Method of DNA extraction of local maize (Zea mays L.) Tana Toraja, South Sulawesi, Indonesia using modification of buffer CTAB (cetyl trimethyl ammonium bromide) without liquid nitrogen

Ramla1, I R Aziz2, M B Pabendon3, and B S Daryono4

1Postgraduate Student of Biology, Faculty of Biology, Universitas Gadjah Mada, Indonesia Jl. Teknika Selatan, Sekip Utara, Bulaksumur, Yogyakarta 55281, Indonesia
2Department of Biology, Faculty of Science and Technology, UIN Alauddin Makassar, Indonesia Jl. H. M.Yasin Limpo No. 36 Samata, Gowa, South Sulawesi, Indonesia
3Indonesian Cereals Research Institute (ICERI) Jl. Dr. Ratulangi, Maros 90514 South Sulawesi, Indonesia
4Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, Indonesia Jl. Teknika Selatan, Sekip Utara, Bulaksumur, Yogyakarta 55281, Indonesia

Email: ramlahganis@gmail.com

Abstract. Maize is one of the cereals strategic with economic value as well as having the opportunity to be developed due to its position as the main source of carbohydrates and proteins after rice. The purpose of this research is to get the efficiency method of DNA extraction which contains good quality and quantity through modification of extraction buffer CTAB without using liquid nitrogen. DNA extraction was the initial part of the molecular analysis process. The extract of DNA was important, it need to be maintained for its quality and quantity by using an appropriate and efficient method. The results showed that DNA extracted from maize using modification of buffer CTAB was efficient method, have good quality and quantity. The product of PCR amplification in local maize using phi032 primers showed clear and thick appearance of DNA bands. Six alleles detected ranging from 175.5 to 369 bp. The accuracy and efficiency of the methods need to be adapted to several things, such as sample form, type of the organism, sample origin, sample condition, and the character of the sample. The description of DNA extraction method was indispensable, regarding to support the maize genetic breeding program in the future.

1. Introduction
Maize is one of the strategic and economic value cereal plants and has the opportunity to be developed because of its position as the main source of carbohydrates and proteins after rice in Indonesia [1]. Maize have a large contribution to increase domestic food production. However, efforts to increase maize production still face various problems so that domestic maize production has not been able to meet national needs. One way to increase domestic maize production is to intensify the program to increase maize production through assembling new high-yielding varieties [2]. Therefore, the maize
plant breeding program needs to be carried out intensively both conventionally and molecularly based to obtain superior varieties of maize with the desired characteristics.

Since the 16th century farmers in various regions began to cultivate maize and make the selection as expected in a still narrow area. Local maize germplasm as a source of plant genes that are able to adapt to the local environment [3]. Gradually local varieties appearing with white seeds, yellow, white and yellow blends, early maturing, pest resistant and able to adapt well in their respective regions. These varieties need to be maintained as germplasm along with the intensive use of superior varieties to increase national production [4]. Local food crop germplasm was a very important asset so it must be preserved, as well as an effort to find, collect and research types of food crops, to secure it as a source of genes in the repair or formation of superior varieties [2]. Efforts to increase maize productivity that can be done was through one of the plant breeding programs with the assembly of superior maize varieties. The effort to obtain superior maize varieties that are specific in accordance with the wishes, requires the availability of informative germplasm, delivered through characterization activities [5]. Today’s food crops are products of relatively new genetic changes that occur due to artificial selection [6].

In maize based molecular plant breeding activities, DNA extraction was an important initial step and determines the next stage [7]. DNA was needed with sufficient quality and quantity for molecular-based activities such as Polymerase Chain Reaction (PCR), Southern blotting, genomic library construction, to sequencing [8]. The presence of contaminated compounds such as polysaccharides and extracted polyphenols often inhibits the action of certain enzymes so they must be avoided [9]. Therefore, an extraction method was needed that was capable of producing DNA with good quantity and quality [10].

