

Phenotypic characters and genetic variations of lurik peanuts (*Arachis hypogaea* L. var. *lurikensis*) with Inter Simple Sequence Repeat

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Abstract. Rosyidi IN, Daryono BS. 2020. Phenotypic characters and genetic variations of lurik peanuts (*Arachis hypogaea* L. var. *lurikensis*) with Inter Simple Sequence Repeat. *Biodiversitas* 21: 629-635. Agriculture is a sector that supports national development, one of its commodities is peanut (*Arachis hypogaea* L.). The needs and demand for peanuts from the food industry sector increased throughout time. However, the current national production level is unable to fulfill the demand, so the number of peanut imports increased sharply. Based on this condition, researches to improve peanuts production using Lurik peanuts should be held as a solution to fulfill the need for national consumption because lurik peanuts have a greater number of pods and seeds. This research was aimed to improve the quality and quantity of Lurik peanuts phenotype and analyze the successes through the variations and its molecular character. This research was done in May - July 2018 in Mutihan, Madurejo, Prambanan, Sleman, Yogyakarta and in the Laboratory of Genetics and Breeding of Faculty of Biology, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia. In this research, Lurik peanuts improvement was conducted by selection and induction with colchicine. The Data was collected qualitatively using Inter Simple Sequence Repeat (ISSR) with ISSR 8, ISSR 9, UBC 825, UBC 841 primers and electrophoresis while the quantitative method was done using plant phenotype measurement and spectrophotometry, then followed by data analysis using one-way ANOVA and MVSP 3.1 program. The results of this research showed that the characters of Lurik peanuts were different from common peanuts such as in having higher plants, different numbers of seeds per pod, and pattern on the seed coat. These data were supported by the molecular results, with similarity index at 84% meaning that the Lurik peanuts and common peanuts are classified as one same species.

Keywords: *Arachis hypogaea*, colchicine, Lurik peanut, Inter Simple Sequence Repeat

INTRODUCTION

Agriculture is one of the most important sectors that support national development. Given the supporting area, geographical conditions, and climate, it is very natural that Indonesia has potential in this sector. Peanut (*Arachis hypogaea* L.) is one of the important commodities of the food crop sub-sector (Ministry of Agriculture 2015). The need and demand for peanuts from the processed food industry sector spurred an increase in the income of farmers in various regions. Processed foods with peanuts raw materials have experienced an increasing demand. However, peanuts production has not been able to meet the demand, so the amount of imports has increased sharply. Based on data from FAO and the 2015 Agricultural Information and Data System Center, Indonesia is the world's number two importer country of peanuts (Ministry of Agriculture 2015).

The higher number of peanut imports compared to the productions potentially led to the emergence of peanut breeding research using Lurik peanut. Lurik peanut has a larger form of pea seed morphology and has more seeds than ordinary peanuts, which is three to four seeds per-pod so the production amount is increased. Peanut breeding is expected to be a solution to meet the needs of peanuts in Indonesia and the world.

Plant breeding is done by selecting superior peanut varieties, which is started by comparing the phenotypic

characters of Lurik peanut originated from Cianjur, West Java, and Lurik peanuts originated from Blitar, East Java. Then the selected results are administrated with a chemical reagent named colchicines, which is one of the plant breeding techniques by chromosome multiplication method. Based on the research conducted by Hetharie, (2003), doubling the number of chromosomes would make a plant more resistant to pests, has a larger size of leaves, stems, flowers, fruits, and the cell nucleus, has increased contents of vitamins and protein, and has lower osmotic pressure. Thus, the implementation of this research is expected to be a solution to increase peanut yields in meeting the consumption needs. In the breeding process, it is also necessary to regard the object classification status, therefore in this study PCR-ISSR analysis was also conducted to see the relationship between Lurik peanuts and common peanuts. Polymerase Chain Reaction (PCR) is a method for DNA replication using polymerase enzymes that synthesize complementary DNA sequences with small fragments of certain (primary) DNA sequences (Valonez et al. 2009). PCR method is used because it is fast, accurate, and can be done using components or materials in small amounts (Kavya 2015). ISSR (Inter Simple Sequence Repeats) is a PCR technique that involves amplification of DNA segments between two identical microsatellites through repeating regions in opposite directions. The length of the sequence used is 16-25 bp. The advantage of ISSR is reproducibility and high polymorphism, there is no

codominant, the precursor sequence does not need to be known in advance, the locus sampling is random, medium costs, simple, fast and efficient.

