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GENETIC DIVERSITY OF EIGHT TYPES OF BAMBOO BASED ON RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

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Abstract

Bamboo is a plant that has been widely known to Indonesian people, generally used as a substitute for wood to supply the needs of the building and furniture raw materials. This study used molecular markers because these markers are stable and not affected by the environment. The purpose of this study was to determine the genetic diversity of eight types of bamboo in the Area of Genetic Resources (ASDG) of 2nd regional of Seed/Seedling Forest Center (BPTH) Bellabori Village, Parangloe District, Gowa Regency, Indonesia. Its usefulness is as information and reference for the development of ASDG Bamboo and also to support the conservation of Bamboo species. The analysis was carried out using 99 DNA samples from 8 Bamboo types. Primer selection using 12 randomly selected DNA samples produced four polymorphic primers out of 20 RAPD primers. RAPD primers produced polymorphic bands were OPP-08, OPA-15, OPC-11, and OPA-05 primers. Based on the analysis of genetic diversity, the average diversity was 0.47. Individuals observed from 99 samples tended to cluster according to their types. Thus genetic diversity for each individual was high.

Keywords: Bamboo, RAPD, Amplification, Polymorphic, and Genetic diversity

Introduction

Bamboo is a plant that has been widely known in Indonesia generally used as a wood substitute to provide the needs of building and furniture raw materials. The community utilizes bamboo's shoots as food (S A Paembonan, 2019). The usage of bamboo as a building material is widely used in the form of poles, beams, walls or partition. Its functions in ecology are to absorb carbon, prevent erosion, and as a protective plant because it has the ability to reduce pollution waste. It is categorized as cultivated plant because it grows in wet to dry climates, in the lowlands to the mountains without a puddle of water (Department of Forestry and Plantation, 1999). Production and cultivation of this plant require superior quality seeds or seedlings. High-quality seeds are obtained by using superior varieties through plant breeding programs (Larekeng, Gusmiaty *et al*., 2019).

Information regarding genetic variation and gene actions expressed in the primary population is needed to determine the appropriate breeding selection method. Knowledge of genetic diversity is required to determine the behavior of genes that control certain traits. An analysis of genetic diversity and genetic relationship is carried out to represent genetic distribution information. It is a crucial step towards genetic conservation efforts in plant breeding strategy (Widyatmoko, 2005).

This study performed molecular markers because these markers are stable and not affected by the environment. Maftuchah (2001) stated that molecular techniques had provided opportunities to develop and identify genetic maps of a plant. The molecular genetic approach using DNA markers has successfully established molecular markers that are capable of detecting specific genes and traits. The ability to distinguish a genotype of individuals within species or some genotypes correctly is highly demanded in breeding programs. The molecular markers are used to arrange the relationship in several individuals within species as well as between species.

The current improvement in molecular markers are isoenzymes, *restriction fragment length polymorphisms* (RFLP), *simple sequence repeat* (SSR) (Halimah Larekeng *et al*., 2018) (Arif *et al*., 2019), *amplified fragment length polymorphism* (AFLP), and *random amplified polymorphic DNA* (RAPD). A widely used method is RAPD that a PCR based molecular marker for identifying diversity in interspecies and intraspecies (Olivia *et al*., 2012). It is also able to detect DNA polymorphisms due to the absence of amplified PCR bands at a locus caused by differences in the nucleotide sequence at the primer attachment site. This marker can be applied to almost all types of plants.

Genetic diversity studies have been performed on several species, such as *Intsia bijuga* (Rimbawanto and Widyatmoko, 2006), *Santalum album,* and *Alstonia scholaris* (Harati *et al*., 2007). The previous study on Parring Bamboo based on RAPD markers only used a bamboo species (Mis'al, 2017). Thus in this study, we evaluated eight bamboo types to determine the difference in DNA sequences generated using RAPD markers.

This study aimed to determine the genetic diversity of eight bamboo types from the genetic resource area of 2nd Regional of Seed/Seedling Forest Center. Its benefits are as information and reference for the development of Bamboo's genetic resource area and also to support the conservation of Bamboo species.

