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Microsatellite (RM20A, RM241, and RM315) Amplification of Panic Grass DNA (*Panicum maximum*)

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Abstrak

Forage is a very important element for livestock development as they need it to survive, produce and reproduce. The forages that is often used is panic grass (*Panicum maximum*) which is a feed grass from the African plains, widely used in various regions of Indonesia. This grass is used for their high nutritional value, the ability to adapt various types of soil and weather, and usage as an ideal or high quality "cut and carry" grass for beef and dairy cattle. Forage cultivation by considering plant genetics can improve the quality of all types of forage inherited from the parent hence increase the productivity of feed crops and support livestock development. This can be done with molecular marker, which is a technology that can confirm target genes effectively, efficiently and accurately and are not influenced by environmental factors. Among the markers or molecular markers currently available, microsatellites or Simple Sequence Repeats (SSR) have become a marker system that is commonly used because of its various advantages, namely that it can be amplified by Polymerase Chain Reaction using primers that combine sequences in the target DNA. In this research, genes that carry leaf-rolling, plant height, and fertility-restoring properties that have previously been identified in rice plants will be tested on panic grass using RM20A, RM241, RM315. After being visualized using electrophoresis gel, it can be concluded that RM241 had the best result as it has clear amplification result and polymorphic bands with effectiveness and polymorphism levels of 75% and 100% respectively.

1. Introduction

Feed is a very important element for animal development. Livestock need feed to survive, produce and reproduce, so there must be sufficient feed with good quality, quantity and continuity. Forage is the main food source for ruminant animals. One of the forages that are often used is panic grass (*Panicum maximum*). Panic grass (*Panicum maximum*) is a type of multipurpose grass from the African plains which is widely used in various regions of Indonesia. This grass has a high nutritional content, adapts to soil with medium fertility levels and rainfall above 600 mm (Jank *et al.*, 2010). This forage is ideal for use as quality "cut and carry" grass for beef and dairy cattle (Aganga and Tshwenyane, 2004; Nakamanee *et al.*, 2008) as well as good quality pasture grass (Carnevalli *et al.*, 2006). Panic grass (*Panicum maximum*) has nutritional value of DM: 23.6%, CP: 10.9%, Crude Fat: 2.43%, Crude Fiber: 32.9%, Ash: 12.47%, Ca: 0.62%, P: 0.27% and TDN: 53.9%.

As the number of livestock increases, the amount of feed needed also increases. One of the problems in providing animal feed is irregular production, which limits the availability of forage for livestock. Efforts that can be made to overcome this problem are by providing quality and easily available feed through forage cultivation. The main requirement for planting animal feed is the selection of quality plant seeds. High quality seeds increase plant productivity and feed availability. Forage cultivation by considering plant genetics can improve the quality of all types of forage inherited from the parent. This can increase the productivity of feed crops and support livestock development. This can be realized with what are called molecular markers. Molecular marker is a technology that can confirm target genes effectively, efficiently and accurately and are not influenced by environmental factors. The discovery of molecular markers can help smooth selection work and can be developed quickly and in larger quantities.

Among the markers or molecular markers currently available, microsatellites or Simple Sequence Repeats (SSR) have become a marker system that is commonly used because of its various advantages, namely that it can be amplified by PCR (Polymerase Chain Reaction) using primers that combine sequences in the target DNA. Primer is a synthetic oligonucleotide consisting of 18 to 24 bases and functions as a precursor for DNA synthesis from a DNA template. To date, many primers have been used to study and determine the genetic background controlling various important traits in plants, such as RM20A, RM241, and RM315. The study conducted by Lee *et al.*, (2007) found that the leaf rolling trait was related to the RM20A marker, which can be used as an indicator that the leaf is tolerant to drought so this primer is very suitable for use in the process of selecting plants that are resistant to drought. Respectively found that RM241 and RM315 could be used to detect plant height and fertility restoration traits in rice plants. Of course, these three markers increase the productivity of the plant itself so it is hoped that these markers can also be used on panic grass.

Microsatellite markers have the potential to characterize genetic material based on control or controlling genes. The use of microsatellite markers to increase forage production based on direct selection at the DNA level that encodes phenotypes needs to be improved. Selection at the DNA level will be more precise than conventional selection, which is only based on plant phenotype, but also involves developing nutritional identification for further research. This research has been carried out with the aim of identifying polymorphic microsatellite markers so that the data obtained can later be used for selection of panic grass (*Panicum maximum*).