This research was carried out using the ISSR (Inter-Simple Sequence Repeat) molecular marker because ISSR had several advantages compared to other molecular markers such as RAPD and RFLP which are often used in the analysis of genetic variation. The strengths of ISSR molecular markers included: more sensitive to genetic diversity than RAPD; easier and more affordable compared to RFLP. The first stage in qualitative and quantitative DNA testing was DNA extraction process, in this stage plant cell walls and membranes were destructed then DNA in the nucleus was removed from the cell (Sharma et al. 2010).

PCR-ISSR analysis is a technique to amplify DNA in regions between microsatellites. The length of the ISSR primer used was 15-30 mers, compared to RAPD which uses 10 mers primer (Reddy et al. 2002). In ISSR there was no need for genomic sequence information but led to multilocus and polymorphism patterns (Abdel-Mawgood 2012). In this study, several primers were used with UBC and ISSR as the codes. UBC primers are commonly used in analyzing the relationship between various plant types (Joshi et al. 2000). There were 4 primers used in this study, namely UBC 825, UBC 841, ISSR 8 and ISSR 9. The primers were selected based on the literature studies on genetic variations of peanuts (*Arachis hypogaea*) with mutagens induction by Tshilenge-Lukanda et al. (2012). After optimization, the obtained optimal concentration was 10 pmol, while the template DNA concentration used was 10 ng / μ L.

The aim of this study was to determine the differences in the phenotypic characters of Lurik peanuts (*Arachis hypogaea* L. var. *lurikensis*) and common peanuts (*Arachis hypogaea* L.), to know the differences in the molecular characters of Lurik peanuts (*Arachis hypogaea* L. var. *lurikensis*) and common peanuts (*Arachis hypogaea* L.) and how much the effect of colchicines mutagens induction on the genetic variations of Lurik peanuts (*Arachis hypogaea* L. var. *lurikensis*), this research is a new study that characterizes the level of production, genetic variation, and the effect of mutagen from unique varieties of peanut namely lurik peanuts.

MATERIALS AND METHODS

Materials

The materials used in this study were Lurik peanuts, common peanuts, NPK fertilizer, pesticides, colchicine, aquadest, DNA extraction kit (Nucleon Phytopure), SYBR Green (10 mg/ml), 10 x TBE 10x (0.89 M Tris Borec Acid, 0.02 M EDTA Disodium, pH 8.4), cold chloroform, ISSR primer in Table 2. PCR Kit Bioline MyTaq, agarose, DNA marker, loading dye, sterile aquabidest, and ice.

Procedures

Colchicine induction

Colchicine induction process began with the selection of seeds to ensure the uniformity of plants in terms of quality. The seeds were weighed using an analytical scale, then a colchicine solution was made with a concentration of 0% (control), 0.025%, 0.050%, 0.1%, 0.15%, and 0.2% (Permadi, 1991). Then the solution was put into a flacon bottle followed by the insertion of 10 peanuts at each concentration. In this study two plantings were carried out, the first planting was done to select the most optimum seeds between Cianjur or Blitar lurik peanuts for breeding, while the second planting was done with a breeding process with colchicine. The first planting was carried out in the greenhouse of Ngombol Village, Purworejo, Central Java. While the second planting was carried out in the village of Mutihan, Berbah, Sleman, D.I. Yogyakarta.

Data collection

Phenotypic data

Phenotypic data were collected by observing and recording, including the length and width of stomata, plant height, number of pods per plant, number of seeds per plant, length of pods, seed length, weight of 100 seeds, age of flowering, maturity of peanuts, and percentage of harvest.

Molecular data

DNA extraction

DNA extraction method according to (Latifah 2016): DNA extraction using Nucleon-Phytopure (brand: 'Illustra DNA Extraction Kit Phytopure TM RPN 1851') consisting of Reagent 1, Reagent 2 and Resin. Followed by a qualitative test performed by electrophoresis method. DNA samples were isolated and loading dye was prepared. The mixture of each DNA sample was loaded on parafilm paper. Then the DNA sample was put into the well on the electrophoresis gel (0.8% concentration) at a voltage of 100 volts. After migration of the DNA reaches 2/3 of the gel (visualized in blue), the machine was turned off. Then, the gel electrophoresis results were observed with UV Transilluminator (Latifah 2016).

