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Bioinformatics and Genetic Analysis of Micropropagation To Preserve the Endemic and Almost Extinct Cibotium Barometz

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ABSTRACT
In vitro culture is very important for the development of increasingly rare Simpei fern (Cibotium Barometz) cultivars. The use of nitrogen can affect the sustainability of breeding traits. This study aims to further investigate the effect of giving variations in nitrogen concentration (KNO3) to the genetic diversity of the Cibotium barometz plantet produced from in vitro culture. Buds were collected and cultured on Murashige and Skoog (MS) medium with different nitrogen concentrations (KNO3) for in vitro culture formation. In this study, RAPD analysis was applied to identify 4 Cibotium Barometz planlets. Five RAPD primers were prepared for this analysis. Our results indicate that the DNA band pattern polymorphism produced from 5 RAPD primers shows very high diversity up to 100%. The results of the analysis of the RAPD band pattern clustering using the UPGMA method on the similarity coefficient of 0.68 and the analysis of the main components were able to be clearly distinguished into 3 groups. Genetic diversity that occurs indicates that nitrogen can cause negligence in Cibotium Barometz. The use of RAPD-derived DNA markers to authenticate Cibotium barometz from in vitro culture can help clarify the genetic diversity that occurs.

Keywords: Micropropagation, Cibotium barometz, Nitrogen, Genetic Diversity, PCR-RAPD;

INTRODUCTION

Simpei fern (Cibotium barometz) is often known as an ornamental fern that has anti-inflammatory and anti-osteoporosis treatment activity (Zhao et al. 2011; Huang et al. 2018). Because it has many benefits, simpei ferns are sought after by many people until finally the number of these plants is increasingly threatened with extinction (Fu et al. 2017). To meet the demand for simpei ferns and maintain their preservation in nature, it is necessary to cultivate plants with tissue culture to be important (Mai, Chen & Li, 2012; Yu et al. 2016; Mikula et al. 2017). MS (Murashige & Skoog) media have been widely used to induce the formation of potato sporophytes (Murashige & Scoog, 1962; Zarrabeitia, 1997), Canna indica (Mishra, 2015) Cyathea delgadii (Mikula et al., 2017), and Cibotium barometz (Rahayuitia, 1997), Canna indica (Mishra, 2015) Cyathea delgadii (Mikula et al., 2017), and Cibotium barometz (Rahayuitia, 1997) et al. 2015; Praptosuwiryo et al., 2017). Tissue culture in Cibotium barometz was carried out by Rahayu et al. (2015) in the efforts to restore this species. Rahayu et al. (2015) showed that the most sporophyte growing was in the 1/12 MS medium for all genotypes. Sporophytes produced from these studies are less fertile and the leaves undergo chlorosis or yellowing. Lack of nutritional sources such as nitrogen is thought to be a factor. Growth and morphogenesis of plant tissue under in vitro conditions is largely influenced by the composition of culture media (Jennifer, Geigerpetra, Ranker, 2013). Gardner et al. (2008) states that N deficiency will limit cell enlargement and division. Symptoms of deficiency include general growth that is abnormal or affected (dwarf) and yellow, especially in older plant parts (Cuong, 2009; Gabryszewska, 2011;). Nitrogen assimilation in plant growth and development plays an important role in building and understanding cell differentiation in plants. Quantitative nitrogen is the most important nutrient for plant growth (Takahashi & Morikawa, 2014). The form and amount of nitrogen in in vitro media has a significant effect on cell growth rate, cell differentiation and totipotency.

Addition of nitrogen is indeed needed for media that still lack of nutrients (Razaq et al., 2017). However, improper addition of nitrogen can affect growth and can even cause abnormalities in sporophyte generation. Abnormalities that have occurred are in the color of the leaves. If the media or soil contains a lot of nitrogen, the leaves will be dark green solid, whereas if the media lacks the nitrogen element, the leaves will be curled, yellowed or brown (necrosis) which means the tissue has been damaged or died (Liu et al., 2014; Rahayu et al. 2015). These symptoms are usually often associated with diseases caused by viruses. In fact, the problem can be caused by a lack of nitrogen in the media or soil. Tissue changes due to nitrogen deficiency can cause genetic changes (Movahedi et al., 2013). In addition, genetic factors also contribute to the qualitative and quantitative characteristics of plants. To analyze the genetic stability of plants derived from tissue culture can be done with Polymerase Chain Reaction (PCR) based on random polymorphic DNA (RAPD) (Mendes et al. 2014). The RAPD

