Polymorphism levels of some SSR markers (Simple Sequence Repeat) for parental line identification on low temperature tolerance

S R Dalimunthe\textsuperscript{1,2}, L A M Siregar\textsuperscript{3*}, L A P Putri\textsuperscript{3}, T Chairunnisa\textsuperscript{3} and A Hairmansis\textsuperscript{4}

\textsuperscript{1}Doctoral Program of Agricultural Science, Faculty of Agriculture, Universitas Sumatera Utara, Medan, Sumatera Utara, Indonesia.
\textsuperscript{2}North Sumatera Assessment Institute for Agricultural Technology, Medan, Indonesia.
\textsuperscript{3}Faculty of Agriculture, Universitas Sumatera Utara, Medan, Sumatera Utara, Indonesia.
\textsuperscript{4}Indonesian Institute for Rice Research, Subang, Indonesia.

E-mail: *luthfi2004@yahoo.com

Abstract. Breeding rice varieties suitable for low temperature areas is very important to support food self-sufficiency. Identification of polymorphic markers assumed to be associated with low temperature tolerant properties is essential for mark-based selection applications in the context of low temperature tolerant rice variety development. The study was conducted to identify 5 rice genotypes to be used as a low temperature tolerant donor hybrid using 11 SSR markers. PCR product amplification was separated using 3\% agarose gel and 8\% electrophoresis polyacrylamide gel (PAGE). The level of information of a mark can be determined by calculating the Polymorphic Information Content (PIC). The study was conducted in May - July 2017 at the Laboratory of Molecular Biology, Centre for Research and Development of Biotechnology and Genetic Resources of Agriculture, Bogor. The results showed that 6 SSR markers were polymorphic and 5 markers were monomorphic. CT234 and CT235 markers have the highest PIC value (0.375) compared to 9 other markers. SSR markers and specific gene markers used to identify low temperature tolerant rice show that Ciherang and Sigambiri Putih crossbreeding can be recommended as a crossing parents because it has the most distant genetic (0.431).

1. Introduction

Indonesia has many potential local rice genetic resources, which can be used as a base for assembling superior varieties in plant preservation. Local rice has a wide genetic diversity and is a source of genes that control important traits.

The use of local varieties as one of the hybrid crosses is highly recommended for obtaining superior genes and to extend the genetic background of the improved varieties to be produced [1]. The use of localized rice that has been characterized and evaluated needs to be further improved and integrated with breeding programs [2] [3].

North Sumatra has Sigambiri Putih and Sigambiri Merah Rice widely grown in Simalungun and Karo communities. Both of the local gogo rice have many advantages such as resistance to blast races
033, 175 and 173, resistance to low temperature and adaptive at altitudes up to 1300 m above sea level, both cultivated in dry and marginal land [4].

The low temperature tolerant farm rice assembly program by utilizing local varieties as gene donors and crosses, coupled with the application of biotechnology is estimated to increase the chances of obtaining low temperature tolerant varieties.

Crosses in rice plants can lead to a combination of alleles that can increase genetic diversity. Determination of the hybrid is a very important stage because it will determine the success of the desired goal of character acquisition. The hybrid used must have the desired character and have a good adaptation. High diversity can be generated using hybrid couples [5].

Advances in the field of molecular biology, allowing the genetic diversity of a population to be observed at the DNA level. These molecular markers are not influenced by environmental factors [6]. The molecular genetic approach using DNA markers has succeeded in forming molecular markers capable of detecting certain genes and traits. The molecular marks on plants can be divided into two: molecular markers based on PCR techniques and molecular markers without the use of PCR techniques. Molecular markers based on PCR techniques include Random Amplified Polymorphic DNA (RAPD), AFLP and SSR while RFLP is a molecular marker that does not use PCR techniques [7].

The polymorphism level of the DNA marker is the production power of the DNA band from the PCR reaction results using a particular marker / primer. The degree of polymorphism is influenced by the variety of genotypes used [8] [9] and it is highly dependent on how primers recognize their complementary DNA sequence in the DNA template used [10]. Markers used for PCR reactions and electrophoresis results show the same banding pattern between each DNA sample of different genetic monomorphic markers whereas if the electrophoresis results show different banding patterns between each DNA sample is a polymorphic marker.