Quantitative Test of DNA Extraction Results

A quantitative test of DNA samples from the extraction was done by measuring the concentration and purity of DNA with a nanodrop spectrophotometer, to ensure the purity of DNA around 1.8 – 2 (Sambrook, 2001). Before measuring the concentration and purity of DNA samples, DNA dilution was carried out, the composition of the solution is 5 μ L DNA and 495 μ L ddH₂O.

DNA Amplification with PCR-ISSR

DNA amplification was carried out by 'BOECO Thermal Cycler TC-PRO' PCR machine. Before the sample was inserted into the PCR machine, Premix PCR was first made based on each primer (Table 1).

Table 1. ISSR primers used in the study (Lukanda 2012)

Primer	Primer sequence 5'- 3'
UBC 841	GAA GGA GAG AGA GAG AYC
UBC 825	ACA CAC ACA CAC ACA CT
ISSR 8	AGA TAG ATA GAT AGA TAG ATG Y
ISSR 9	GAT CGA TCG ATC GC

The tube containing the components of the PCR formula was then amplified with the 'BOECO Thermal cyclor TC-PRO (Germany)' PCR machine according to the time and temperature procedure in each step (Predenaturation, Denaturation, Annealing, Elongation, Postelongation, Endless). The temperature orders were predenaturation at 94°C for 2 minutes, denaturation at 94°C for 45 seconds, annealing at 44-52°C for 1 minute, elongation at 72°C for 90 seconds, post elongation at 72°C for 10 minutes, and 4°C ∞ endless.

Data analysis

Phenotypic data analysis was carried out by measuring all parameters, then processed with SPSS 23 software. While molecular data analysis was performed with DNA band scoring and processed with MVSP 3.1 software.

RESULTS AND DISCUSSION

Morphological characters

Morphological analysis was done through calculation, measurement, and observation of existing characters.

Based on Table 2. it can be seen, the qualitative phenotype between Lurik peanuts and common peanuts are different, the seeds in both peanuts are very easy to distinguish., where the Lurik peanuts contain purplish color

accents as their characteristic, the Lurik peanuts flowers have red accents in the area shown by arrow, while the normal peanuts in this area are only pale orange. In leaf observation, data collection was carried out over a period of and 60 days after planted with a difference in size, leaves from Lurik peanuts are much longer than common peanuts. In the phenotypic appearance of peas can be seen in the size of Lurik pod is much bigger than common peanuts, besides that in one Luriks pod in usually contain 3 seeds or even 4 seeds, in common peanuts only 2 seeds are available in general (Muarifin 2015).

Quantitative morphological comparison of Lurik peanuts and common peanuts use some character include plant height, leaf length, leaf width, weight of 10 seeds, weight of 10 pods, weight of 160 seeds, number of pods, flowering time (dap), average length of pods, average width of pods, average seed length, and average seed width, were shown in the following table 3.

The quantitative morphological character analysis is presented in Table 2. It could be observed that almost all of the parameters in the results were significantly different after being analyzed by SPSS 23 BMI, except for the parameters of seed width and weight of 10 seeds. This situation was very sufficient to background the need to do an analysis of the existing genetic variations, to determine the relationship between the two.

Molecular characteristics

Based on Figure 1. which is the visualization of ISSR PCR products using ISSR 8 primer, 10 DNA bands with a size of 244-1946 bp were obtained. Of the 10 bands formed there were 6 monomorphic bands and 4 polymorphic bands. There was 2 band which were specifically owned by common peanuts (non-striated) with the sizes of 1946 and 1422 bp. Both bands could be a specific DNA band marker for common peanuts.