technique is indeed an old analysis, but it is still feasible to use because it is simple, easy to do quickly done, requires a small amount of DNA and does not require prior genome information. DNA fingerprints with RAPD have been widely used to detect polymorphisms among micropropagation of medicinal plants in vitro such as Canna indica (Mishra, 2015), Linum usitatissimum (Kumari, 2017), Morus alba (Sheet, 2018), Solanum cultivar (Susilo & Setyaningsih) , 2018), Xylocarpus granatum, Excoecaria agallocha, and Phoenix paludosa (Dasgupta et al, 2018) and Dendrocalamus strictus (Goyal, 2015). However, reports on the genetic structure of Cibotium barometz in vitro culture results with nitrogen treatment are still rarely investigated. This research is important to ensure an appropriate protocol for the combination of nitrogen with culture media in general. The results of this study are expected to help understanding plant physiology regarding growth, differentiation, restoration of plant properties, and their development for researchers in the laboratory.

This study aims to determine the effect of adding nitrogen to the diversity and genetic distance of the Cibotium barometz plantet. In this study, we administered concentrations (KNO_3) to the Cibotium barometz prothallus growth media in vitro. To help with this analysis, we use the RAPD technique to examine genetic variations that emerge.

METHOD

Plant Collection

Our sample collection from the Center for Plant Conservation, Bogor Botanic Garden, Bogor Indonesia. In the micropropagation stage, we treated nitrogen concentrations (1/6 KNO_3, 1/3 KNO_3, 1/1 KNO_3 and one parent as a control) on MS media. We used four samples of Cibotium baromet prothallus in vitro (F1) culture aged 10 weeks for genetic stability testing. To prepare the explants that will be used is by selecting the source of explants that will be used in this study, namely the mass of the Cibotium baromet prothallus which has been selected for its quality and uniformity.

DNA Extraction

DNA isolation in this study refers to the procedure carried out by Banting (2017). The steps work as follows: 100 mg of tissue sample is added with enough liquid nitrogen to the mortar. The mixture is crushed until the tissue turns into dry powder. The cell powder was transferred into an eppendorf tube and added with 600 µl CTAB solution. The solution was then added 600 µl CI solution (24: 1) and turned back and forth 8 to 10 times. Sentifugate the tube at 10000 rpm for 10 minutes at 4oC. The formed supernatant is separated into a new tube and PCI (25: 24: 1) is added 1 time the volume of the supernatant. The tube is turned back up to homogeneous and re-centrifuged at 10,000 rpm for 10 minutes at 4oC. the supernatant is separated into new tubes. Supernatant added absolute alcohol twice the volume and NaOAc as much as 0.1 times the volume. The tube is incubated in the refrigerator for at least 2 hours. After incubation, the tubes were centrifuged at 10000 rpm for 15 minutes at 4oC. The supernatant is removed while the pellet is added 500 µl of 70% alcohol. Centrifugation returns with a speed of 10000 rpm for 5 minutes at 4oC. The supernatant is removed and the pellet is dried. After the pellet dried, 20 ddH_2O and 0.4 µl RNAse were added. The tubes were incubated at 37oC for 10 minutes and 70oC for 10 minutes.

Proses RAPD *(Random Amplified Polymorphic DNA)*

As much as 5 µl of genomic DNA is added 1 µl of Loadyng dye until mixed. Amplification of DNA segments is carried out using a single primer decekanucleotide. 15 µl of total DNA sample (0.2 nM dNTPs; 5 primary pmole; 1.5 ml reaction buffer; 2 mM $MgCl_2$; 10 ng DNA sample; and 1 unit of Taq DNA polymerase) were amplified for 45 cycles in the Thermalcycler (Takara Gradient PCR) unit. The PCR for RAPD was carried out as follows: first heating at 940C (5 minutes), denaturation at 940C for 1 minute with 45 cycles, 360 annealing (1 minute), and 720C extension (2 minutes). After 45 cycles are completed, then the DNA fragment extension process continues at 720C for 4 minutes. Next, a dye loading solution is added to add DNA molecular weight. The results of PCR amplification were visualized using horizontal electrophoresis with 1% agarose gel in a TEA buffer (Tris-EDTA). Agarose gel is dipped in 1ml / 100 ml EtBr for 10 minutes. The results of splitting DNA fragments were detected using a UV transluminator under Geldoc ultraviolet, then drawn using a polaroid camera. As a standard measure of DNA, 100 bp DNA ladder (Promega) is used to determine the tape size of DNA amplification results (Krizman, 2007; Susilo et al. 2018).

