Optimization of DNA extraction and amplification of Kikuyu (Pennisetum clandestinum Hochst. ex Chiov) for molecular identification

W Nawfetrias1*, J I Royani1, I S Bidara1, DP Handayani1, M Surahman1, Herdis1, R Herry2, Sarmedi2 and Mansyur3

1 Center for Agricultural Production Technology, Agency for the Assessment and Application of Technology (BPPT) Gd LABTIAP No 610-614-Puspiptek Serpong, Tangerang Selatan, Banten, Indonesia
2 PT Bio Farma, Jalan Kolonel Masturi No. 1 Kabupaten Bandung Barat Jawa Barat Indonesia
3 Faculty of Animal Husbandry University of Padjajaran Jalan Raya Bandung-Sumedang Hegaranmah Jatinangor, Kabupaten Sumedang, Jawa Barat, Indonesia

*Corresponding author: winda.nawfetrias@bppt.go.id

Abstract. Kikuyu (Pennisetum clandestinum Hochst. ex Chiov) is an important forage containing high crude protein for livestock. Molecular analysis of kikuyu relies on high yields of pure DNA and suitable PCR conditions. This research aimed to extract DNA from kikuyu based on weight of the sample and amplify the DNA of Burangrang accession using specific primers. 100 grams and 200 grams leaves of 3 accessions of kikuyu from Burangrang, Bukit Tunggul, and Tangkuban Perahu were extracted by Qiagen Mini Kit Plant. Concentration and purity of DNA were analyzed by NanoDrop Spectrophotometer 2000. DNA from Burangrang accession was amplified using six specific primers at different annealing temperatures. The result showed that the yield of DNA ranged 2.2 µg/µl to 21.4 µg/µl and the purity (ratio) were 1.08 to 2.01. Bukit Tunggul and Burangrang accession showed the same interaction pattern on the sample weight for concentration and purity. One hundred grams of leaves from Burangrang accession produce the highest concentration and the best purity of DNA, but no difference between other weight and accession. Reproducible amplifiable products were observed in all PCR reactions except primer K2. These results indicated that optimized protocol is suitable for further work on molecular identification of kikuyu.

1. Introduction
Kikuyu (Pennisetum clandestinum Hochst. ex Chiov), the indigenous grass from East and Central Africa (Ethiopia, Kenya, Uganda, Tanzania, Rwanda, Zaire, and Congo), grows at highland with low temperature and high humidity. Kikuyu is a tropical creeping grass and used as a pasture species for horses in South Africa. It responds well to fertilization and irrigation with yields of 9 – 30t DM/Ha [1], although quality varies vastly with the season and growing conditions [2,3]. As invasive grass with high levels of fertility character, kikuyu was introduced to different countries to improve pastures, especially for livestock feed [4]. Kikuyu grass has high crude protein content, which important nutrient for livestock [5]. Young leaves have the highest crude protein content, which declines rapidly to below 10%
within 12 weeks [6]. Based on its potency, kikuyu deserves to develop as fodder forage, especially for livestock in Indonesia. The genetic diversity of kikuyu can be explored for basic genetic information of indigenous kikuyu from Indonesia.

DNA extraction is an important step to obtain high-quality DNA, which is required for various molecular activities, such as molecular markers analysis and genetic sequence database [7]. Optimization of DNA extraction process including preparation of weight of samples was required to produce DNA with high quality and quantity. DNA with high yields and quality was needed for some analytical techniques for plant breeding and genetic identification based on molecular hybridization or Polymerase chain reaction (PCR) [8].

PCR is a widely used technique capable of screening a large number of samples in a short time with high specificity. A single PCR cycle comprises three steps: denaturation, annealing, and extension. The annealing temperature is critical for primer-template pairing for the success of PCR. An optimal temperature suppresses mismatched annealing, thereby reducing non-specific products. The determination of critical annealing temperature is developed using preliminary gradient PCR [9]. High sensitivity and specificity primers are important factors to obtain good quality of PCR product. This research aimed to extract DNA from kikuyu based on the weight of the sample and amplify the DNA of Burangrang accession using specific primers. Six specific primers were used to check the quality of DNA from kikuyu.

2. Materials and methods

This research was done at the Laboratory of Plant Production of BPPT at Puspiptek Serpong. All kikuyu accessions are plant collections from Master Seed Farm owned by PT Biofarma Persero.

2.1. Plant material

Plant material used in this study were leaves of kikuyu from three accessions (Burangrang, Bukit Tunggul and Tangkuban Perahu). Young, healthy leaves were collected from each accession and stored at -80 °C.

