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Genetic diversity of mutant napiergrass using Expressed Sequence Tag Simple Sequence Repeat (EST-SSR)

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Abstract. *Mansyur, Karti PMH, Abdullah L, Husni A, Lestari P. 2019. Genetic diversity of mutant napiergrass using Expressed Sequence Tag Simple Sequence Repeat (EST-SSR). Biodiversitas 20: 2403-2409.* Napiergrass is one of the tropical grasses which has a very important role in developing ruminant livestock, its productivity is high and its nutritional content is quite good. Plant breeding to produce new varieties that have better productivity continues. One of them is through mutation breeding and in vitro culture. The purpose of this research was to look at the genetic diversity among napiergrasses using the Expressed Sequence Tag Simple Sequence Repeat (EST-SSR). This study used 14 SSR molecular markers. The results showed that mutant DNA of napiergrass can be clearly amplified by all the EST-SSR primers used. The average number of alleles was 4.57, the average frequency of the main allele was 42%, and the average value of gene diversity was 0.66. While the PIC average value was 0.60. There were five markers that were very informative and have PIC values above 0.7, among others, namely ICMP3045, ICMP3018, PSMP2090, PSMP2209, and PSMP2019. Phylogenetic analysis shows that 37 numbers of napiergrass mutants split into two main clusters at a coefficient of 0.56. The first cluster consists of 26 lines while the second cluster consists of 11 mutants. The parent napiergrass is in the first cluster. There are two pairs of mutants that have the same diversity, namely R20-11 with R 20-20-3 and R100-1 with R100-3.

Keywords: EST-SSR, forage, genetic diversity, napiergrass

INTRODUCTION

Napiergrass (*Pennisetum purpureum* Schum) is one of the grasses accepted by farmers in the tropical area, including in Indonesia as a very economical forage. This grass is used because it has high biomass production, has wide and wide adaptability, is easily breed and managed, and has a high risk of pests and diseases (Smith et al. 1993; Lowe et al. 2003; Bhandari et al. 2006; Sundaram et al. 2009; Struwig et al. 2009). Even in the latest developments this grass has been used for energy sources (Anderson et al. 2008a; Strezov et al. 2008; Jakob et al. 2009; Morais et al. 2009; Lee et al. 2010), and also as paper raw material (Zhou et al. 2007).

The wave of introduction of napiergrass to Indonesia has entered through two generations. First, the inclusion of Hawaiian and African cultivar grasses which focused on production, and then the inclusion of Taiwan and Mott cultivars that focused on production and quality, as a genetic modification result of forage crops. Existing forage crops cannot provide satisfaction to farmers who want to combine productivity, quality, and adaptation to the environment. Continued development efforts are focused on agronomic preformance and use by livestock (Anderson et al. 2008b).

Efforts to improve both through conventional and molecular breeding are developed continuously. Reproductive characteristics of each plant are observed to facilitate breeding. Techniques such as cell culture and tissue culture are good techniques for the selection and breeding of feed plants (1987), or even combined with mutation breeding. Cheng (1991) has reviewed the breeding process in tropical food plants, such as napiergrass and pangola grass. The development of phenotypic and genotypic appearance based forage crop with various methods has been and will continue to be developed (Humpreys 2005). Molecular breeding is an important method and will be used more in the future in the process of breeding feed plants, as well as for the development of other energy sources of plants as cellulose source for livestock (Yamada 2014). Among the putative content of breeding results will have diversity and different genetic information. To see that diversity, the fingerprint method can be used.

The DNA fingerprint terminology which was found previously (Jeffreys et al. 1985) is unique to each individual so that it can be used to identify specific individuals (Henry 2001). Many molecular markers using the PCR technique are utilized for DNA fingerprint analysis such as random amplified polymorphism DNA (RAPD) (Pan et al. 2003), sequence tagged microsatellite (STMS) and simple sequence repeat (SSR) (Pan et al. 2007). Variations of single nucleotide polymorphism (SNP) based sequencing have also been frequently used

because of the ease of application in high throughput (Silva and Bressiani 2005). Efficient fingerprinting has also been obtained using a sequencing platform target with high throughput (Monden et al. 2014). Simple Sequence Repeat (SSR) or commonly called microsatellite is one of molecular markers, which consists of repetition units 1-6 DNA base pairs with high variation (Gupta et al. 1996; Senior et al. 1998).