Materials and Methods

The research was conducted in two stages consisted of research sampling and genetic diversity analysis. Samplings were taken from the Genetic Resources Area of 2nd Regional of Seed/Seedling Forest Office, Bellabori Village, Parangloe District, Gowa Regency. Analysis of genetic diversity was carried out at the Laboratory of Biotechnology and Tree Breeding, Faculty of Forestry, Universitas Hasanuddin, Makassar. The sample used was leaves taken from 8 types of

bamboo in the research area. The number of leaves collected for each species were varied and based on the code of trees in the field. Samples collected from each type of bamboo were put in a container/Coolbox containing ice gel to keep the leaves in good quality and preventing damage. The leaves were stored in the freezer until the DNA extraction process.

DNA isolation was performed using the CTAB (Cetyl Trimethyl Ammonium Bromide) method (Larekeng *et al*., 2018). The DNA extraction was carried out with the following steps: a bamboo leaf without leaf bone was weighed 0.3 grams and then crushed until powder. The 800 µ1 CTAB extraction buffer (100 mM Tris HCl pH 8.0; 20 Nm EDTA (Ethylene Diamine Tetra Acetic Acid), 2% CTAB; 1.4 M. NaCl) were added and vortexed for 15 seconds. The cell wall lysis process in the sample happened by incubating a tube containing leaf samples into a water bath with a temperature of 65ºC for 120 minutes. Each incubated sample was added isoamyl alcohol: chloroform (24: 1) 100 µ1 and mixed slowly, then centrifuged at 10,000 rpm for 5 minutes. The supernatant was transferred into a new tube, and 800 µ1 isopropanol was added. The solution centrifuged at 10,000 rpm for 10 minutes, and the DNA pellet was dried overnight. The DNA pellet was purified by adding 500 µ1 TE buffer 1x (10 mM Tris-HCl pH 7.5 mM EDTA), then centrifuged for 10 minutes at a speed of 10,000 rpm. The supernatant was transferred into a new 2 ml tube, and 100 µ1 chloroform was added. The supernatant solution was then centrifuged at 10,000 rpm for 10 minutes. The supernatant was taken, then 100 µ1 sodium acetate 3 M and 800 µ1 isopropanol were added, then centrifuged for 10 minutes at a speed of 10,000 rpm. The precipitation was taken and dried overnight. The 100 µ1 TE buffer was added into the DNA and stored then in a -20°C freezer.

Table 1 : Primer Name and RAPD Primer Sequence

No	Primer Name	Nucleotide Sequence	TM (ºC)
1	OPG-06	5'-GTG CCT AAC C-3'	31,8
2	OPAD-11	5'-CAA TCG GGT C-3'	32,1
3	OPA-05	5'-AGG GGT CTT G-3'	32,6
4	OPA-15	5'-TTC CGA ACC C-3'	34,2
5	OPZ-05	5'-TCC CATGCT G-3'	34,3
6	OPG-19	5'-GTC AGGGCA A-3'	34,7
7	OPAE-11	5'-AAG ACCGGG A-3'	35,5
8	OPO-14	5'-AGC ATG GCT C-3'	35,1
9	OPAA-20	5'-TTG CCT TCG G-3'	35,6
10	OPA-18	5'-AGG TGACCG T-3'	36,2
11	OPC-11	5'-AAA GCTGCG G-3'	36,9
12	OPA-09	5'-GGG TAA CGC C-3'	37,4
13	OPP-08	5'-ACA TCGCCC A-3'	37,6
14	OPAC-12	5'-GGC GAG TGT G-3'	38,1
15	OPK-20	5'-GTG TCG CGA G-3'	38,5
16	OPQ-07	5'-CCC CGATGG T-3'	38,5
17	OPD-20	5'-ACC CGGTCA C-3'	39,1
18	OPA-02	5'-TGC CGAGCT G-3'	40,7
19	OPD-03	5'-GTC GCC GTC A-3'	40,8
20	OPY-09	5'-AGC AGC GCA C-3'	42,5

The selected primers are polymorphic primers that able to produce clear and bright bands. The optimum conditions and the level of band variation produced from each primer require several different primers under the same conditions and use different DNA samples (Larekeng, Dermawan *et al*., 2019). The selected primers are presented in Table 1.