2. Method

2.1 Time and Location

This research was carried out in the Biotechnology Research and Testing Lab which is located in the Faculty of Animal Husbandry, Padjadjaran University, building 3 on October 9, 2023 to December 7, 2023. Another research was carried out on May 16, 2024 to May 20, 2024.

2.2 Materials and Tools

Materials used in this research include liquid nitrogen (N2), panic grass samples, chloroform, buffers (PAL, GWP, DW1, GW2, AE, TAE), agarose, ethidium bromide solution (ETBR), ladder, loading dye, green mix, forward and reverse primers (RM 241, 20A, 315), PCR water. The tools used in this research include mortar, spatula, analytical balance, eppendorf tubes, water bath, vortex, centrifuge, micropipettes and tips, electrophoresis machine, refrigerator microwave, PCR machine (thermocycler), and gel documentation UV transilluminator.

2.3 Procedure DNA Extraction

The DNA extraction procedure used the procedure available in the *Magen HiPure HP Plant DNA kit*. Grind 150 mg of grass samples with the help of liquid nitrogen (N2). Then, put the powdered samples into a 2 ml tube. Add 700 µl PAL (preheated to 65℃) into the tubes and vortex until mixed. Incubate the samples for 20 minutes at 65℃. Add 700 µl of chloroform to the tubes then vortex for 15 seconds. Centrifuge the samples at 12,000 for 5 minutes. Take the supernatants and transfer them into 1.5 ml tubes. Repeat this step. Add 700 µl of GWP then homogenize manually for 10-15 times. Take 750 µl of samples and transfer them to the blue column. Centrifuge the samples at 10,000 for 1 minute. Once they're done, discard the liquid in the columns. Repeat this step until the samples run out. Add 500 µl of DW1 to rinse. Centrifuge at 12,000 for 1 minute then discard the liquid. Add 500 µl of GW2. Centrifuge at 12,000 for 1

minute then discard the liquid. Repeat this step. Centrifuge at 12,000 for 2 minutes to dry the columns. Transfer the columns to new 1.5 ml tubes and add 50 µl AE (preheated to 65℃). Let them sit at room temperature for 2 minutes. Centrifuge at 12,000 for 1 minute. Repeat this step. Discard the blue columns and store the tubes containing the DNA in the refrigerator.

2.4 DNA Amplification (PCR)

Make a 24 µl PCR Mix containing: 10 µl Green Mix, 1µl of each primers (forward and reverse), 4 µl PCR Water, and 8 µl grass sample DNA. Mix by pipetting and name the Eppendorf tubes. Insert the PCR Mix into the PCR machine (thermocycler) then set the desired temperature and cycle. PCR was carried out with an initial denaturation temperature of 94℃ for 5 minutes, denaturation 94℃ for 1 minute, annealing 56℃ (RM 241, 20A) or 61℃ (RM 315) for 1 minute, and extension 72℃ for 1.5 minutes for 40 cycles). When finished, put the PCR Mix in plastic and store it in the refrigerator.

2.5 Agarose Gel Making

Weigh out 2.25 grams of agarose then mix it with 150 ml of TAE buffer. Microwave for 3 minutes then add 8 µl ETBR and homogenize. Pour the solution into the mold and wait for it to set for 45-60 minutes.

2.6 Visualization of DNA (Electrophoresis)

Remove and thaw DNA samples of panic grass along with loading dye and ladder. Place 2 µl of loading dye into a 0.5 ml Eppendorf tube. Add 10 µl of DNA samples then homogenize by pipetting. For each tube, you must replace the micropipette. Place 7 µl of ladder and DNA samples in agarose well. Turn on the electrophoresis machine and set the time and voltage (2 hours, 100 volts). Visualize and check the results using gel documentation UV transilluminator.

2.7 Observed Variables

The variables observed in this study were variations in the microsatellite markers RM 241, 20A, and 315 along with their allele frequencies in panic grass (*Panicum maximum*).