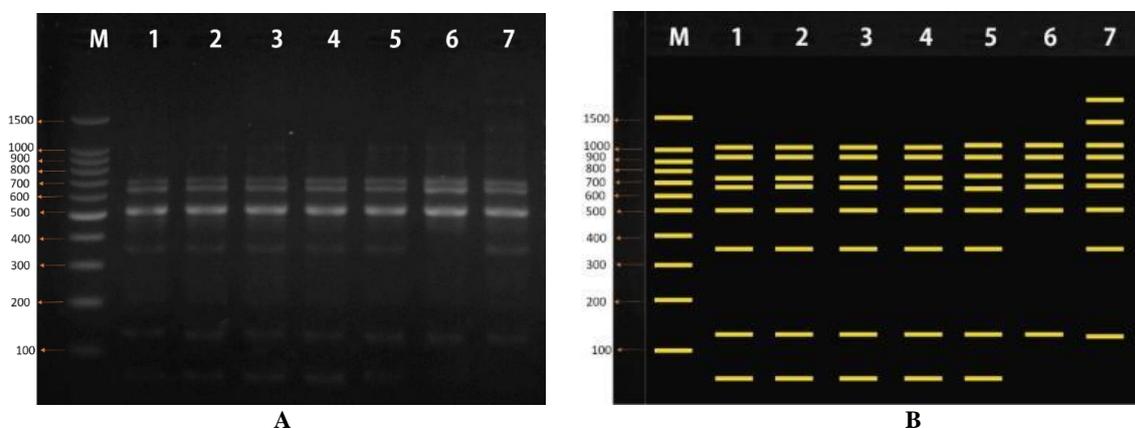


Figure 1. Electrophoresis results (A) and electrophoregram (B) 6 samples of Lurik peanuts and one sample common peanuts using ISSR 8 primer. (M=100 bp marker (Kappa); 1: Control of Lurik peanuts; 2: Lurik peanuts 0,025M; 3: Lurik peanuts 0,05M; 4: Lurik peanuts 0,10M; 5: Lurik peanuts 0,15M; 6: Lurik peanuts 0,2M ; 7: Common peanuts)

Tabel 2. Morphological comparison of Lurik peanuts and common peanuts qualitatively

Character	Lurik peanuts	Common peanuts
Seeds		
Flower		
Leaf		
Pod		

Based on Figure 2. which is the visualization of ISSR PCR products using ISSR 9 primer, 6 DNA bands of 244–957 bp were obtained. Of the 6 bands formed, there were 4 monomorphic bands and 2 polymorphic bands. There were 2 bands that were specifically owned by Lurik peanuts with sizes of 957 and 244 bp. Both bands could be a specific DNA band marker for Lurik peanuts when conducting a comparative analysis with common peanuts.

Based on Figure 3. which is the visualization of ISSR PCR products using UBC 825 primer, only 4 DNA bands of 684 - 1691 bp were obtained. Of the 4 bands formed, there was not one polymorphic DNA band. Therefore, UBC 825 primer was less effective in the study of genetic variations between the control of Lurik peanuts and the colchicine-treated peanuts, as well as compared with the common peanuts. The absence of this difference was due to the attachment of UBC 825 primer to a DNA segment that was owned by all the control and treated Lurik peanuts, and the common peanuts. Polymorphism was a form of genetic diversity which in this study was shown by the difference in the bp sizes of DNA bands.

Figure 4 is the visualization of electrophoresis results with UBC 841 primer, 6 DNA bands of 244-1557 bp were obtained, with 2 polymorphic bands and 4 monomorphic DNA bands. A DNA band at a size of 537 bp was only owned by common peanut samples. This DNA band could be referred to as a specific band that differentiates Lurik peanuts and peanuts in general. Likewise, a band with a size of 223 bp was only owned by Lurik cultivar.

Based on the electrophoresis results of PCR-ISSR results using the four primers above, it could be observed that the majority of samples from 1 to 6, which were the control Lurik peanuts, Lurik peanuts with 0.025M colchicine; Lurik peanuts with 0.05M colchicine; Lurik peanuts with 0.1M colchicine; lurik peanuts with 0.15M colchicine; and lurik peanuts with 0.2M colchicine; all six of them had high monomorphic levels and very low polymorphic levels. This was because the six samples were one species and even in the same cultivar, with not too different phenotypic appearances.

Table 3. Quantitative morphological comparison of Lurik peanuts and common peanuts

Parameter Plant	Peanut varieties	
	Lurik peanuts	Common peanuts
Plant height (cm)	62.75**	48.5**
Leaf length (cm)	6.93**	3.41**
Leaf width (cm)	2.67**	1.57**
Number of pods	32.5**	17.87**
Flowering time (dap)	25**	27.25**
Pod (random) 10 pods/plant		
Average length of pods (cm)	4.13**	2.88**
Average width of pods (cm)	1.37**	0.83**
Weight of 10 pods (gram)	6.97**	2.79**
Seeds		
Average seed length (10 KL random seeds) (cm)	1.59**	1.38**
Average seed width (10 KL random seeds) (cm)	0.81	0.73
Weight of seeds (10 KL random seeds) (gram)	4.19	4.31
Weight of 160 Lurik peanut seeds (cm)	67.18**	69.02**