According to Tenriulo et al. [11], the basic principle of DNA extraction was a series of processes to separate DNA from other components. The extraction results are important stages for the next rare and must be done well and free of contamination. Cell extraction or thinning in this study was carried out with a slight modification, namely replacing the use of liquid nitrogen with CTAB extraction buffer. This process was cheaper without reducing the quality of DNA obtained. The process of separating DNA from other cell components or unwanted contaminants, including cell debris by centrifugation.

The DNA extraction method that also can be used liquid nitrogen, which functions to destroy plant cell walls and deactivate certain cell chemical components [12]. The weakness of using liquid nitrogen will appear if the research location was far from the city so that liquid nitrogen was difficult to obtain [13]. According to Ahmed et al. [14] liquid nitrogen was an insecure component that requires special storage. The purpose of this study was to obtain a local maize DNA extraction method that has good quantity and quality using CTAB buffer without liquid nitrogen.

2. Method
The research was conducted at the Laboratory of Molecular Biology and Greenhouse, Cereals Research Institute (ICERI), in Maros, South Sulawesi, Indonesia. Plant material used consisted of 4 Local populations (Bebo, Kandora, Purple and Dalle Pondan) derived from Tana Toraja of South Sulawesi, Indonesia where each population consists of 25 individuals.

All local Maize genotypes were grown on polybags (15x20 cm) containing a mixture of compost and soil (1: 1). Plants are kept in greenhouses and watered daily according to planting conditions. Plants used for DNA extraction 10–15 days after planting. Part of the plant taken was a young leaf that has been opened perfectly, cut into small pieces, then weighed 0.4 g/sample. The process of extraction, electrophoresis and visualization of DNA follows the procedure of George et al. (2004) and Khan et al. (2004) with some modification. Modifications are made through the addition of β-mercaptoethanol. The ingredients for making extraction buffers consist of 2% (w / v) CTAB (Cetyl trimethyl ammonium bromide), 100 mM Tris-HCL pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0. The quantity and quality of DNA extracts were measured by λ DNA standards through a horizontal electrophoresis process using 1% agarose gel and spectrophotometer.
2.1. Primer and PCR Amplification

Each plant DNA sample was analyzed using the Polimerase Chain Reaction (PCR) PTC-200 Peltier Thermal Cycler machine using simple sequence repeat (SSR) primer phi 032, melting temperature 56°C, bin no.9.04, sequence primer Forward : CTCCAGCAAGTGATGCGTGAC; Reverse : GACACCCGGATCAATGATGGAAC, repeat type AAAG. Total reaction for PCR analysis was 10 μl, consisting of 1 μl of Maize DNA, 1 μl of primary (forward and reverse), PCR buffer 1X (10 Mm Tris HCl pH-8, 50 Mm KCl, 0.01% gelatin, 1.5 Mm MgCl2, 0.125 dNTP and 1 unit of DNA Taq polymerase. The amplification process was 30 cycles, consisting of the initial denaturation at 94°C for 5 min, denaturation at 94°C for 45 seconds, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, then cycle cycling back to step II, 29 times, final extension at 72°C for 5 minutes and final hold 4°C.

The amplification results were then separated using a mini vertical electrophoresis machine (Dual Mini-Verticals Complete System MGV-202-33 CBG Scientific Co.) using an 8% non-denaturation polyacrylamide gel in 1X buffer TBE, 1 g/l marker and 20 g NaOH solution, added formaldehyde 1000 μl. The results are visualized on a white glass table to be seen clearly, next to the marker given a small ruler to make it easier to read the size of DNA band.

The result of visualization of DNA disk banding pattern by way of alleles is calculated molecular weight based on the position of the DNA band fragments against known DNA markers (PhiX174 markers).