2.8 Data Analysis

The research was conducted using descriptive methods. The collected research data was analyzed by scoring bands resulting from DNA visualization. Each band that appears on the gel represents a specific allele of the marker used. These alleles are translated into binary data which is given a value based on the presence or absence of an allele (Hairinsyah, 2010). A value of one or (+) will be given if there is an allele, and a value of 0 or (-) if there is no allele**.** Analysis of the percentage of polymorphic alleles is calculated to see what percentage of polymorphic alleles are formed in each primer used. To calculate the percentage of polymorphic bands, the formula is used:

$$Polymorphism\ (\%) = \frac{amount\ of\ polymorphic\ allels}{total\ allels} \times 100\%$$

3. Results And Discussions

3.1 Genom Electrophoresis

Before being used for PCR, DNA that has been successfully isolated is first electrophoresed for 1 hour to test the quality of the genome. The sample used in this research were samples of fresh panic grass which was taken and put in the refrigerator for running on the next day. This genome electrophoresis activity is carried out to see whether there is DNA in the isolation results that we have carried out and to predict whether the DNA is pure or not by looking at the "smear" band pattern. The more visible the "smear" band pattern, the more it indicates that the DNA is contaminated or impure.

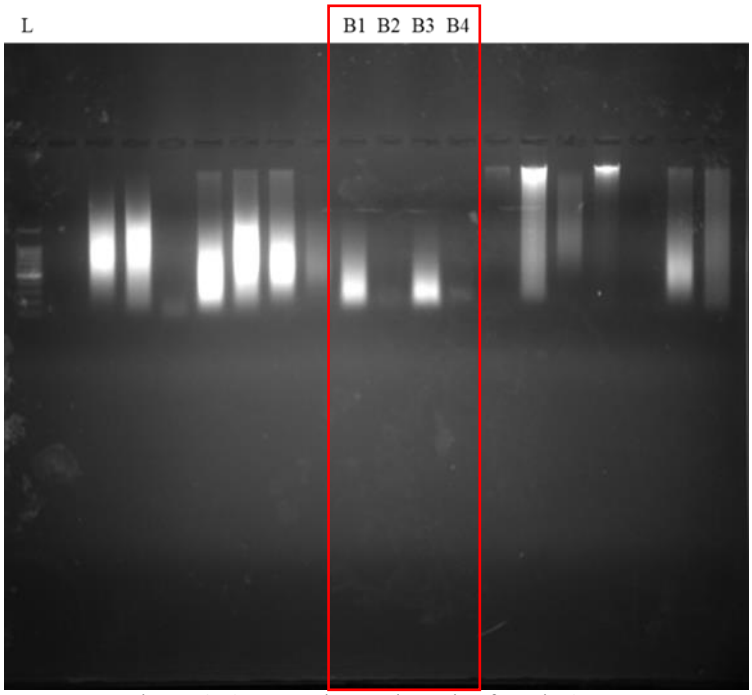

Figure 1. Genom Electrophoresis of Panic Grass

Based on the results of the electrophoresis visualization in Figure 1, it can be seen that the four panic grass samples had varying results. Samples B1 and B3 showed clear and bright DNA bands, indicating that a large amount of DNA was detected in these samples. However, in the middle of the sample there is a "smear" pattern. This indicates that the DNA in the samples are not completely pure. This "smear" pattern can be caused by several things, such as contamination from protein, polysaccharides and other debris. Apart from that, this pattern may also be caused by RNA identified as an impurity being isolated during the DNA extraction procedure (Eticha *et al.*, 2013). In contrast, sample B2 showed a "smear" DNA band with sample B4 showing a little at the bottom. However, the absence of a band indicates that the concentration of the two samples can be confirmed to be below 50 ng/µl. There is also a big possibility that samples B2 and B4 are not in the good DNA purity range, namely, 1.8 to 2.0, whereas samples B1 and B3 can be tested using a spectrophotometer to obtain the concentration and purity of the DNA.

3.2 Amplification of Microsatellite Markers RM20A, RM241, and RM315 on Panic Grass

SSR markers are DNA markers that are widely used in assessing genetic diversity, phylogenetic relationships, and population structure in plants (Pandey *et al.*, 2016). Microsatellite markers can also be used to analyze genomic DNA, looking at the DNA band patterns created after the genomic DNA is amplified with primers. This research used a type of panic grass called *Panicum maximum* cv

Gatton. *Panicum maximum* cv Gatton is a type of Guinea grass that originates from Zimbabwe and is a small type cultivar. It has dark green leaves and smooth stems. This cultivar can grow quickly and well compared to green panic (Panicum maximum var Trichoglume) and produces good production in its first season, and is easier to maintain and harvest forage compared to large or tall types of *Panicum maximum*. The primers used in this research included RM 20A, 241, and 315 which were used to detect leaf rolling, plant height, and fertility restoring genes. The RM20A marker is located on chromosome 12 while RM241 and RM315 are located on chromosomes 4 and 1 as mentioned in Table 1.