Data Analysis

The results obtained were analyzed descriptively qualitatively. This data is based on the scoring of DNA bands that appear on electrophoresis results, both agarose 1.2% and agarose 1%. The ribbons seen in agarose are considered as one allele. DNA bands that have the same migration rate are assumed to be homologous loci. At the same migration rate, each band that appears is given a value of 1, while the tape that does not appear is given a value of 0 so that the result of the band scoring is binary data. Scoring result data were analyzed using Sequential Agglomerative Hierarchial And Nested (SAHN) -UPGMA (Unweighted Pair-Group Method with Arithmetic) programs on software using NTSYS version 2.1. The results of the analysis are presented in the form of a dendogram.

RESULT AND DISCUSSION

We selected four Cibotium barometz planlets as samples to be analyzed by RAPD using five primers selected for the PCR process. The primers that we use are OPT01, OPT02, OPT03, OPT04 and OPT05. The PCR process in this study only requires a little DNA template to be able to multiply DNA fragments. DNA detection is done by mixing agarose media 5 µl of genomic DNA plus 2 µl of loading dye. The mixture is inserted into each hole ependoft contained in agarose 1.2% and then run for 20 minutes. The agarose is removed from the electrophoresis device and then immersed in Etidhium Bromide solution for 10 minutes, then soaked with water for 5 minutes. DNA movement was observed under UV light using Chemidoc.

We conducted DNA quality and concentration testing using Nanodrop which uses UV light with wavelengths of 260 and 280 nm. Comparisons in testing using UV light with a wavelength of 260/280 nm provide a measure of protein contamination in a DNA. Meanwhile, the use of UV light with a length of 260 nm serves to calculate the concentration of a DNA sample to be tested for its concentration and purity. 2 µl

of DNA genome of each plantlet was placed above the 2010 Nanodrop hole which had previously been inserted 2 µl of

TE. The results of testing the quantity of DNA with Nanodrop turned out to show mixed results. The results of DNA quantity testing using the Nanodrop are presented in table 1. The highest DNA quantity and purity results were found in CB2 samples with a purity of 1.93 µg / µl and concentrations of 168.3 nm while CB3 showed the lowest values of purity of 1.79 µg / µl and a concentration of 118.1 nm.

Table 1. Results of DNA quantity tests using the 2010 Nanodrop

EXAMPLE	PURE (µg / µl)	CONCENTRATION (nm)
CB0	1,81	125,4
CB1	1,88	154,2
CB2	1,93	168,3
CB3	1,79	118,1

Information: CB0: Cibotium barometz parent plant; CB1: Cibotium barometz Golden haired; CB2: Cibotium barometz with brown hair; CB3: White-haired Cibotium barometz

Polymorphism formed by RAPD markers

PCR amplification with five RAPD primers succeeded in producing 27 DNA bands with sizes ranging from 100 to 500 bp (Table 2). Twenty bands are polymorphic and seven bands are monomorphic. The highest polymorphic band is OPT04 primers with fragment sizes between 100 bp to 400 bp with 100% polymorphic percentage followed by OPT02 with fragment sizes between 91 to 340 with polymorphic percentage of 90%. Polymorphic primers are found on primers OPT01, OPT02, OPT04, and OPT05 while OPT03 are monomorphic which produce 4 bands.

Table 2. Nucleotide sequences and percentage polymorphisms formed by random amplified polymorphic DNA (RAPD) markers.

