this primer can distinguish the pattern of aromatic and non-aromatic rice bands [20]. This primer differentiates aromatic from non-aromatic rice based on the DNA size, where [21]) used primer RM223 to detect aromatic rice based on the presence of 151 bp band, and non-aromatic marked with a 145 bp.

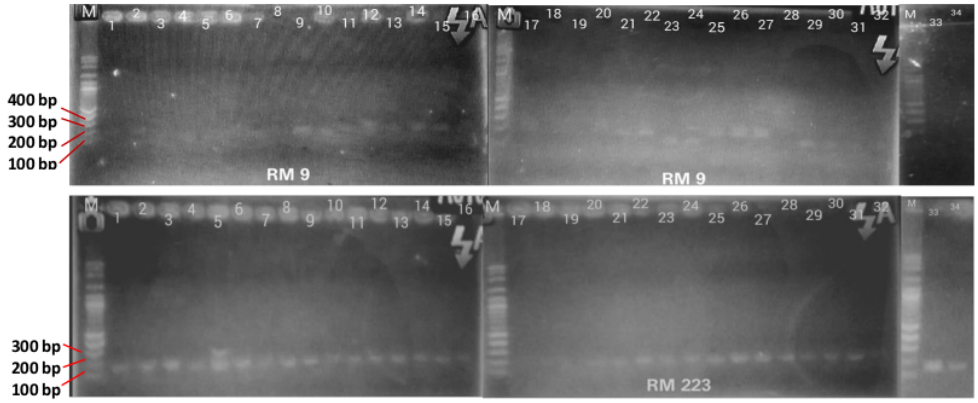

Figure 1. Band of primers RM 9 and RM 223.
Notes: (1) Rabeg, (2) Rumbah, (3) Cere Lintang, (4) Pare Emas (5) Pare Racik, (6) Manikam, (7) Kapundung, (8) Cao, (9) Carogol, (10) Care Wari, (11) Care Wari, (12) Care Beureum, (13) Cokrom, (14) Seungkeuhan, (15) Waren, (16) Jawara Hawara, (17) Pare Caok, (18) Godok, (19) Mayang, (20) Jalawara, (21) Pare Jaketra, (22) Tambleng, (23) Seren, (24) Cireh Hideung, (25) Konjal, (26) Pondok leger, (27) Segubal, (28) Tampai beureum, (29) Kewal Bulu hideung, (30) Maninjau, (31) Sidenuk, (32) Ciherang, (33) Mira, and (34) Rojolele. M: 100 bp.

Genetic Diverisity of the 34 Rice Germplasms Based on Aromatic Gene

The dendrogram (Fig. 1) generated by NTSYS-program indicates an intersection of 0.57-0.79 or a level of genetic diversity of 21-43% (genetic similarity as much as 57-79%).

Table 4. Genetic similarity of the 34 rice accessions based on 6 SSR-marker primers

Group	Sub-group	Number of Accessions	Selected Accessions	Genetic Similarity
A	**A1**	**20**	Rabeg, Rumbah, Cere Lintang, Manikam, Caok, Jalawara Hawara, Jalawara, Rojolele, Pare Racik, Godok, Mayang, Carogol, Segubal, Konjal, Beureum Batu, Tambleg, Pare Jaketra, Pondok Leger, Seungkeuhan and Waren.	0.68-0.79
	A2	**3**	Pare Emas, Kapundung and Cireh Hideung.	0.64-0.79

B	**B1**	**10**	Care Wari, Care Beureum, Cokrom, Ciherang, Kewal Bulu Hideung, Maninjau, Seren, Sidenuk, Tampai Beureum and Mira.	0.59-0.79
	B2	**1**	Pare Caok	0.59
Total		**34**		

There were 2 main groups. Group A consisted of 2 sub-groups, i.e. A1 and A2. The former had a genetic similarity coefficient (GSC) of 0.68-0.79 or a genetic distance of 21-32% and was divided into 2 other sub-groups. The first sub-group obtained a genetic similarity coefficient of 0.77-0.79 or a genetic distance of 21-23%. There were 11 accessions of rice germplasm in this sub-group, while the second sub-group gained a genetic similarity coefficient of 0.68-0.79 or a genetic distance of 21-32%. There were 9 accessions of rice germplasm in this sub-group. The A2 main sub-group obtained a genetic similarity coefficient of 0.64-0.79 or a genetic distance of 21-36%, with 3 accessions of rice germplasm.