The use of SSR in forage crops is commonly used, as in napiergrass (Kawube et al. 2015; Wang et al. 2017; Kandel et al. 2015; Lopez et al. 2018, Wang et al. 2018), *Trifolium* (Malaviya et al. 2019), *Dactylis glomerata* (Hirata et al. 2011), *Opuntia* sp (Casas et al. 2017), Kentucky bluegrass (Yuan et al. 2018), and Alfalfa (Wang et al. 2013). The research is to measure the diversity from breeding results or from the results of mutation selection naturally due to geographical distribution. Based on the considerations above, it is necessary to conduct a study to see the level of genetic diversity among putative napiergrasses as a result of mutation breeding with their parents using SSR molecular markers.

MATERIALS AND METHODS

Plant and primary material

A total of 37 mutant lines were molecularly characterized in this study (Table 1). These mutant lines are mutants that were successfully acclimatized from previous research processes, namely mutation breeding and in vitro culture. Young and healthy leaves were collected to isolate genomic DNA. Molecular analysis was carried out based on 14 markers of expressed sequenced tagged-simple sequence repeat (EST-SSR) adopted from the public domain. The list of primers and sequences is shown in Table 2.

Genomic DNA extraction

DNA was extracted using the modified Doyle and Doyle (1990) method. A total of 0.5 grams of leaf were crushed with liquid nitrogen on the mortar until it was crushed into powder using pestle and transferred to a 2 ml eppendorf tube. Furthermore, 800 µl of extraction buffer was added (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% (w/v) CTAB (*cetyltrimethylammonium bromide*), 2% (w/v) PVP (*Polyvinylpyrrolidone*), and 0.38% (w/v) sodium disulfite) into the 2 ml eppendorf tube. The mixture was then incubated at 65°C for 15 minutes and homogenized by flipping the tubes every 5 minutes. Furthermore, the addition of 800 µl of chloroform solution: isoamyl alcohol (24: 1) into each sample followed by centrifugation at a speed of 12 000 rpm for 10 minutes at 20°C. The supernatant formed was transferred to the new Eppendorf tube. Next, 3M sodium acetate pH 5.2 was added as much as 1/10 of the volume of the supernatant followed by the addition of isopropanol to one volume of the supernatant. The mixture was slowly turned and then incubated at-20^0 C for one hour. After that centrifugation was carried out at a speed of 12 000 rpm for 10 minutes at 20°C. Then the supernatant was removed and the DNA pellet formed, washed with 500 µl of 70% ethanol solution. The pellets were then kept at room temperature for 5 minutes and then centrifuged for 5 minutes at a speed of 12,000 rpm at 20°C. The supernatant was discarded and the pellets were dried to remove the remaining ethanol. The dried pellets were dissolved in 100 µl TE solution (10 mM Tris pH 8.0 and 1 mM EDTA) plus RNAse A (10 mg /ml). Then the DNA solution stock was incubated at 37°C for 1 hour and stored at-20°C until ready for use.

Qualitative and quantitative tests of DNA

The elephant DNA solution stock was measured quantitatively and qualitatively. Quantitative tests were carried out using a nanodrop2000 spectrophotometer (Thermo Scientific, USA) while the qualitative test was carried out by electrophoresis on agarose gel with a concentration of 1% in a tank containing 1x TAE buffer, with a voltage of 90 volts for 30 minutes. The electrophoresis results were then observed under UV light in the *UV TransIlluminator* device.