Primers were selected from 12 random samples because the PCR machine has 12 temperature gradients; thus, the specific temperature of each primer could be determined. One PCR reaction consisted of 3 µl DNA working, 1.5 µl RAPD primers, 5 µl Hostaq PCR mix, 1 µl Cl, and 3 µl ddH2O with a total reaction of 13.5 µl. DNAs were amplified using a PCR machine. The stages of PCR amplification were as followed:

1. Initial denaturation, with a temperature of 95ºC for 5 minutes
2. The denaturation with a temperature of 94ºC for 1 minute
3. Specific primer attachment (temperature adjusted to each primer pair) for 60 seconds
4. Primer elongation at 72ºC for 1 minute
5. Step 2 to step 4 were repeated 35 times
6. Final elongation at 72ºC for 10 minutes
7. The storing at 4º C.

The electrophoresis stages were:

1. 4 grams of Agarose was weighed, and 200 ml of TAE buffer 1 x in 500 ml size Erlenmeyer were added
2. The solution was heated using a microwave for 5 minutes
3. After dissolving, 1.5 µl of Gelred was added, and it stood until warm.
4. The solution was poured into an agar mold, then combed and allowed to stand until it hardened.
5. The comb then removed and put in a tank containing 1x TAE buffer solution
6. The samples were inserted into the wells. The last well contained the leader
7. Electrophoresis carried out for 60 minutes at a voltage of 100 volts
8. The agar was removed, and the template was placed inside Geldoc to be documented.

RAPD Data Analysis

The results obtained are bands that appeared on agar. The bands present the alleles at a specific locus. Each primer used presents a certain locus. The bands that appeared then scored according to the band size. Band with the largest size was given with number 1, and if there was no band, then it was given with number 0. An assessment of the presence or absence of the band was done by observing the electropherogram manually. The data then tabulated and processed using Darwin 6.5 software to determine the relationship and the genetic variation. Heterozygosity value is calculated using the following formula (Wallace, 2003):

Heterozygote:

$$Pi = 1 - qi$$

$$He = 1 - pi^2 - qi^2$$

Note: qi= zero allele frequency
Pi= allele dominant frequency

The polymorphic information content (PIC) value is calculated using the following formula (Guo *et al.*, 2014) :

$$PIC = 2\ fi\ (1 - fi)$$

Note: PIC = Polymorphic information content

Fi = allele frequency

Results

Primer selection was done to determine the primers that generate polymorphic, clear, and bright bands. The success of DNA sample amplification with the primers using a PCR machine can be seen from the suitability of the primers used. Primer selection was carried out on 20 RAPD primers. Four primers could amplify bamboo DNA, namely OPP-08, OPA-15, OPC-11, and OPA-05 primers. At the same time, other primers produced smear amplification band products with no polymorphic bands, namely OPO-14, OPA-02, OPA-18, OPG-06, OPK-20, OPAE-11, OPZ-05, OPAA-20, OPA-09, OPAC-12, OPQ-07, OPD-20, OPD-03, and OPY-09 primers.

The primers used for further analysis are primers that produce clear, bright, and polymorphic bands (Larekeng *et al*., 2015). Polymorphic primers are needed in analyzing the genetic diversity of plant populations by showing the variation of band patterns produced. The accomplishment of DNA amplification using specific primers is based on the similarity of sequences between genomes and primers (Larekeng, Siti Halimah., Restu, Muh., Gusmiaty, Gusmiaty., Rismawati, 2016). Photos of four primers that produced polymorphic bands are depicted in Figures 1, 2, 3, and 4.