The level of DNA marker polymorphism can be assessed by looking at the value of PIC (polymorphism information DNA). The PIC value is the value of the DNA production power produced by the PCR reaction using a particular primer. The value of PIC 0.00 means that the marker used in the PCR reaction produces the same banding pattern on all sample DNAs, which means monomorphic. Markers that have PIC values> 0.00 are called polymorphic markers. The higher the PIC value means the resulting DNA band patterns are more informative. The PIC value > 0.50 is the expected value for DNA fingerprint identification [11]. Quantification PIC is the number of alleles that can be generated by a marker and the frequency of each allele in the set of genotypes tested. The value of polymorphism is determined by the frequency of occurrence of alleles [12].

Thousands of SSR markers scattered throughout the genomes of rice plants have been investigated and validated [13] [14] [15]. However, not all of the validated markers can be used. As well as marker election results in [16] indicating that of the 16 SSR markers used, only 7 can produce polymorphic bands.

Analysis of PIC marker DNA value is one of a series of activities in germplasm fingerprint identification. This analysis serves to determine the available markers for use in the analysis of the degree of genetic diversity / distance / clustering among the rice genotypes used. The level of polymorphism (PIC) is required to select a marker that can distinguish the used hybrids.

The aim of this research is to know the level of DNA band polymorphism based on PIC value (Polymorphism Information Content) generated by markers used in fingerprinting of germplasm DNA, so that the data obtained later can be used for low temperature tolerant rice selection. In addition, selected varieties can be used as a crossing hybrid for low temperature tolerant rice assembly.

2. Materials and methods
The study was conducted in May - July 2017 at the Laboratory of Molecular Biology, Indonesian Centre for Agricultural Biotechnology and Genetic Resources Research and Development, Bogor. DNA samples used were 5 DNA samples from germplasm genotypes consisting of 2 local rice and 3
high yielding varieties (HYV) rice. The SSR Marker applied and researched the polymorphism level of 11 markers related to the low temperature tolerance properties (Table 1).

Table 1. List of SSR Markers used in the study

| No | SSR Marker | Forward Primer (5’-3’) | Reverse Primer (3’-5’) | Base Range (bp) | Source |
|----|------------|------------------------|------------------------|-----------------|--------|
| 1  | Ctb1       | AATAAACACCTGGA ACATA   | ATAAACAAGCAATCTATC AC  | 56              | [17]   |
| 2  | CT220      | AAGTGGTTTCATG TTATGCTAATTTT | GAAAGCAAGCGGATTA GC  | 250             | [18]   |
| 3  | CT221      | GTTTTGCTGTCGTT TGGATT  | GGCAACCATGTTTTGGG TAT  | 246             |        |
| 4  | CT224      | CAATACATAATA ATGCGGACTCTG | TGACTATACGGAATGC AAGAA  | 234             |        |
| 5  | CT232      | GGCTCGGGTTTAA GGCTCGGA | TCTCGTATGTTCACAGGA GGAATCT  | 154             |        |
| 6  | CT233      | GGACGGATTTTCT CATCCTATGC | GACATACTGAACGATG CCAAT  | 253             |        |
| 7  | CT234      | TTGAAGAAAGAT TTGATACCG | GCTGGGGTTGTGGTTGG T  | 235             |        |
| 8  | CT235      | GTACCAGCTGCTG TTGATACCG | GGAATAGGGCGGTATGT TGT  | 138             |        |
| 9  | CT236      | AGTCGGCTATTGT CGTCGTT  | AGTCGGCTATTGTGC TGT  | 114             |        |
| 10 | CT245      | CAAAAATTTAAG CAAAAACACC | AGATGGGTGCGTCTCC TAA  | 453             |        |
| 11 | CT6860     | ACCATGCCAAGTG CCGCCCTGT | AAGTGGGACCCACATGT CAT  | 470             |        |

2.1. Molecular analysis

DNA was isolated from young leaf of 21 HSS. DNA isolation on rice leaves was done using CTAB [19]. The plasma leaf germ plasma samples were collected as much as +5 grams from the seedlings. DNA quality was measured using 3% agarose gel electrophoresis method and 8% polyacrylamide gel electrophoresis (PAGE), while the quantity of DNA was measured using spectrophotometer NanoDrop™ 2000 (Thermo Scientific, USA).