Table 1. List of 37 mutant napiergrass lines used in this study

No.	Mutant line	No.	Mutant line	No.	Mutant line
1	R20-7	13	R20-10	25	R20-20-2
2	R100-9	14	R40-2	26	R20-1
3	R40-4	15	Parent	27	R20-4
4	R40-8	16	R20-5	28	R20-3
5	R40-7	17	R20-6	29	R20-2
6	R40-6	18	R40-5	30	R20-20-4
7	R100-6	19	R20-11	31	R0-1
8	R100-10	20	R20-8	32	R100-2
9	R0-2	21	R100-7	33	R100-5
10	R100-8	22	R20-9	34	R20-20-5
11	R40-3	23	R100-4	35	R100-1
12	R40-1	24	R20-20-3	36	R100-3
				37	R20-20-1

Table 2. List of 14 EST_SSR primers used in this study

Primer id	Sequence	SSR Motif
ICMP3020	F: GTTCCATGGAGCTGGAAGC	(CGTG)5
	R: GCTAGAACAGGGCCGTTACA	
ICMP3045	F: ACAAGGACGACAAGGACCAC	(AAG)5
	R: CCTCTCCAAGCACATGTTTC	(CAG)5
ICMP3066	F: GGCCCCAAGTAACTTCCCTA	(AG)7
	R: TGTCAGACACAGATGCCACA	
ICMP3068	F: CTGGCAAAGTTGTAGCGTGA	(GCT)5
	R: ATGTCGCTCTCTGCCAAGAT	
ICMP3017	F: CACCAAACAGCATCAAGCAG	(CAG)7
	R: AGGTAGCCGAGGAAGGTGAG	
ICMP3018	F: ACGAGGACAAGCTCTTGGAA	(CATG)4
	R: ACGGCGCATACTCGATCATA	
ICMP3021	F: GCCGACAGGAAGATTACGAT	(CGTG)5
	R: AGCAAAACGCAGAACAACAG	
ICMP3026	F: GTGAGGCCTCGAACAAACAC	(CTC)6
	R: GCCGACCAAGAACTTCATACA	
ICMP3080	F: CAAACAGCATCAAGCAGGAG	(AGC)8
	R: GCGTAGACGGCGTAGATGAT	
PSMP2090	F:AGCAGCCCAGTAATACCTCAGCTC	(CT)12
	R:AGCCCTAGCGCACAACACAAACTC	
PSMP2209	F: TTGGACGATTTGGAAGCATAG	(GT)6
	R: GAGGAAAAGAGCCATACAGAGAC	(CT)7
PSMP2210	F: CAATGATGACCGTAATCTGGGTG	(GT)12
	R: GGGCAAGATATGTGAAATCAAG	
PSMP2237	F: TGGCCTTGGCCTTTCCACGCTT	(GT)8

	R:CAATCAGTCCGTAGTCCACACCCCA	
PSMP2019	F: TGTGCCACAGCTTGTTCCTC	(CA)38
	R: CAAGCAGCCAGTTCCTCATC	

PCR amplification

DNA amplification by Polymerase Chain Reaction (PCR) was carried out with a total reaction of 10 µl consisting of 10 ng of 1µl template DNA; 20µl Kapa2G Fast ReadyMix (Kapa Biosystems, USA); Forward and Reverse primers as many as 2µl respectively, and sterile ddH_2O. The PCR reaction was carried out in the *T1 Thermocycler* PCR machine (Biometra, Germany) with PCR conditions as follows: initial denaturation was carried out at 95°C for 5 minutes, followed by 35 cycles of denaturation process at 94°C for 30 seconds, *annealing* (primary attachment stage) at temperature of 55°C for 1 minute, and elongation at a temperature of 72°C for 1 minute. The PCR reaction ends with a *final extension* cycle (the final stage of base extension) at a temperature of 60°C for 15 minutes. As a standard DNA band size, 100 bp DNA ladder was used. The PCR results were electrophoresed on 6% polyacrylamide gel in a tank containing TBE 1x buffer, with a voltage of 90 volts for 70 minutes. The electrophoresis results were immersed in a solution of *ethidium bromide* and the amplicon was visualized under UV light.