Table 1. Microsatellite Markers Used in This Research

Primer	Chromosome	Traits	Base Sequence	Annealing Temperature
RM20A	12	Rolling Leaf	F: ATCTTGTCCCTGCAGGTCAT R: GAAACAGAGGCACATTTCATTG	56℃
RM241	4	Plant Height	F: GAGCCAAATAAGATCGCTGA R:TGCAAGCAGCAGATTTAGTG	56℃
RM315	1	Fertility Restorer	F: GAGGTACTTCCTCCGTTTCAC R:AGTCAGCTCACTGTGCAGTG	61℃

F = Forward, R = Reverse

Simple sequence repeats (SSR) are one of the most popular molecular markers used because they have a degree of polymorphism which is high, abundant, and widely distributed throughout the genome. The advantage of using SSR is that it is easily automated, has high reproduction and is non-radioactive (Miah *et al.*, 2013). SSR markers have been used for viewing genetic variation and genetic diversity in *Poaceae* family such as Cockspur Grass (*Echinochloa sp.*) (Lee *et al.*, 2016), wild rice (Van Oosten *et al.*, 2016), *Brachypodium* (Shiposha *et al.*, 2016), Mulato Grass (*Brachiaria*) (Trivino *et al.*, 2017) and Corn (Ramlah *et al.*, 2017). SSR markers are widely used to study and knowing the genetic background of various important properties controller, especially in plants such as rice (*Oryza sativa L.*) because of its codominant nature, multiallelic, can be used on *indica* and *japonica* correctly, as well as easy, quick, and economical in application because it is based on PCR technique (McCouch *et al.*, 2002). Rice SSR markers have also been used for amplification of other species such as sorghum (Krupa & Shashidhar, 2017), finger millet (Babu *et al.*, 2017) and Barnyard millet (Babu *et al.*, 2018). Polymerase Chain Reaction (PCR) is a technique used to amplify certain DNA base sequences. Since it uses microsatellites, it requires less DNA to perform compared to other techniques.