Selection of rice plants was performed with PCR-based marker with 11 primers. The PCR reaction is performed by the PTC-100 PCR machine using the CKX-fast program. Total volume used is 20 μL, contains 8.0 μL ddH2O, 10μL mix PCR Kapa 2G™ Fast ReadyMix (2X) from KAPA Biosystems, 1 μL each primer (F and R). The amplification reaction was carried out for 34 cycles, consisting of a 5-minute denaturation at 94°C, 30-second denaturation at 94°C, primary attachment for 30 seconds at 55°C, and a primary extension for 45 seconds at 72°C. The last primary extension occurred for 7 minutes at 72°C. Run electrophoresis of PCR results was performed using 1% agarose gel with 100 volt for 30 minutes. DNA staining was performed with ethidium bromide and documented using ChemiDoc™ EQ System (Bio-Rad, USA).

Molecular data analysis was performed on the basis of DNA scaling results that appeared on the gel manually in Ms. Excel media. A value of 1 is given if there is an allele and a value of 0 when there are no alleles. The scores were then analysed by PIC using Power Marker software version 3.23 [20] to determine the polymorphism level of the banding pattern.
The percentage analysis of polymorphic alleles was calculated to see how many percent of the polymorphism alleles formed in each primer were used. To calculate the percentage of polymorphic bands, the formula is used:

\[
\text{Polymorphic Percentage} = \frac{\text{Number of polymorphic alleles}}{\text{Total of allele}}
\]

(1)

The PIC value is standardized for evaluating genetic markers based on DNA bands of PCR amplification results, therefore PIC values are divided into three classes: PIC > 0.5 = highly informative, then 0.25 > PIC > 0.5 = moderate, and PIC < 0.25 = low.

3. Results and discussion

Plant breeding with the aim of improving plant genetics always begins by selecting a superior hybrid as a source of gen donor. Expected source of genes may be in populations that have wide genetic diversity. Without the availability of gen donor, it is impossible for breeding purposes to succeed. Differences in diversity in a population can be observed phenotypically and genetically. The weakness of the phenotype is the limited character observed, the characters appearing influenced by the environment and often genetic differences do not appear in phenotypic characters.

Observations of genetic diversity in this study used molecular markers with SSR primers. The results of the analysis using genetic markers are not affected by the environment. Polymorphic shows the diversity in the plant genome. The more primers that can amplify the polymorphic band, the greater the diversity in the genome.

The results showed that from 11 kinds of SSR primer used, polymorphism band from 5 primers are obtained. Primers showing the polymorphism are: CT221, CT224, CT232, CT233, and CT234 (Figures 1 and 2). The Ctb1 and CT236 primers do not appear. The absence of the bands from the two primers is suspected due to the incompatibility of these primers for the tested rice crops.

Primers that have not been able to amplify well may be due to its improper PCR program. In addition, primers that are inconsistent with the studied rice sequences of DNA, may also cause the product not to be amplified as there is no complementary match between the rice DNA and the primary sequences used. Informative primers are shown by PIC values ≥ 0.5 [15], whereas primers with larger PIC values are the best primers that can be used as molecular markers.

![Figure 1](image.png)

**Figure 1.** Appearance of DNA polymorphism tape from 5 elders using CT221 (a), CT224 (b) and Ctb1 (c) markers using agarose.
The level of polymorphism (PIC) is required to select markers that can differentiate between the lines/hybrid used. Quantification PIC is the number of alleles that can be generated by a marker and the frequency of each allele in the set of genotypes tested. The value of polymorphism is determined by the frequency of occurrence of alleles [12]. Marks that produce fewer alleles have a smaller ability to distinguish samples tested. The high PIC value is shown on the mark that generates multiple alleles.

The result of PIC value analysis shows 5 SSR markers are polymorphic, 4 are monomorphic and 2 are not amplified. The average PIC value of the 11 SSR markers used is 0.2045 with the highest value 0.3750 and the lowest 0.000 (Table 2).