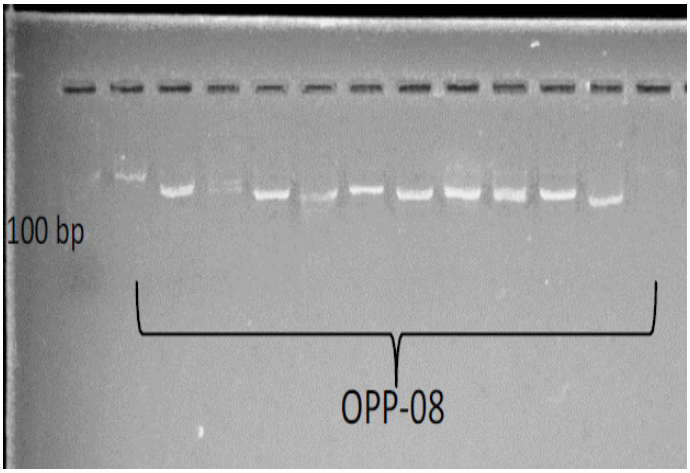

Fig. 1 : Electropherogram of DNA amplification band using OPP-08. Note: bp= base pair, 1-12 = bamboo sample

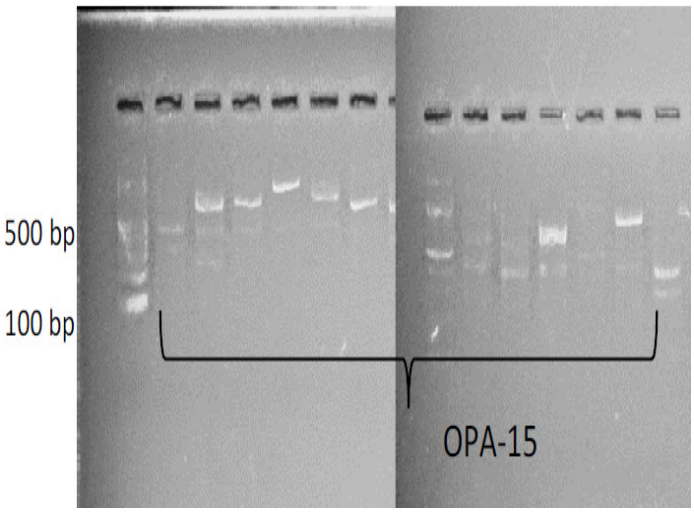

Fig. 2 : Electropherogram of DNA amplification band using OPA-15. Note : bp=base pair, 1-12 = bamboo sample

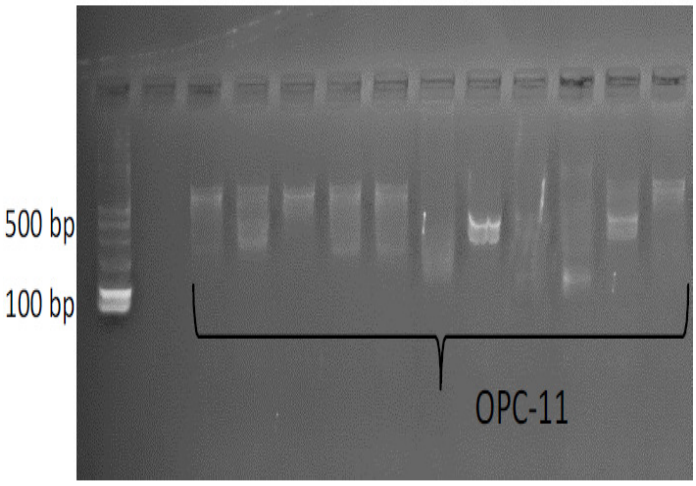

Fig. 3 : Electropherogram of DNA amplification band using OPC-11. Note: bp= base pair, 1-12 = bamboo sample