3. Result and Discussion

3.1. Quantity and Quality of DNA

| Maize line | DNA concentration (ng/μl) | Purity (OD) 260/280 | Maize line | DNA concentration (ng/μl) | Purity (OD) 260/280 |
|------------|--------------------------|---------------------|------------|--------------------------|---------------------|
| Bebo-1     | 56                       | 1.718               | Kandora-1  | 234                      | 1.088               |
| Bebo-2     | 52                       | 1.199               | Kandora-2  | 195                      | 1.027               |
| Bebo-3     | 68                       | 1.249               | Kandora-3  | 190                      | 1.051               |
| Bebo-4     | 79                       | 1.068               | Kandora-4  | 185                      | 1.135               |
| Bebo-5     | 57                       | 1.384               | Kandora-5  | 205                      | 1.278               |
| Bebo-6     | 219                      | 1.178               | Kandora-6  | 232                      | 1.035               |
| Bebo-7     | 233                      | 1.034               | Kandora-7  | 215                      | 1.121               |
| Bebo-8     | 230                      | 1.019               | Kandora-8  | 208                      | 1.006               |
| Bebo-9     | 210                      | 1.04                | Kandora-9  | 204                      | 1.015               |
| Bebo-10    | 201                      | 1.051               | Kandora-10 | 225                      | 1.031               |
| Bebo-11    | 177                      | 1.052               | Kandora-11 | 214                      | 1.097               |
| Bebo-12    | 187                      | 1.075               | Kandora-12 | 190                      | 1.051               |
| Bebo-13    | 194                      | 1.069               | Kandora-13 | 214                      | 1.059               |
| Bebo-14    | 203                      | 1.04                | Kandora-14 | 233                      | 1.034               |
| Bebo-15    | 215                      | 1.072               | Kandora-15 | 210                      | 1.065               |
| Bebo-16    | 190                      | 1.069               | Kandora-16 | 219                      | 1.061               |
| Bebo-17    | 221                      | 1.105               | Kandora-17 | 202                      | 1.718               |
| Bebo-18    | 225                      | 1.031               | Kandora-18 | 228                      | 1.234               |
| Bebo-19    | 208                      | 1.04                | Kandora-19 | 215                      | 1.083               |
| DNA concentration (ng/μl) | Purity (OD) 260/280 | DNA concentration (ng/μl) | Purity (OD) 260/280 |
|--------------------------|---------------------|--------------------------|---------------------|
| Bebo-20                  | 195                 | 1.007                    | Kandora-20          | 221                 | 1.32                    |
| Bebo-21                  | 228                 | 1.08                     | Kandora-21          | 214                 | 1.249                   |
| Bebo-22                  | 211                 | 1.012                    | Kandora-22          | 222                 | 1.425                   |
| Bebo-23                  | 232                 | 1.023                    | Kandora-23          | 230                 | 1.199                   |
| Bebo-24                  | 215                 | 1.121                    | Kandora-24          | 221                 | 1.32                    |
| Bebo-25                  | 214                 | 1.097                    | Kandora-25          | 220                 | 1.089                   |