Primer	Primer sequences	Tape		Total Tape	Presentase Polimorpik
		Polimorpik	Monomorpik		
OPT01	GAG GAT CCC T	2	1	3	75%
OPT02	GGG CCA CTC A	4	1	5	90%
OPT03	GGT GAT CAG G	0	4	4	0%
OPT04	GGG TGT GCA G	12	0	12	100%
OPT05	CCT GAT GAC C	2	1	3	75%

The results of the analysis using the GelAnalyzer program clearly show that DNA appears in many primers OPT02, OPT03, OPT04 (Figure 2). DNA bands are dominated by OPT04 primers, this shows that the compatibility of primers can be seen from the number of DNA bands that appear.

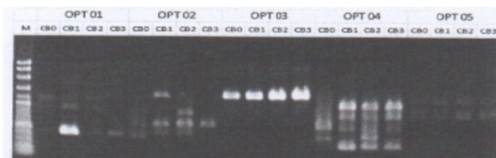

Figure 2. Amplification results and polymorphic levels on 4 Cibotium barometz planlets. (CB0 = Cibotium barometz parent (control); CB1 = Cibotium barometz with Golden Hair; CB2 = Cibotium barometz with Brown Hair; CB3 = Cibotium barometz with Hairy Caucasians).

The OPT02 and OPT04 primers can clearly amplify all Cibotium barometz planlets. Whereas primers OPT01 and OPT05 show vague bands. This study shows that the presence of DNA bands that appear in a primer is directly proportional to the suitability of a simpei fern sample with that primer. This means that the primer can be a reference for simpei fern plants or the like. The results of the scoring are done by looking at the pattern of the results of the PCR then analyzed using the NTSYS program to display the dendogram. The results of the DNA band scoring are shown in table 3.

Table 3. Scoring results from DNA fragment print profiles.

Primer	ALEL	Example				MV
		CB0	CB1	CB2	CB3	
OPT-01	1	0	1	0	0	286
	2	0	1	0	0	168
	3	0	0	0	1	152
OPT-02	1	0	1	0	0	340
	2	0	1	0	0	254
	3	0	0	1	0	207
	4	0	1	0	0	200
	5	0	0	0	1	193
OPT-03	1	0	1	0	0	325
	2	1	0	1	1	333
OPT-04	1	0	1	1	1	275
	2	0	2	0	0	234
	3	0	0	0	1	227
	4	1	0	0	0	190
	5	0	1	0	0	177
	6	0	0	1	0	133
	7	0	0	1	0	97
	8	0	0	0	1	94
	9	0	1	0	0	91
OPT-05	1	0	0	1	0	297
	2	0	0	1	0	240
	3	0	0	0	1	237

Scoring analysis shows the separation of simpei ferns into primers which are grouped based on whether or not DNA appears. Whether or not it is marked marked with the symbol '1' means appears and '0' means does not appear. Each DNA that appears has a varying magnitude (MV). The value of genetic similarity coefficient of four accessions of the simpei fern ranges from 25% to 100%. Electrophoresis results on simpei fern using primary OPT-02, OPT-03, and OPT-04 showed that CB1 was different from other samples, whereas CB0 and CB2 samples tended to be the same and CB03 samples showed differences in the three primers. In the primary OPT-01 and OPT-05 did not show any difference between the samples that have been tested. The results of the scoring are then analyzed using the NTSYSPC, SAHN, SIM, and NTED programs. The program is widely used to find out the closeness between samples. The results of the dendogram analysis in this study formed three clusters (I, II, and III). cluster I consisted of CB1 simpei fern (gold fern), cluster II consisted of CB2 (brown fern) and CB0 (parent

fern), and cluster III consisted of CB3 (Caucasian fern). To see the closest kinship between CB0, CB1, CB2, and CB3 can be seen in the dendogram Figure 3.

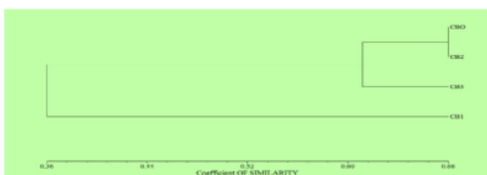

Figure 3. UPGMA grouping dendogram according to the level of kinship in 3 Cibotium barometz accessions with the NTSYSPC, SAHN, SIM, and NTED programs.