CT220, CT233, CT236, CT245 and Ctb1 markers are markers that have the lowest PIC value of 0.000. This may be due to two possibilities, namely (1) the absence of DNA bands due to the base of the marker sequence which cannot recognize the complementary DNA sequence in the DNA template of each of the germplasm genes used, or (2) the product yield PCR shows the same band pattern on all germplasm DNA used. The low PIC values also occur in several studies such as [16] [21] [22] used the same primary PIC value with this research that is 0.000. This indicates that the marker is suspected to be a monomorphic marker.

| No | Marker | Total of alleles | Availability | GeneDiversity | PIC  |
|----|--------|-----------------|--------------|---------------|------|
| 1  | Ctb1   | 1               | 0.1667       | 0.0000        | 0.0000 |
| 2  | CT220  | 1               | 0.1667       | 0.0000        | 0.0000 |
| 3  | CT221  | 2               | 0.8333       | 0.4800        | 0.3648 |
| 4  | CT224  | 2               | 1.0000       | 0.1528        | 0.1411 |
| 5  | CT232  | 2               | 1.0000       | 0.4444        | 0.3457 |
| 6  | CT233  | 1               | 0.3333       | 0.0000        | 0.0000 |
| 7  | CT234  | 2               | 0.3333       | 0.5000        | 0.3750 |
| 8  | CT235  | 2               | 1.0000       | 0.5000        | 0.3750 |
| 9  | CT236  | 1               | 0.1667       | 0.0000        | 0.0000 |
| 10 | CT245  | 1               | 1.0000       | 0.0000        | 0.0000 |
| 11 | CT6860 | 2               | 1.0000       | 0.2778        | 0.2392 |
|    | Mean   | 1.6667          | 0.7407       | 0.2617        | 0.2045 |
The results show that CT234 and CT235 are the best polymorphic markers because they have the highest PIC value of 0.3750. Based on Botstein et al. (1980) PIC values are good and informative for DNA fingerprint activity is PIC> 0.5. According to [23] [24], the PIC value> 0.50 indicates a high degree of polymorphism. Based on this, that is, CT234 and CT235 markers are the most available SSR markers and have the ability to distinguish between the characters (gen diversity) best compared to others. CT234 and CT235 resulted in a diversity gene of 0.5000 and the highest compared to other markers. This marker is related to the tolerance to low temperatures based on the results of studies [18] which maps qCTS4 and is located on chromosome no 4.

The value of PIC becomes important to be observed. [25] states there are some problems in using microsatellite/SSR markers. These problems can be grouped into practical problems and data problems. The practical problems are: a) Primary selection for microsatellite, many primary types have been designed for microsatellite analysis on plants. The primers need to be screened and optimized before they are applied to certain types of plants, because each plant has specific characteristics that differ from one another. b) Slippage during the amplification process, thermopolymerase can slip to produce different products in size. The term slip here means the process of attaching a sequence of bases is likely missed against the template DNA. c) The size of the amplification product is different from the actual product size. The size of the amplification product is probably most affected by the quality of the marker by looking at the PIC value.

Marks that produce fewer alleles have a smaller ability to distinguish samples tested. The high PIC value is shown on the mark that generates multiple alleles. The highest PIC values of polymorphic markers are shown by CT234 and CT235 (0.3750) and lowest on CT233, CT236, CT245 (0.000). According to the classification of PIC values performed by Botstein et al. (1980), no markers are included in the highly informative category (PIC> 0.5), four markers have a moderate PIC value of CT221, CT232, CT234, and CT235 while markers with low PIC values (<0.25) five markers are CT224, CT233, CT236, CT245, CT6860.

The genetic distance coefficient analysis shows that the range of genetic variations ranges from 0 to 0.431. The longest genetic distance coefficient is between Ciharang and Sigambiri Putih, and Situbagendit with Sigambiri Putih, of 0.431. That is, Ciharang and Situbagendit have genetic difference of 43.1% to Sigambiri Putih. The second and third furthest genetic distance is between Ciharang and Situbagendit with Sigambiri Merah of 0.4321 and between Mekongga and Sigambiri Putih of 0.3172. The closest genetic distance even similar is 0.000 (Table 3).