| Maize line | DNA concentration (ng/μl) | Purity (OD) 260/280 | Maize line | DNA concentration (ng/μl) | Purity (OD) 260/280 |
|------------|--------------------------|---------------------|------------|--------------------------|---------------------|
| Purple-1   | 229                      | 1.079               | Dalle-     | 200                      | 1.05                 |
| Purple-2   | 205                      | 1.08                 | Pondan-1   |                        |                     |
| Purple-3   | 214                      | 1.06                 | Dalle-     | 227                      | 1.049               |
| Purple-4   | 177                      | 1.052               | Pondan-2   |                        |                     |
| Purple-5   | 202                      | 1.047               | Dalle-     | 230                      | 1.093               |
| Purple-6   | 217                      | 1.055               | Pondan-3   |                        |                     |
| Purple-7   | 196                      | 1.008               | Dalle-     | 229                      | 1.075               |
| Purple-8   | 215                      | 1.069               | Pondan-4   |                        |                     |
| Purple-9   | 213                      | 1.055               | Dalle-     | 211                      | 1.058               |
| Purple-10  | 227                      | 1.088               | Pondan-5   |                        |                     |
| Purple-11  | 224                      | 1.054               | Dalle-     | 219                      | 1.052               |
| Purple-12  | 230                      | 1.071               | Pondan-6   |                        |                     |
| Purple-13  | 215                      | 1.027               | Dalle-     | 224                      | 1.151               |
| Purple-14  | 217                      | 0.957               | Pondan-7   |                        |                     |
| Purple-15  | 205                      | 0.971               | Dalle-     | 220                      | 1.025               |
| Purple-16  | 203                      | 1.017               | Pondan-8   |                        |                     |
| Purple-17  | 200                      | 1.021               | Dalle-     | 215                      | 1.027               |
| Purple-18  | 224                      | 1.337               | Pondan-9   |                        |                     |
| Purple-19  | 217                      | 1.048               | Dalle-     | 234                      | 1.041               |
| Purple-20  | 212                      | 1.066               | Pondan-10  |                        |                     |
|             |                          |                     | Dalle-      | 219                      | 1.118               |
|             |                          |                     | Pondan-11  |                        |                     |
|             |                          |                     | Dalle-      | 231                      | 1.115               |
|             |                          |                     | Pondan-12  |                        |                     |
|             |                          |                     | Dalle-      | 226                      | 1.039               |
|             |                          |                     | Pondan-13  |                        |                     |
|             |                          |                     | Dalle-      | 203                      | 1.017               |
|             |                          |                     | Pondan-14  |                        |                     |
|             |                          |                     | Dalle-      | 213                      | 1.037               |
|             |                          |                     | Pondan-15  |                        |                     |
|             |                          |                     | Dalle-      | 238                      | 1.063               |
|             |                          |                     | Pondan-16  |                        |                     |
|             |                          |                     | Dalle-      | 229                      | 1.13                |
|             |                          |                     | Pondan-17  |                        |                     |
|             |                          |                     | Dalle-      | 232                      | 1.085               |
|             |                          |                     | Pondan-18  |                        |                     |
|             |                          |                     | Dalle-      | 221                      | 1.384               |
|             |                          |                     | Pondan-19  |                        |                     |
|             |                          |                     | Dalle-      | 201                      | 1.021               |
The concentration of DNA (ng/μL) varies from 52 ng/μL to 238 ng/μL and purity varies from 0.957 to 1.718 (Table 1). The lowest DNA concentration was owned by the Bebo-2 population, while the highest concentration was owned by the Dalle pondan-16 population. The amount of DNA needed for PCR process was equivalent to 50 ng / μL. Thus, DNA that was still high, was diluted using the formula:

\[ M_1 \cdot V_1 = M_2 \cdot V_2 \]  \hspace{1cm} (1)

Note:
M1 = concentration of stock DNA
V1 = volume of stock to be dissolved
M2 = concentration of work solution
V2 = volume of work solution prepared

The purity of DNA was slightly low due to RNA contamination, but it doesn’t affect the PCR amplification (figure 1). Quality of DNA was also a component that was quite influential in DNA amplification on PCR process, the DNA with good quality was not contain of contaminant from other components of the cell. According to Tenriulo et al. [11], DNA that has good quality was clean and uncontaminated DNA. Contamination by phenols and other organic materials can be seen with the appearance of a smear background along the path of the DNA band [15].

DNA contaminants that were usually found in presence of polysaccharides that can interfere with advance processes such as PCR, where there were barriers to the activity of enzyme taq polymeration or other contaminants namely polyphenols which in oxidized form will bind covalently of DNA. To avoid this, the related components during the extraction process were kept cool. As a basic material for the PCR process, the DNA used must be clean of contaminants (high purity). Dirty DNA can be caused by the remnants of ethanol during incomplete drying and the presence of secondary metabolite content in extracted plant organs.