Data analysis

Data analysis was performed using a scoring method for DNA bands that appeared on 6% polyacrylamide gel electrophoresis. The ribbons seen in the visualization results were considered as one allele. DNA bands that have the same migration rate were considered as the same locus. At the same migration rate, each visible band is given a score of 1 while the invisible band is given a score of 0 so that the results of the ribbon scoring were binary data. To facilitate the determination of the position of the ribbon, the scoring activity was assisted by the *Gel Analyzer* software. Scoring results data were then analyzed using the *Equential Agglomerative Hierarchial and Nested* (SAHN)-UPGMA (*Unweighted Pair-Group Method with Arithmetic*) program on the NTSYS version 2.1 software. (Rohlf 2000). The results of the analysis were presented in the form of dendrograms. Furthermore the scoring data were also analyzed statistically using PowerMarker 3.25 software (Liu and Muse 2005) to determine the value of the main allele frequency, genetic diversity, and PIC (*Polymorphic Information Content*) produced by the primers used in this study. The similarity matrix was calculated to ensure genetic proximity between mutantts and controls.

RESULTS AND DISCUSSION

DNA of mutant napiergrass can be clearly amplified by all primary EST-SSRs, meaning that 14 primers can produce 100% polymorphic bands. An example of the total mutant banding pattern of electrophoresis on a 6% polyacrylamide gel is shown in Figure 1. The polymorphism of the DNA banding pattern was seen in 14 primers which were observed on the mutant napiergrass.

Genetic diversity can be detected from 37 mutant lines used in this study. As many as 64 alleles were detected based on 14 polymorphic markers. The average number of alleles was 4.57 alleles per mark with a range between 2-11 alleles per locus. The highest number of allele (11) was found with PSMP2090, and the smallest (2) was found in ICMP3066, ICMP3017, and PSMP2210 (Table 3). Markers that produce fewer alleles have a smaller ability to provide differences on the tested samples. The average number of alleles in this study was higher than that of Kandel et al. (2015) with an average of 3.74 pairs of alleles per marker and lower than the study of Azevado et al. (2012) with 10 pairs of alleles per marker.

The PIC (*Polymorphism Information Content*) value reflects the number of polymorphism that is produced. This study shows that the PIC values of 14 markers ranged from 0.28 to 0.79 with an average of 0.60. The lowest PIC value (0.28) was produced by ICMP3017 markers and the highest value (0.79) was generated by the PSMP2090 mark. Furthermore, from the 14 polymorphic markers, 10 markers showed the value PIC ≥0.5 and the rest had the value PIC<0.5 (Table 3). This shows that the ten EST-SSR markers were informative markers and were very useful for use in distinguishing the future napiergrass mutantts. The high PIC value produced shows the ability of these markers to differentiate between and within individuals in the population (Kawube et al. 2015). According to the opinion of DeWoody et al. (1995) which states that PIC≥0.5 molecular markers were efficient markers in discriminating genotypes and were very useful in detecting the level of polymorphism on these loci.

PIC values can vary from one marker to another marker, this can be caused by the influence of the marker itself and the clones used in the study (Elibariki, et al. 2013), and were determined by the frequency of appearance of allele (De Vicente and Fulton 2003). This level of polymerism is needed to select markers that can distinguish between the lines/parents used. Markers who have values above 0.7 indicate that the marker is informative (Hildebrand et al. 1992) and has a high ability to differentiate between clones or inhybrides (Legasse et al. 2007). In this study there were five markers which have values above 0.7, among others, namely ICMP3045, ICMP3018, PSMP2090, PSMP2209, and PSMP2019. Then there were three markers that have a value closer to 0.7, namely ICMP3020 (0.68), ICMP3068 (0.69) and ICMP3080 (0.69). However, if using the PIC value classification used according to Zhang et al. (2011), the value of PIC ≥0.5 was an informative marker, the ten markers used were very informative because they have values above 0.5 except for ICMP3017 markers (0.37), ICMP3026 (0.45), ICMP3017 (0.28), and PSMP2210 (0.37).