Simpei ferns (Indonesia) or golden chicken fern (Cibotium barometz) are ecologically rare plants because of their limited numbers and are sought after by many people for medicines (Huang et al, 2018). Therefore, the business of plant recovery with micropropagation is very important to meet market needs. Cibotium barometz from the Dicksonia family has a difficult development depending on environmental conditions and stages of development. In our research we have done the multiplication of Cibotium barometz by differentiating nitrogen concentrations in the media and producing 3 planlet genotypes. We planletlets from 3 genotypes and 1 parent to test using RAPD to see the possibility of genetic variations. PCR-RAPD amplification on 4 planlets was successfully carried out using five selected primers. In this work, we use the same primers reported by Escandón et al. (2007), who identified six new varieties of Nierembergia linariaefolia.

The results of this study indicate that RAPD markers can determine intra-genetic relationships in the Cibotium barometz genotype. The results of UV chemidoc beam irradiation with five primers in the GelAnalyzer program can be scaled clearly. DNA polymorphic bands were detected in each primer with markers ranging from 100 to 500 bp. Polymorphic properties appear on the primer OPT01, OPT02, OPT04, and OPT05. OPT03 primers have monomorphic properties, this shows that the OPT03 primers are not able to distinguish one sample from another so it is not suitable for simpei fern application. In Figure 2 it can be seen that the least appearing DNA bands are found in the primer OPT01 compared to OPT03. But seen from its nature, the OPT02 primer is even better than the OPT03 primer. OPT02 primers are polymorphic, so they can differentiate DNA in these primers compared to OPT03 which emit more DNA but cannot distinguish CB0 to CB3 planlets.

Regarding the genetic relationship of the Cibotium barometz genotype, the assessment of the genetic similarity coefficient and the incorporation of genotypes resulted in the exposure of genetic variability among the genotypes of the four planlets. Figure 3 is an explanation of the kinship between CB0, CB1, CB2, and CB3 samples with the help of the NTSYSPC, SAHN, SIM, and NTED programs in the form of dendograms. In the similarity correlation coefficient (r = 0.68), the dendogram was drawn using the UPGMA cluster method which produced three groups. One large group consists of CB2 simpei ferns (brown ferns) and CB0 (parent ferns), and two independent individual accesses namely CB3 (Caucasian ferns) and CB1 (golden ferns). Morphologically CB1, CB2, and CB3 samples have almost no difference, only the color of the feathers that distinguishes brownish, golden, and white. Judging from the DNA band pattern they have different sizes, bp locations and properties. Morphologically, CB1, CB2, CB3 have in common with one another, but by testing DNA diversity and kinship levels, they actually have differences from one another.

Genetic similarity between CB0 and CB2 tends to be closer (Figure 3), although it has different morphological characteristics in its genotype. The same genotype turns out to have differences even though they are from the same ecotype (Susilo & Setyaningsih, 2018). This proves that the survival of every plant population can be influenced by genetic diversity. In addition, hereditary multiplicity in the population is influenced by factors such as genetic, gene, selection, mating, and distribution of complex genetic changes.

Our study supports this view to some extent. However, given the small number of genotypes and markers used for this analysis, research with various types of markers and a higher number of genotypes needs to be done. The results of this study, validate once again that RAPD is a useful marker in the study of genetic diversity, because of the degree of polymorphism that can be detected by primers (Susilo & Meitiyani, 2018). In addition, when the genetic basis of the species or genus to be analyzed is not well known and fast and strong results are needed, this technique is very useful because it is simple and has low cost. With this technique, the characterization of each individual is examined so that it offers a promising perspective for the identification of varieties and applications of breeding programs.

Conflicts of interest

The authors declare that they have no conflict of interest.

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CONCLUSIONS

The results showed that the four Cibotium barometz sporophyte samples which were treated with nitrogen turned out to have different DNA variations. With five RAPD primers, four accessions are able to show 27 DNA band patterns ranging from 100 bp to 500 bp dominated by OPT02 and OPT04 primers. The results of primary GelAnalyzer analysis OPT01, OPT02, OPT04, OPT05 are polymorphic, while OPT03 is monomirpis. Our study reveals the potential to produce new Cibotium barometz varieties as plant breeding efforts. This is an opportunity for further research to exploit specific gene markers and the unique properties of Cibotium barometz.

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