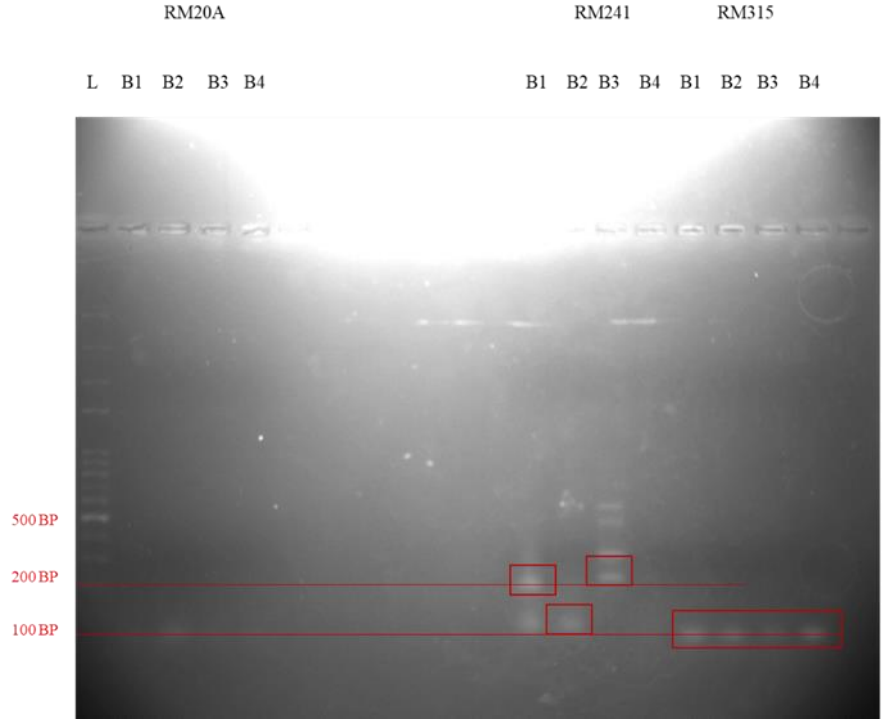

Figure 2. Amplification Results of Panic Grass

Based on the electrophoresis results, it can be seen that none of the samples subjected to PCR using RM20A succeeded in showing the allele. As we know, there are several factors that influence the success of DNA amplification, namely primer annealing temperature, primer suitability, polymerase enzyme concentration, and number of amplification cycles (Simarmata *et al.*, 2014). Therefore, we can conclude that the RM20A primer we are using is not suitable for panic grass. This incompatibility is thought to be due to an error in designing the RM20A primer. Thus, panic grass cannot respond well to the RM20A primer (Panaud, 1996). Apart from that, it is very likely that an error occurred during pipetting or making the PCR mix with RM20A which caused the amplification process to fail because the primer attached to the wrong DNA sequence or the number of bands that came out was too small. Furthermore, the initial denaturation and denaturation time is also considered too long because generally it only takes 3 minutes and 30 seconds respectively for a temperature of ± 95℃. A denaturation time that is too long will reduce the effectiveness of the Taq polymerase enzyme, causing PCR to fail because the genome cannot be cut and the primers cannot attach. To calculate the annealing temperature of RM20A, it is found to be 55℃ for forward and 57℃ for reverse so that the annealing temperature of RM20A is correct, namely 56℃. However, the optimum annealing temperature can still be adjusted depending on the length of the nitrogen base sequence used and how much G and C the sequence contains.

For the amplification results with RM241, the four samples tested produced three bands that were clearly amplified, namely in samples B1, B2, and B3. Even though the correct annealing temperature for RM241 is 53℃, the primer still adheres well. Apart from that, it can be seen that Bengal grass PCR with RM241 produces polymorphic bands. Polymorphic bands are DNA bands that have variations in shape or have different amplification sites. This is caused by the absence of amplification at this locus which is triggered by differences in the sequence of nucleotide bases at the primer attachment point. The presence of polymorphic bands indicates that the diversity of panic grass is still very high. Therefore, selection still needs to be done.

Then, as a result of amplification with RM315, four clearly amplified bands were obtained. So, it can be confirmed that the panic grass genome DNA template is suitable for the RM315 primer because amplification can only occur if there is a complementary nitrogen base sequence match between the primer and the DNA template. Previously, the first amplification results with RM315 failed because the procedure was less than optimal so that what was obtained during visualization was only a whole, uncut genome. However, after optimizing the procedure, a clearly amplified band has now been obtained. Therefore, it can be concluded that increasing the DNA concentration and PCR cycles affects the success of DNA band amplification because enough cycles but not enough DNA will not make the bands clearly visible. All of these bands show a monomorphic band pattern, namely bands that are the same size or have one shape so that they have no variations (Williams and Ronald, 1990). The existence of these monomorphic bands proves that the fertility restoring genes in the panic grass samples are uniform so there is no need for further selection.

The results of amplification with RM241 resulted in PCR products of 200 BP in B1, around 130 BP in B2, and around 220 BP (between 200 and 300 BP) in B3. Meanwhile, the results of amplification with RM315 all obtained PCR products of 100 BP. The different bands resulting from amplification of the RM241 marker in the grass tested showed good polymorphism properties. This shows that the SSR marker RM241 can be used as a tool to differentiate species and test the varietal purity of the four species tested. Dwiwandana *et al.*, (2013) stated that polymorphic loci would be better for detecting genetic variation between populations. However, keep in mind that these results may not be accurate because the electrophoresis procedure used agarose gel, not polyacrylamide gel.

3.3 Level of Effectiveness and Polymorphism

The visualization results show that of the four panic grass samples that were PCR using RM241, there were three samples that were amplified, resulting in an effectiveness level of 75%. Furthermore, from these three samples, the three alleles that were amplified had polymorphic patterns so the polymorphism was 100%. According to Grattapaglia *et al.* (1992), the amplification process can occur in various places, but only a few bands can be identified as bands after amplification. Furthermore, the PCR results with RM315 showed a very good level of effectiveness because all the bands were successfully amplified in each sample so that an effectiveness level of 100% was obtained. However, because it produced a monomorphic band pattern, the polymorphism cannot be calculated from the amplification results with RM315.

Proper primer design for a particular forage will produce better results. According to Zulfahmi (2013), ideal markers must have features such as being neutral, evenly distributed in the genome, having sufficient resolution of genetic differences, codominant inheritance, and a high level of polymorphism. In this study, rice markers RM20A, RM241, and RM315 were used on panic grass because the two types of plants have almost the same classification. The results showed that of the three SSR markers, the RM241 marker had the best level of effectiveness and polymorphism for panic grass, namely 75% and 100% respectively.

4. Conclusion

There are two conclusions in this research: 1.SSR RM241 forms polymorphic bands so it can be used as a tool to detect genetic diversity in panic grass, 2. Both B1 and B3 have decent amplification results using RM241. Therefore, they can be selected for further research.

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