2.2. DNA extraction

One hundred grams and 200 grams of leaves were ground in liquid nitrogen using mortar and pestle. The pulverized leaves were quickly transferred to a tube and extracted by Qiagen Mini Kit Plant. After extraction, DNA samples were stored at -20°C until use for molecular identification analysis. The yield of DNA was measured using NanoDrop Spectrophotometer 2000. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm and 280 nm. The value of A260/A280 should be between 1.8 and 2.0 for pure DNA samples. Each DNA sample was applied to an agarose gel electrophoresis system to investigate the quality of DNA. 1% agarose gel with Gel Rad addition was used in the TBE buffer system electrophoresis. Gels were visualized by Gel Doc UV.

2.3. PCR amplification and gel electrophoresis

To optimize of primers used, one accession of Kikuyu, Burangrang accession was used as the sample. DNA from Burangrang accession was amplified using six specific primers at different annealing temperatures (Table 1). The total volume of PCR mixture was 10 µl and comprised of 5 µl TopTaq Master Mix Kit, 0.2 µl of each primer Forward and Reverse, 1 µl CoralLoad concentrate 10x, 1 µl DNA template, and 2.6 µl water. The PCR reaction was performed in an Extragene 9600 thermal cycler using PCR conditions (Table 2). The PCR products were analyzed on 1% agarose gel with Gel Rad addition in the TBE buffer system electrophoresis.
Table 1. Six specific primers of Kikuyu (*Pennisetum clandestinum* Hochst. Ex Chiov) used for PCR-based amplification in this study

| Primer code | Primer sequence | % GC content |
|-------------|-----------------|--------------|
| K1          | F : GNGGGNAGA GTANA | 52           |
|             | R : TANTCNTCN TGNCGCNTC | 45           |
| K2          | F : NCTCTGN CNTGNTCT CN | 47           |
|             | R : ANCTCNCCANCAN CTTNCA | 42           |
| K3          | F : TNATA CANGCAN CANC NTNTG | 40           |
|             | R : ACNAGNAGNAGNA GNG | 50           |
| K4          | F : NCTA NTCAA A ACNAG TCA CN | 42           |
|             | R : AANCCNATG NGC TNA TAN | 50           |
| K5          | F : GTAN TGAN CNTNGNT N GG | 45           |
|             | R : NGATTNGA ANGG A N TCA NA A | 40           |
| K6          | F : CANCTNTNTTNTG ANC AN TTT | 42           |
|             | R : GGNTANACGA A NTA TTNTNAC | 40           |

Table 2. Annealing temperature and condition for Gradient PCR

| Primer code | Annealing temperature and PCR condition | Cycles |
|-------------|----------------------------------------|--------|
| K1          | Initial denaturation : 95 °C for 2 minute Denaturation : 95 °C for 30 second Annealing : 50±6 °C for 30 second Extension : 72 °C for 50 second Final extension : 72 °C for 7 minute | 35     |
| K2          | Initial denaturation : 95 °C for 2 minute Denaturation : 95 °C for 30 second Annealing : 51±6 °C for 30 second Extension : 72 °C for 50 second Final extension : 72 °C for 7 minute | 35     |
| K3          | Initial denaturation : 94 °C for 5 minute Denaturation : 94 °C for 30 second Annealing : 55.6±6 °C for 1 minute Extension : 72 °C for 30 second Final extension : 72 °C for 7 minute | 34     |
| K4          | Initial denaturation : 94 °C for 4 minute Denaturation : 94 °C for 1 minute Annealing : 54.1±6 °C for 1 minute Extension : 72 °C for 2 minute Final extension : 72 °C for 7 minute | 34     |
| K5          | Initial denaturation : 94 °C for 5 minute Denaturation : 94 °C for 30 second Annealing : 55.3±6 °C for 1 minute Extension : 72 °C for 30 second Final extension : 72 °C for 7 minute | 34     |
| K6          | Initial denaturation : 95 °C for 2 minute Denaturation : 95 °C for 30 second Annealing : 57.9±6 °C for 30 second Extension : 72 °C for 50 second Final extension : 72 °C for 7 minute | 35     |
2.4. Statistical analysis
All concentration and purity data were analyzed using R Studio 1.4.1106. One-way ANOVA analyses were used to compare the concentration and purity of DNA from two different weights of sample and three different accessions. As a post-hoc analysis, Duncan’s test was performed to determine variation between all samples. A p value < 0.05 was considered significant. Estimated marginal means (emmeans) were used to analyze the interaction of DNA weight of sample and three different accessions.