The average frequency of the main alleles produced was 42% with the lowest value of 30% in the PSMP2090 markers and the highest value of 78% in the ICMP3017 markers (Table 3). While Kawube et al. (2015) found the frequency of alleles on napiergrass as a collection of the International Livestock Research Institute, ILRI and

several countries in Africa in the range of 16-64% with an average of 44%.

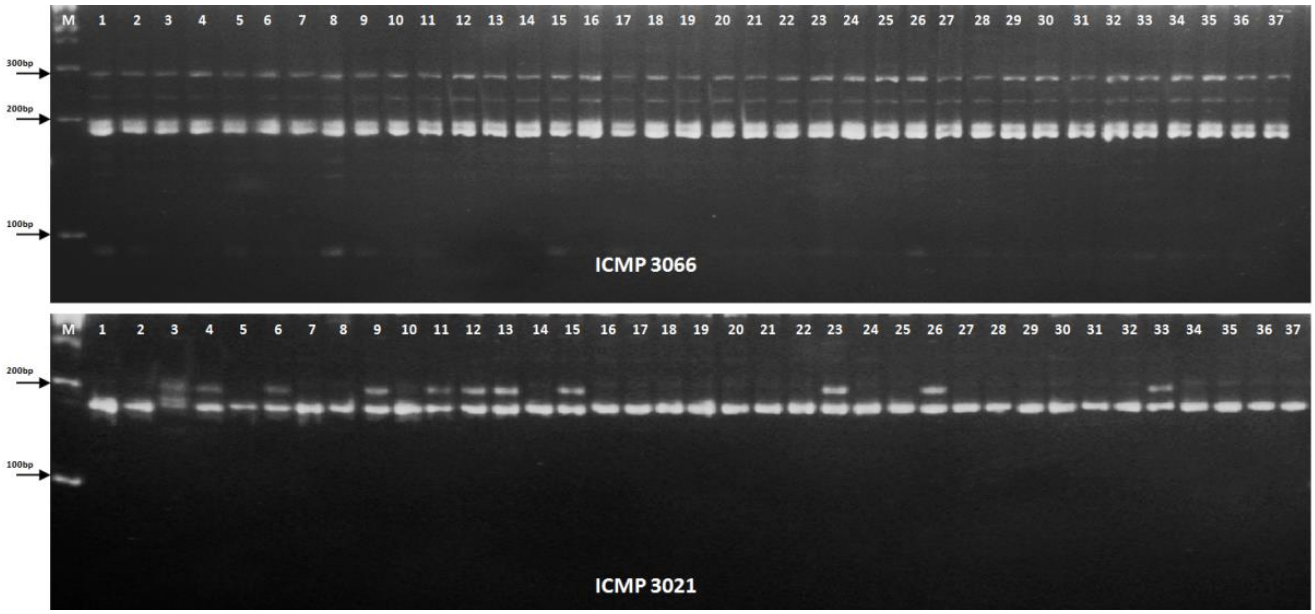

Figure 1. Examples of the ribbon pattern of PCR results from 6% polyacrylamide gel electrophoresis on 37 mutant lines using ICMP3066, and CMP3021 primers. Note: M: DNA ladder 100bp

Table 3. The number of alleles, the main allele frequency, gene diversity and the level of polymorphism (*polymorphism information content*, PIC) produced from 37 napiergrass mutants.

Marker	Number of alleles	Main allele frequency	Gene diversity	PIC
ICMP3020	4	0.35	0.73	0.68
ICMP3045	6	0.33	0.75	0.71
ICMP3066	2	0.51	0.50	0.37
ICMP3068	5	0.34	0.74	0.69
ICMP3017	2	0.78	0.34	0.28
ICMP3018	6	0.33	0.76	0.72
ICMP3021	6	0.51	0.621	0.56
ICMP3026	3	0.49	0.55	0.45
ICMP3080	4	0.32	0.74	0.69
PSMP2090	11	0.30	0.81	0.79
PSMP2209	5	0.38	0.75	0.71
PSMP2210	2	0.59	0.48	0.37
PSMP2237	3	0.35	0.66	0.59
PSMP2019	5	0.31	0.76	0.72
Total	64			
Average	4.57	0.42	0.66	0.60