Note: ** = Significantly different

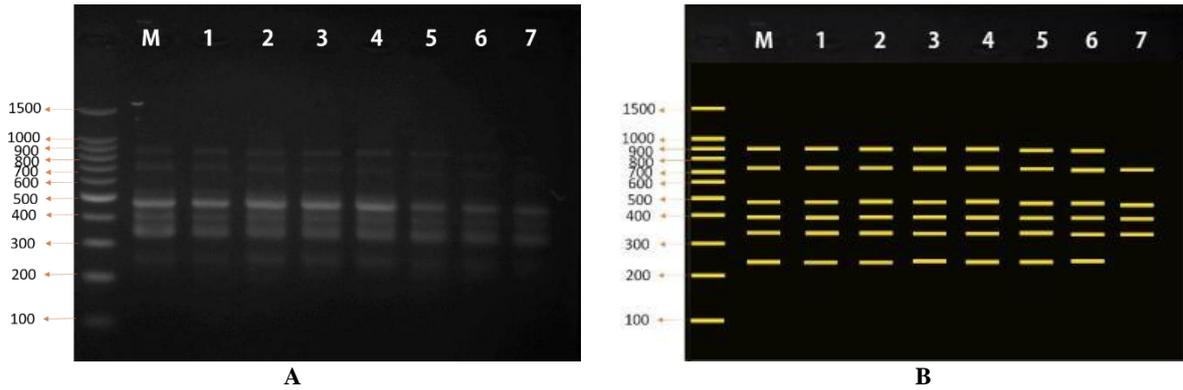


Figure 2. Electrophoresis results (A) and electrophoregram (B) 6 samples of Lurik peanuts and one sample common peanuts using ISSR 9 primer. M=100 bp marker (Kappa); 1: Control of Lurik peanuts; 2: Lurik peanuts 0,025M; 3: Lurik peanuts 0,05M; 4: Lurik peanuts 0,10M; 5: Lurik peanuts 0,15M; 6: Lurik peanuts 0,2M ; 7: common peanuts

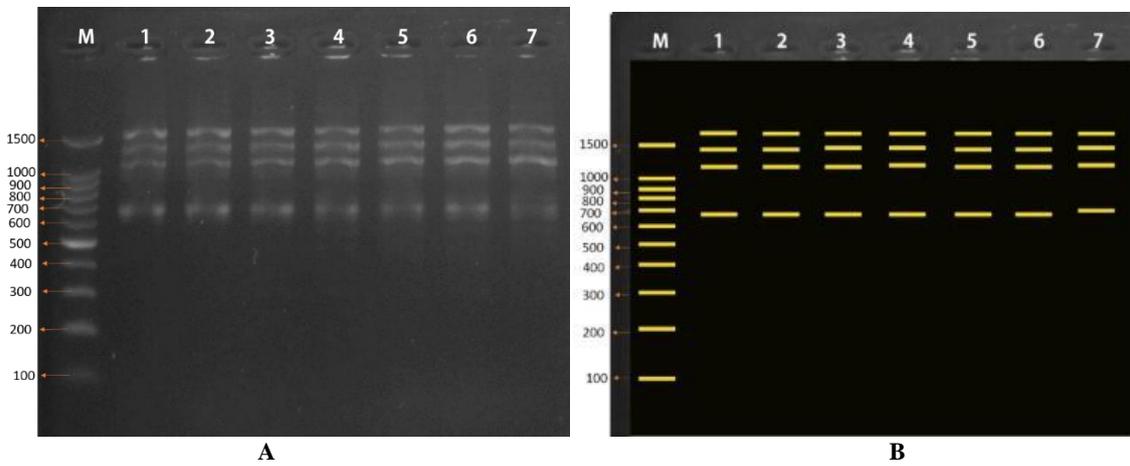


Figure 3. Electrophoresis results (A) and electrophoregram (B) 6 samples of Lurik peanuts and one sample common peanuts using UBC 823 primer. M=100 bp marker (Kappa); 1: Control of Lurik peanuts; 2: Lurik peanuts 0,025M; 3: Lurik peanuts 0,05M; 4: Lurik peanuts 0,10M; 5: Lurik peanuts 0,15M; 6: Lurik peanuts 0,2M ; 7: common peanuts