Contamination can occur due to several things, namely the process of taking and storing samples that are not good, errors in supernatant collection techniques that are contaminated by buffer lysis and DNA digestion processes that may be imperfect because during incubation, the sample is not shaken so that when taking phenol, some proteins are not bound and remain attached to the DNA in the sample. Restu et al. [16], revealed that each type of plant has a different secondary compound content so that it requires optimum extraction. Appropriate extraction techniques determine the quality and quantity of DNA produced.

| Purple-21  | 201 | 1.031 | Dalle- Pondan-21 | 222 | 1.145 |
| Purple-22  | 187 | 0.963 | Dalle- Pondan-22 | 210 | 1.104 |
| Purple-23  | 206 | 1.004 | Dalle- Pondan-23 | 212 | 1.066 |
| Purple-24  | 176 | 1.101 | Dalle- Pondan-24 | 202 | 0.968 |
| Purple-25  | 231 | 1.111 | Dalle- Pondan-25 | 225 | 1.059 |
3.2. PCR Amplification

Figure 1. Visualization of DNA bands result from PCR process using phi032 SSR primers

Table 2. Variation in the number and size of alleles in four local maize population

| Local Maize | Bin.no | Temperature (Tm) °C | Number of allele | Ukuran alel (bp) |
|-------------|--------|---------------------|------------------|-----------------|
| Bebo        | 9.04   | 56                  | 4                | 175.5-340       |
| Kandora     | 6      | 175.5-369           |                  |                 |
| Purple      | 4      | 175.5-340           |                  |                 |
| Dalle Pondan| 4      | 175.5-340           |                  |                 |

Visualization of DNA bands result from PCR amplification using phi032 primers showed that the entire local maize population was very well amplified, six alleles detected ranging from 175.5 to 369 bp. Good amplification of 4 local maize populations shows that the use of the CTAB buffer method for DNA extraction in local maize from Tana Toraja, gives very efficient and good results.

The existence of several genotypes of maize undergoing poor amplification was indicated by missing data. Missing data was marked by the emergence of a DNA band resulting from visualization, which can be caused by the DNA template not finding an appropriate base pair on the primer used. According to Ruwaida et al. [16], the difference in the number of bands produced by each primer was different, this was due to differences in the order of the primary nucleotide bases or the interaction between the primer and printed DNA. The primers used are not well amplified in certain base pairs causing DNA bands to be invisible so that only a little DNA can be observed. According to Haris et al. [17], the presence of band fragments that are not visible on the visual or very thin bands on the gel can be caused by DNA concentrations that are too low [18]. According to Pabendon et al. [19], other factors causing the missing data include the primary quality that has been used because it has been stored for a long time, the annealing temperature that was not right at the time of PCR amplification, and technical factors related to personal that directly handle the characterization activities. States that annealing temperature was the primer will stick to the DNA template, the amount of temperature can be calculated based on the melting temperature (Tm) value of each primer [20]. The search for optimal conditions of annealing temperature was very important, because it relates to the specificity and sensitivity of the PCR product.

The existence of a thicker DNA banding pattern compared to the others can be caused by the quality and quantity of DNA that is not good. Wati et al. [21], states that the quantity and quality of DNA can affect the intensity of the DNA band amplification results in each primer. The result of the amplification of thin DNA bands can be caused by the presence of compounds such as phenolic and polysaccharides in the DNA template. Haris et al. [17] also added that the presence of DNA band fragments that look thick makes it difficult to compare with other bands due to high DNA concentration.

4. Conclusion

The DNA extraction method for local maize (Zea mays L.) Tana Toraja, South Sulawesi, Indonesia using modification of buffer CTAB without liquid nitrogen was efficient method, have good quality and quantity. The results of PCR amplification in local maize using phi032 primers showed clear and thick appearance of DNA bands. Six alleles detected ranging from 175.5 to 369 bp. The accuracy and
efficiency of the methods need to be adapted to several things, such as sample form, type of the organism, sample origin, sample condition, and the character of the sample. The description of DNA extraction method was indispensable, regarding to support the maize genetic breeding program in the future.

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