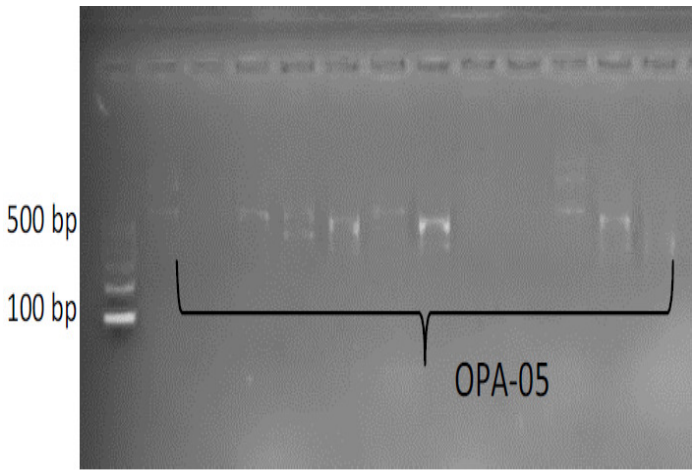

Fig. 4 : Electropherogram of DNA amplification band using OPA-05. Note : bp= base pair, 1-12 = bamboo sample

Primer selection was performed to select polymorphic primers and determine the appropriate annealing temperature. Determining the proper annealing temperature can affecting the entire sample amplification results because all samples amplification using the same temperature. Polymorphic primers and annealing temperatures can be seen in Table 2.

Table 2. Polymorphic Primer and Annealing Temperature

No	Primer	Annealing (°C)
1	OPA-15	30,5
2	OPP-08	41,3
3	OPA-05	29,8
4	OPC-11	39,7

The parameters of genetic diversity are in the form of heterozygosity value, number of bands, and detected polymorphic bands. These parameters were obtained from the overall scoring of PCR amplification bands manually and tabulated into the Heterozygosity and the Polymorphic Information Content (PIC) formulas.

The number of alleles per primer produced in OPP-08 primers was 112 alleles, OPA-15 were 24 alleles, OPC-11 were 133 alleles, and OPA-05 were 41 alleles. The highest number of alleles produced by the OPC-11, while the lowest was OPA-15. The highest heterozygote was obtained from the Talang bamboo species, while the lowest heterozygote was obtained from the Black bamboo species. In total, eight types of heterozygous values were varied.

These parameters were obtained from the scoring results of overall PCR amplification manually and tabulated in the Heterozygosity formula. Heterozygosity values for all types can be seen in Table 3.

Table 3 : Number of Bands and Heterozygosity Value

No.	Type	Primer	Number of Bands	Heterozygosity
1	Chinese Bamboo	OPP-08 OPA-15 OPC-11 OPA-05	39 2 26 1	0,46
2	Tropical Black Bamboo	OPP-08 OPA-15 OPC-11 OPA-05	9 0 9 1	0,47
3	Oldhamii Bamboo	OPP-08 OPA-15 OPC-11 OPA-05	7 0 9 4	0,46
4	Pring Tutul	OPP-08 OPA-15 OPC-11 OPA-05	26 2 30 8	0,47
5	Talang Bamboo	OPP-08 OPA-15 OPC-11 OPA-05	8 0 9 6	0,48
6	sp. 1 Bamboo	OPP-08 OPA-15 OPC-11 OPA-05	7 1 8 2	0,48
7	sp.18 Bamboo	OPP-08 OPA-15 OPC-11 OPA-05	5 9 11 12	0,47
8	sp.16 Bamboo	OPP-08 OPA-15 OPC-11 OPA-05	11 10 31 7	0,46
Average Heterozygosity				0,47

Based on the value of the Polymorphic Information Content (PIC), OPC-11 primer had the highest PIC value, which was 0.44, or close to 0.5 thus, it was indicated that the markers are more effective in distinguishing between individuals. Guo (2014) stated that a PIC value close to 0.5, illustrating that the marker is more effective in distinguishing each individual.