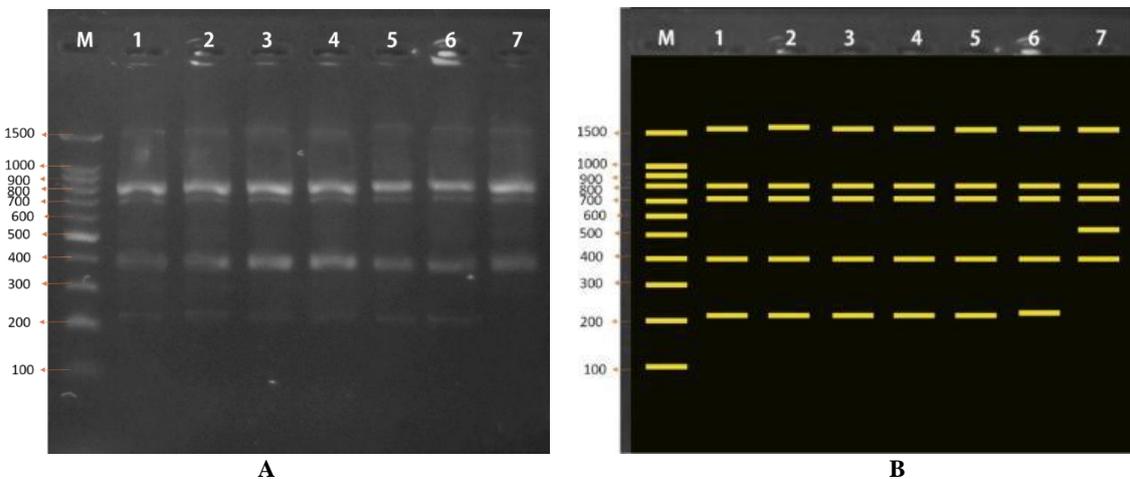


Figure 4. Electrophoresis results (A) and electrophoregram (B) 6 samples of Lurik peanuts and one sample common peanuts using UBC 841 primer. M=100 bp marker (Kappa); 1: Control of Lurik peanuts; 2: Lurik peanuts 0,025M; 3: Lurik peanuts 0,05M; 4: Lurik peanuts 0,10M; 5: Lurik peanuts 0,15M; 6: Lurik peanuts 0,2M ; 7: common peanuts

Therefore it could be ascertained in this study, that colchicine induction which was intended as a mutagen, in fact, did not change the gene compositions from the samples. The following is a summary table of polymorphism levels of the seven samples, the presence of polymorphic bands shows there are variations. In addition, the ISSR analysis method can also be used to detect the success of a mutagen. Mutations result from all types of material change derived. DNA, which is the main component of genes as a carrier of genetic information from generation to generation, is the main target of mutagen delivery. DNA changes that occur due to mutations, will cause new genetic variations that will be inherited in the descent. Genetic changes that occur due to irradiation can be seen phenotypically but can also not be expressed. The success of mutations can be observed through changes in morphology, anatomy, and at the DNA level (Widiastuti et al. 2013).

The polymorphism table above shows that all four primers produced very low levels of genetic diversity, which were below 50%. Whereas with ISSR 8 primer only 40% percentage of polymorphic DNA was found, with ISSR 9 and UBC 841 only 33.3% were found, while with UBC 825 a polymorphic DNA was not found at all. These results were very reasonable since out of the 7 samples, 6 samples were the same species and in the same cultivar, the difference lied only in the treatments of colchicine induction with various concentrations. Therefore in this study, induction of colchicine as a mutagen did not have an impact on improving genetic diversity of Lurik, meanwhile according to Tshilenge-Lukanda et al. (2012) induction of mutagens such as colchicine, gamma-ray, and other mutagens can often improve genetic diversity, cause mutations, and produce significant changes compared to the broodstocks. The artificial mutation process has been widely used to improve the phenotype quality in plants that have low genetic variability where it is impossible for phenotypic crosses (Widiastuti 2013). The presence of ISSR molecular marker plays an important role as one of the steps to monitor the success of mutagen induction. Information about genetic diversity obtained from DNA analysis is also useful in determining the kinship between individuals or between populations studied. This

information can be used as a basis for improving plant genetic quality through well-programmed plant breeding. Genetic diversity can occur due to variations in the nucleotides that make up DNA. This variation can affect the individual phenotypes of organisms that can be observed directly (Yuliani 2017).