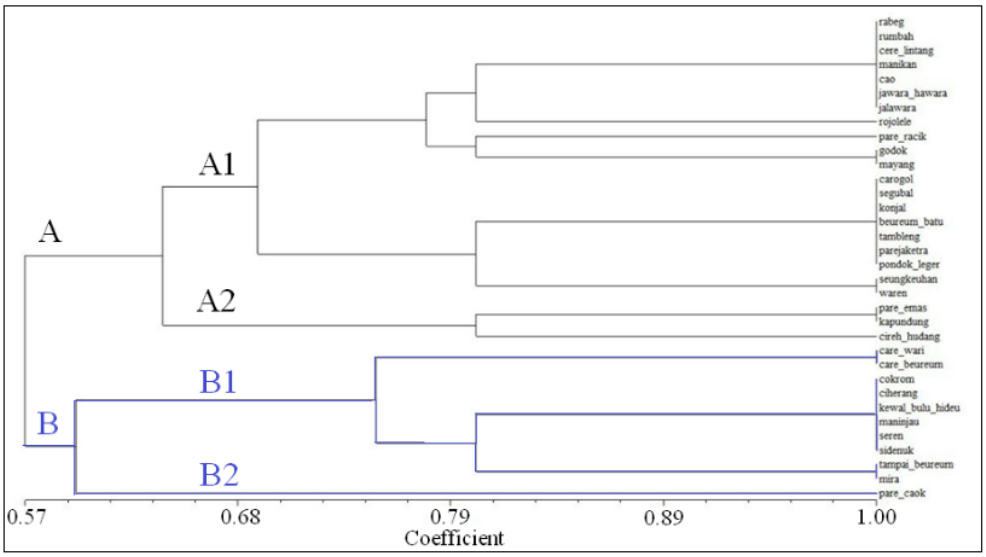

Figure 2. Dendrogram of 34 rice accessions based on 6 SSR-marked link to aromatic gene

Group B was also divided into 2 other sub-groups, i.e. B1 and B2. The former obtained a genetic similarity coefficient of 0.59-0.79 or a genetic distance of 21-41% and was subdivided again into 2 other sub-groups. The first sub-group gained a genetic similarity coefficient of 0.59-0.75% or a genetic distance of 25-41%. There were 2 accessions of rice germplasm in this sub-group. Meanwhile, the second sub-group gained a genetic similarity coefficient of 0.75-0.79 or a genetic distance of 21-25%, having eight accessions of rice germplasm. The B2 main sub-group got a genetic similarity coefficient of 0.59 or a genetic distance of 41%, having one accession of rice germplasm. The transparencies can be seen in Table 4.

The magnitude of the genetic distance between the evaluated clones is important in the utilization of the clones for plant breeding. Two clones that have a high genetic distance when crossed will produce highly varied derivatives. In contrast, two clones having a low genetic distance, when crossed, will produce low-varied derivatives. According to Fig. 2, the highest genetic distance value was found in the B2 sub-group for as much as 41%, followed by the B1 sub-group for 21-41%.

It can be assumed that group A belongs to a rice class with an aromatic-linked gene, while group B belongs to that with a non-aromatic-linked gene. The dendrogram shows the closeness of the genetic similarity intersection points. Between A1 and A2 sub-groups, 0.68 and 0.64, the GSC value was only 0.02. On the other hand, B1 and B2 sub-groups had the same GSC value of 0.59. In group A, there was an accession with an aromatic gene, namely Rojolele, while in group B there was Ciherang which is non-aromatic rice accession. Both can be utilized as good examples of both aromatic and non-aromatic rice.

Figure 2 also shows that Caok belongs to group A. According to the field study, this accession has a distinctive aroma, but not too sharp like Rojolele. The difference between aromatic and non-aromatic rice is not based on the presence or absence of 2-AP but based on its quantity. The aromatic rice contains a higher 2-AP compound (0.04-0.07 ppm) than the non-aromatic one (0.004-0.006 ppm). Besides genetic factors, the contributing factors of the content and concentration of 2-AP are environment, cultivation method, and post-harvest process [13]. Therefore, some things need to be considered before the implementation of rice planting in identifying the aromatic genes.

4. Conclusion

Based on the PCR process with the primers used, the rice accessions associated with aromatic characters were Rabeg, Rumbah, Cere Lintang, Manikam, Caok, Jawara Hawara, Rojolele, Pare Racik, Godok, Mayang, Carogol, Segubal, Konjal, Beureum Stone, Tambleg, Pare Jaketra, Pondok Leger, Seungkeuhan, Waren, Gold Pare, Kapundung, and Cireh Hideung. Meanwhile, the rice accessions associated to non-aromatic characters were Care Wari, Care Beureum, Cokrom, Ciherang, Kewal Bulu Hideung, Maninjau, Seren, Sidenuk, Tampai Beureum, Mira, and Pare Caok.

All SSR primers used were highly informative, can be recommended as the markers to track the linkage with aromatic genes. RM 223 obtained a heterozygosity value of 0.03 and the second highest diversity value of 0.89. On the other hand, the highest PIC value was obtained by primer RM 9 of 0.91. Genetic diversity of the 34 rice accessions had divided in 2 main groups: Group A (linked to aromatic) and group B (linked to the non-aromatic). Group A consists of 2 sub-groups in which the A1 (consists of: Rabeg, Rumbah, Cere Lintang, Manikam, Caok, Jara Hawara, Jalawara, Rojolele, Pare Racik, Godok, Mayang, Carogol, Segubal, Konjal, Beureum Stone, Tambleg, Pare Jaketra, Pondok Leger, Seungkeuhan, and Waren); the A2 sub-group (consists of Pare Emas, Kapundung, and Cideh Hideung). Meanwhile, group B consists of 2 sub-groups. The B1 sub-group (consists of Care Wari, Care Beureum, Cokrom, Ciherang, Kewal Bulu Hideung, Maninjau, Seren, Sidenuk, Tampai Beureum, and Mira); and the B2 sub-group (consists of Pare Caok).

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