Discussion

Random primers in RAPD analysis, causing the need for primer selections to detect polymorphisms of amplified DNA fragments that useful for the genetic diversity analysis among plant individuals (Y. F. Syahri *et al*., 2019). The primers generated different amplification results, some were not amplified, and some were either clear or smear amplified. The primer sequences can cause the absence of amplification bands in some of the primers that are not complemented with the nucleotide of the DNA template. As a result, the primers are unable to amplify DNA fragments (Sulistyawati, 2014).

The number of primers producing clear and bright polymorphic bands on eight DNA types of bamboo was four primers, namely OPP-08, OPA-15, OPC-11, and OPA-05 primers. The study by Nurhidayatullah (2018) that examined white Jabon (*Anthocephalus cadamba* (Robx) Miq.) using 21 RAPD primers and obtained three polymorphic primers. The OPP-08 primer was the highest number of bands with a value of 10. While the previous study by Mis'al (2017) in parring bamboo (*Gigantochloa atter*) using 31 primers showed that OPP-08 and OPC-11 did not produce polymorphic bands while 16 primers either produced smear band or no band and no primers produced a monomorphic band.

Amplification without producing bands in some primers can be caused by several factors, such as during the PCR process, DNA is not well mixed in the PCR mix solution; thus, primer is not attached to the DNA template. Other factors are the purity and concentration of DNA template containing compounds such as polysaccharides and phenolic compounds, and DNA template concentrations are too low, so the resulting DNA amplification is unclear or smear (Yolanda Fitria Syahri *et al*., 2020).

The study on genetic diversity shows the ability of RAPD markers as an appropriate method in determining the level of genetic diversity in various plants. RAPD markers have some disadvantages, such as low reproducibility and dominance, but the development of data analysis and sophisticated equipment make this technique more reliable (Yolanda Fitria Syahri *et al*., 2020). This technique can provide valuable information in breeding and conservation programs and has been widely used in the analysis of population genetic diversity (Widyatmoko, 2005).

The number of polymorphic bands in the analysis of genetic diversity mainly determines the level of diversity in a population. The difference in the number of bands produced by each primer is caused by the primer sequences or interaction between the primers and the DNA template. Genetic diversity reflects a plant population, the higher the genetic diversity, the higher the level of population adaptation (Mis'al, 2017).

The number of bands produced from the four amplified RAPD primers was 310 bands, and the OPC-11 produced the highest number of bands among the four selected primers. The OPC-11 was the best in detecting the diversity of eight types of bamboo because it had a PIC value of almost 0.5, which was 0.44. This polymorphism primer value was the highest compared to the four selected primers. Guo *et al*. (2014) stated that the dominant markers had a maximum PIC value, which was 0.5. In contrast, the OPA-15 had the lowest polymorphism, with a value of 0.11. This is different from the study by Nurhidayatullah (2018) obtained a PIC value of 0.14 as the highest polymorphism value.

The heterozygosity (He) value for each population of eight types of bamboo almost the same, ranging from 0.46 to 0.48, with an average value of He was 0.47. This heterozygote value is higher than previous researches on genetic diversity of sengon conducted with RAPD markers by Siregar *et al*. (2012) with a value 0.23, study by Poerba (2008), which obtained He value of 0.20, study on bamboo parring by Mis'al (2017) with a value of 0.38 and study on white jabon by Nurhidayatullah (2018) obtained almost the same value, which was 0.46.

For future tree breeding strategy, genetic variations within and between populations need to be maintained (Halimah Larekeng *et al*., 2018). These efforts can be done

by establishing seed orchard from different populations and with the source from selected plus trees (superior tree), to maintain genetic variation in the population and produce good quality and genetically superior plants. High genetic variation supporting a population in adapting with a change in the surrounding environment and biodiversity will be maintained (Mulyadiana, 2010).

Conclusion

1. Primers that can be used for genetic diversity analysis of eight types of bamboo were OPP-08, OPA-15, OPC-11, and OPA-05 out of 20 selected primers.
2. The heterozygosity value obtained from eight types of bamboo had a high average value of 0.47, genetic diversity of eight types of bamboo in the Genetic Resources Area at Gowa Regency categorized as high.

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