Phenetic relationship

Phenetic relationship in 7 samples, the binary data was used to determine the similarity level using dendrogram, with UPGMA method (Unweighted Pair Group Method with Arithmetic Average). Dendrogram construction using MVSP 3.1A software can be seen in Figure 5.

Based on the dendrogram in Figure 5., it could be seen that the control Lurik peanuts, Lurik peanuts 0.025 M, 0.05 M, 0.1 M, and 0.15 M were in a straight line with 100% similarity on phonetic relation level because the five samples were one species and one cultivar. The concentration variations of colchicine did not affect the genetic characters of Lurik peanut cultivar, indicated by the similarity of DNA bands between control Lurik peanuts and colchicine-treated peanuts. Meanwhile, Lurik peanut sample with 0.2 M colchicine induction was not included in a straight line also have far phonetic relation with other concentrations and the control with a similarity index of 93.02%, this indicated that colchicine induction with a concentration of 0.2 M for 6 hours can affect the molecular character of Lurik peanuts. In addition, the dendrogram formed also showed that the Lurik peanuts and common peanuts were not in a straight line but met at a similarity point of 84.44%.

Table 4. DNA polymorphism in Lurik peanut samples with and without colchicine, and 1 sample of common peanuts

Primer	Number of DNA bands	Poly-morphic DNA	Mono-morphic DNA	Percentage of polymorphic DNA (%)
ISSR 8	10	4	6	40 %
ISSR 9	6	2	4	33,3 %
UBC 825	4	0	4	0 %
UBC 841	6	2	4	33,3%

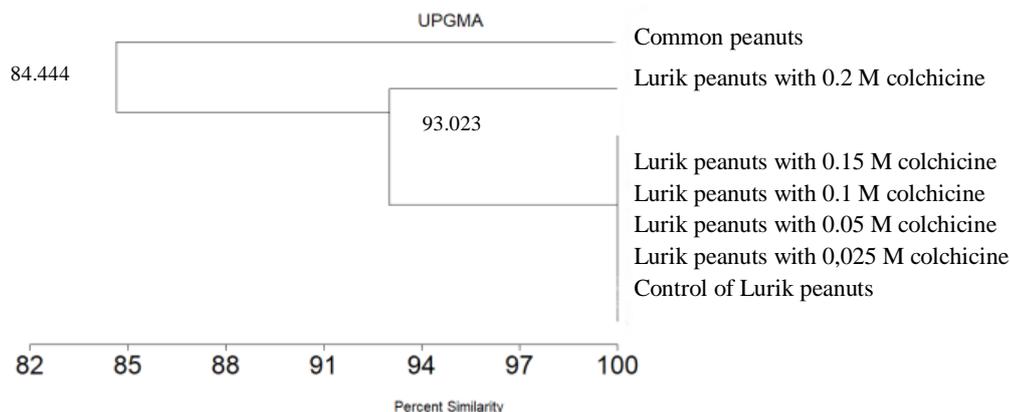


Figure 5. Dendrogram of the phenetic relationship between Lurik peanuts (control and colchicine-treated) and common peanuts based on the ISSR marker with UPGMA method

According to Singh (1999), a taxon is said to be a genus if it has a similarity index of $\geq 65\%$ and $\pm 80-100\%$ for one species, while the greater the similarity index, the closer the relationship between varieties or cultivars. This was supported also by research conducted by Purnomo (2004) about the relationship of *Piper* species which stated a similarity index of $\geq 60\%$ belonging to one species.

Genetic diversity can also be caused by mutations (Agisimanto and Supriyanto 2007). Mutations are changes in the DNA nucleotide sequence of an organism that produces genetic diversity (Campbell et al. 2008). The difference in the expression of lurik genes in lurik peanuts may be due to the presence of transposon gene elements that need to be investigated further. Molecular markers could be used to describe relationships that were more accurate than using only morphological characters. This was because molecular markers are less influenced by environmental factors (Pandin 2009). Based on this study, the ISSR primer could analyze genetic variations between common peanuts and Lurik peanuts by producing 8 polymorphic bands. The use of the ISSR analysis method was faster, easier and did not require a specific locus because this primer would search for every place in the genome that contained microsatellite motives (Fang & Roose, 1997). Induction of colchicine mutagen in lurik peanuts with a concentration variation of 0.025 M; 0.05M; 0.1 M; 0.15 M does not affect its molecular character, whereas at the concentration of 0.2 M can shift the value of similarity so that by using primers and molecular markers a change due to mutagens can be detected.

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