To see genetic diversity by using DNA markers is a very appropriate method for feed plants (Harris et al. 2009; Xie et al. 2009). This genetic diversity is very important and is useful for determining the traits desired and maintaining them in the population, as well as to eliminate unwanted traits. To assess genetic diversity can be seen using the diversity value of gene (Lestari et al. 2016). The highest value of gene diversity was indicated by PSMP2090 marker which were 0.81, while the lowest gene diversity values was indicated by ICMP3017 marker which were 0.34. The average value of gene diversity in the total mutants were 0.66. The value of the genetic diversity of napiergrass resulted from mutation breeding and the studied tissue culture is quite high.

Phylogenetic analysis shows that 37 numbers of mutant napiergrass split into two main clusters at a coefficient of 0.56 (Figure 2). The first cluster consists of 26 lines while the second cluster consists of 11 mutants. The first cluster was divided into two subclusters, namely subcluster Ia and subcluster Ib. The parent line were grouped in the Ib subcluster along with mutants R100-9, R40-1, R100-8, and R20-5, which indicate their genetic proximities. This clustering demonstrated clearly that the original parent was closer to the non-irradiated first clone (R0-1) that another non-irrariated clone (R0-2). Less numberof mutant in cluster II compared to that in cluster I indicated the changes occured in nucleotide due to mutation. The differentiated mutants in the two clusters did not reflect the dose of gamma ray radiation. Based on the genetic similarity matrix (Table 4), there are some numbers that begin to be genetically separated from their parents with genetic similarity values of less than 50%, such as in mutants R100-3, R100-1, R20-20-5, R100-6, R100-2, R0-1, and R20-2. These mutants have the potential to be developed in the future while still considering the results of the analysis in the field so that this genetic diversity data can act as supporting data to select which cultivars can continue to be multiplied in the future. There were two pairs of mutants which have the highest genetic similarity, namely R20-11 with R 20-20-3 and R100-1 with R100-3 with a coefficient of genetic similarity of 0.93.