**Table 3. Genetic distance coefficient between superior germplasma based on eleven SSR markers**

| OTU          | Ciharang | Mekongga | Situbagendit | Sigambiri Merah | Sigambiri Putih |
|--------------|----------|----------|--------------|----------------|----------------|
| Ciharang     | 0.0000   | 0.0000   | 0.0000       | 0.3821         | 0.4310         |
| Mekongga     | 0.0000   | 0.0000   | 0.0000       | 0.2586         | 0.3172         |
| Situbagendit | 0.0000   | 0.0000   | 0.0000       | 0.3821         | 0.4310         |
| Sigambiri Merah | 0.3821 | 0.2586   | 0.3821       | 0.0000         | 0.3676         |
| Sigambiri Putih | 0.4310 | 0.3172   | 0.4310       | 0.0366         | 0.0000         |

Selection of hybrid crosses that have a long genetic distance allows the emergence of transgressive segregation in the resulting progeny. Transgressive segregation is a segregation pattern with the resulting offspring having superior properties over two hybrid [26]. The recommendation of the crossing parents in Table 3 is expected to be used as a reference for obtaining transgressive segregation so that the chances of obtaining offspring that have the superior properties of tolerant to low temperatures exceeding the hybrid will be greater. The greatest chance of obtaining the transgressive segment is the Ciharang x Sigambiri Putih and Situbagendit x Sigambiri Putih descent because of the most distant genetic distance compared to other couples. Some assembly-based varieties based on the selection of elliptical hybrid based on distant genetic distances have superior properties, such as high yield potential, disease resistance properties, and other properties that are better than those of hybrid. As is the case of [27] obtained F2 crops crossed from IR64 × O. rufipogon
superior rash-resistant 001, [26] that the MTL 98 of Vietnam has superiority highly resistant to WBC over its hybrid, which is descended from two very distant genetic distant hybrids, namely Oryza sativa × O. officinalis (wild species) and Matatag 9 of the Philippines (superior properties are highly resistant to tungro) of the cross O. sativa × O. Rufipogon (wild species).

Crosses between hybrids with far-reaching genetic distances are most likely to produce offspring that have a high degree of diversity and there is a chance of obtaining a superior genetic strain better than both heterosis. [28] reported that genetic distance values based on molecular markers are useful in screening large numbers of inbreds, based on the genetic distance values between inbreds tested and test inbreds in order to reduce the test material. However, according to [16], the effect of heterosis on rice is controlled by many genes so that heterosis is not adequately explained only through genetic distance. However, breeders can utilize germplasm with a long genetic distance so that the choice of more lines.

In this study the kinship of Sigambiri local rice accession with popular varieties of Ciherang are analysed. Ciherang closest kinship with Mekongga and Situbagendit with a coefficient of genetic distance of 0.000. Based on the results of this research, the assembly of high yielding rice varieties with tolerant to low temperature can be obtained by crossing Ciherang with local varieties of Sigambiri. Ciherang is a very popular variety, both by farmers and rice traders, has properties resistant to WBC biotypes 1 and 3, and HDB resistant. While Sigambiri is a local gogo rice that has resistance to blast races 033, 175 and 173. Sigambiri also resistant to low temperatures and adaptive at an altitude of up to 1300 m asl, both cultivated in dry land and marginal. In addition, other advantages are the potential yield can reach 4.5 to 4.8 tons per hectare. These two genotypes have different clusters so the chances of obtaining genetic diversity of offspring will be great in order to obtain an effective superior trait.

4. Conclusions
The results concluded that of the 11 markers used there were 5 SSR markers polymorphic, 4 monomorphic and 2 not amplified. The CT234 and CT235 markers are the most informative markers with the highest PIC values (0.375) compared to the other 9 markers. The genotypes selected by molecular markers related to tolerant to low temperatures are Sigambiri Putih and Sigambiri Merah. Assembling of rice superior varieties with low temperature tolerant properties can be obtained by crossing Ciherang with local varieties of Sigambiri Putih.

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