3. Results and discussion
Extracted DNA from the different weights of sample and accession of kikuyu was evaluated in terms of DNA purity and concentration. DNA concentration and purity obtained from each accession and different weight of sample were shown in Figure 1. DNA extracted from 100 grams leaves of Bukit Tunggul accession showed higher concentration and good purity of DNA than others. DNA from 100 grams of leaves showed higher purity than DNA from 200 grams of leaves for all extracted accessions, and this research showed that increasing the weight of the sample is not related to the purity of DNA. The limitation of reagents from commercial extraction kit could extract only 100 grams of kikuyu leaves to produce good DNA purity at 1.8 – 2.0. The addition of reagent from a commercial extraction kit was needed for DNA extraction with 200 grams leaves. The use of commercial extraction kits caused unstable DNA yield. Commercial extraction kits effectively isolate contaminant-free DNA from recalcitrant plant species, though there is still a loss of significant amounts of DNA [10].

![Figure 1. Box plot of DNA concentration and purity on difference weight of the sample and kikuyu accessions](image)

The differences of accessions and weight of sample showed significantly different concentrations between 100 grams of leaves from Burangrang accession and 200 grams of leaves from Bukit Tunggul accession, 100 grams of leaves from Tangkuban Perahu accession, and 200 grams of leaves from Bukit Tunggul accession (Figure 2). DNA quality was estimated by measuring the 260/280 UV absorbance ratio, which varied between 1.08 and 2.01, but the purity of DNA did not differ in all accessions and weight of the sample (p<0.05). The high quality of DNA is characterized by predominantly high molecular weight fragments with an A260/280 ratio, between 1.8 and 2.0, and the lack of contaminating substances, such as polysaccharides and phenols [11].

One hundred grams and 200 grams of leaves from Bukit Tunggul and Burangrang accession showed the same interaction pattern on DNA purity, while Tangkuban Perahu accession showed a different pattern of interaction on DNA concentration (Figure 3). One hundred grams of leaves from Tangkuban Perahu accession produce DNA purity higher than 200 grams of leaves, but 100 grams of leaves from Tangkuban Perahu produce DNA concentration lower than 200 grams. This result showed that increasing weight from extracted sample was not related to increasing of DNA concentration, but successful amplification influenced by purity and concentration of DNA. Sufficient purity does not guarantee the successful amplification of a gene. There are also other factors such as concentration that also need consideration [12,13].
Figure 2. Duncan’s test of DNA concentration and purity on difference weight of the sample and kikuyu accessions

Figure 3. Interaction of DNA concentration and purity on difference weight of the sample and kikuyu accessions

The quality and purity of extracted DNA on different weights and accession was checked on electrophoresis gel (Figure 4). DNA from Burangrang dan Bukit Tunggul accession showed better results than DNA from Tangkuban Perahu accession on all sample weight. Two hundred grams of leaves gave a very weak band for Tangkuban Perahu accession. There is no smear view in all extracted DNA, which means there is no DNAse contamination during isolation. Significant smearing on DNA samples indicates degradation of the sample [10].

Figure 4. The quality of DNA extracted from leaves on difference weight of sample and kikuyu accessions. TP (Tangkuban Perahu), BT (Bukit Tunggul), BR (Burangrang)
Burangrang accession was used to optimize specific primers for PCR amplification to check the quality of the genomic DNA extracted. DNA fragments were obtained from PCR following agarose gel electrophoresis from fresh leaves of Burangrang accession. This was an indication that the DNA was free from plant secondary metabolites, e.g., flavones, terpenes, and phenolic compounds, which interfere with the yield and quality of the DNA. These secondary metabolites were successfully removed during the extraction process. One hundred grams and 200 grams leaves of Burangrang accession produce good quality PCR product using six specific primers except for K2 primer (Figure 5). Gradient PCR showed that different annealing temperatures for each primer follow K1: 54.3 °C, K3: 48.1 °C, K4:53.4 °C, K5:55.4 °C, K6:53.6 °C. PCR product from K6 showed increasing annealing temperature would decreasing PCR products, nevertheless, PCR products from K5 showed decreasing annealing temperature would decreasing PCR products. The annealing temperature is an important factor for PCR amplification results because the right condition on the amplification process would produce high-quality products.

![Image of DNA gel electrophoresis](https://www.tropicalforages.info)

**Figure 5.** The optimization of annealing temperature of six specific primers for DNA from Burangrang accession. K1(A), K2(B), K3(C), K4(D), K5(E), K6(F)

4. Conclusion
One hundred grams of leaves from Burangrang accession produce the highest concentration and the best purity of DNA, but no difference between other weight and accession. Reproducible amplifiable products were observed in all PCR reactions except primer K2. These results indicated that optimized protocol is suitable for further work on molecular identification of kikuyu.

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