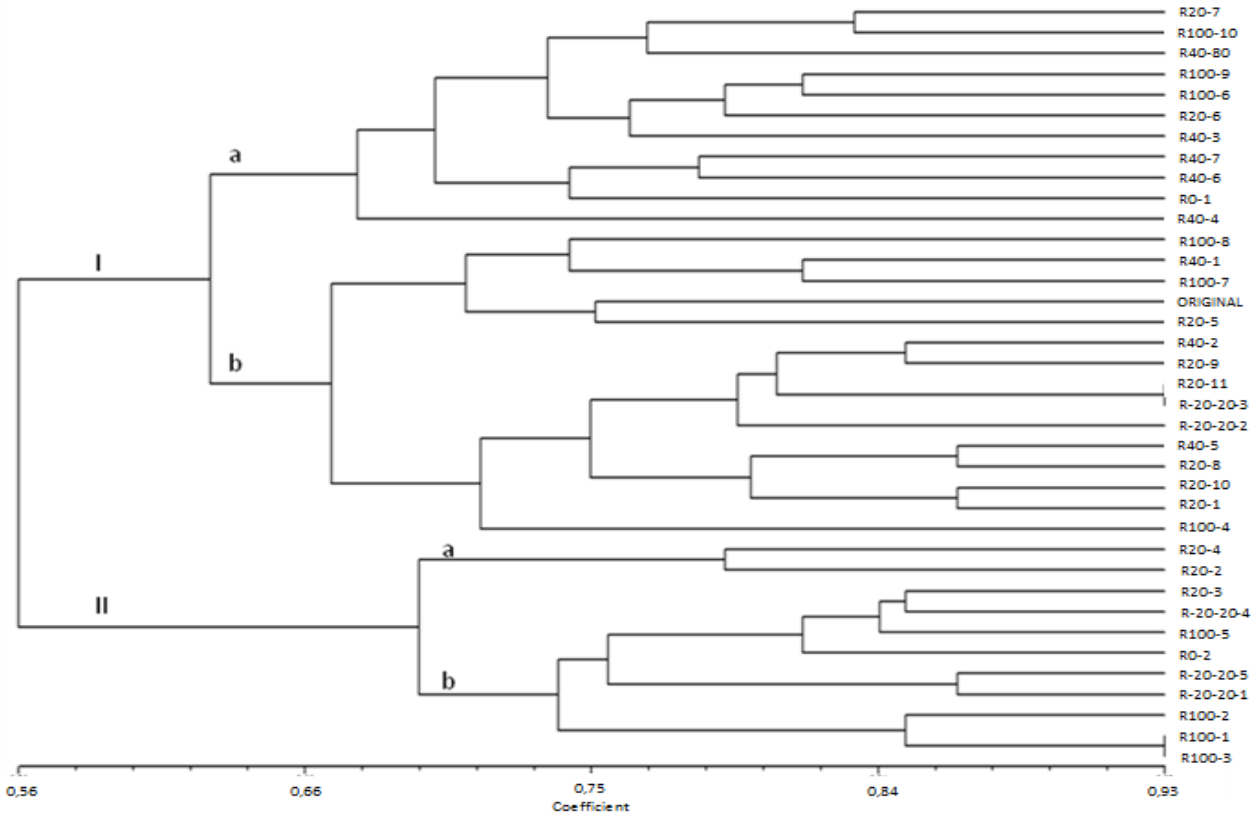

Figure 2. The dendogram of 37 napiergrass mutant lines based on polymorphism produced from 14 EST-SSR markers

Table 4. The genetic similarity matrix value of the 37 napiergrass mutantt lines

	R20-7	R100-10	R40-8	R100-5	R100-6	R20-6	R40-3	R40-7	R40-6	R0-1	R40-4	R100-8	R40-1	R100-7	Original	R20-2	R40-2	R20-9	R20-1	20-3	20-2	R40-5	R20-8	R20	R20-1	R100-4	R20-4	R20-2	R20-3	R20-20-4	R100-5	R0-2	R20-20-5	R20-20-1	R100-2	R100-4	R100-3
R20-7	1,00																																				
R100-10	0,73	1,00																																			
R40-8	0,78	0,75	1,00																																		
R100-5	0,73	0,67	0,68	1,00																																	
R100-6	0,65	0,68	0,70	0,75	1,00																																
R20-6	0,70	0,70	0,62	0,73	0,78	1,00																															
R40-3	0,82	0,82	0,73	0,78	0,77	0,75	1,00																														
R40-7	0,83	0,77	0,68	0,80	0,68	0,73	0,78	1,00																													
R40-6	0,73	0,63	0,58	0,67	0,72	0,77	0,65	0,80	1,00																												
R0-1	0,63	0,77	0,62	0,63	0,72	0,63	0,72	0,73	0,70	1,00																											
R40-4	0,70	0,73	0,62	0,73	0,75	0,77	0,82	0,80	0,67	0,73	1,00																										
R100-8	0,63	0,77	0,62	0,77	0,72	0,67	0,75	0,77	0,70	0,80	0,73	1,00																									
R40-1	0,62	0,72	0,67	0,72	0,73	0,62	0,67	0,72	0,72	0,68	0,62	0,82	1,00																								
R100-7	0,67	0,67	0,58	0,63	0,55	0,63	0,55	0,67	0,60	0,63	0,50	0,67	0,68	1,00																							
Original	0,65	0,78	0,63	0,58	0,60	0,58	0,67	0,68	0,62	0,72	0,68	0,75	0,70	0,68	1,00																						
R20-2	0,50	0,73	0,55	0,53	0,62	0,60	0,58	0,57	0,53	0,73	0,57	0,70	0,65	0,80	0,75	1,00																					
R40-2	0,70	0,80	0,58	0,63	0,65	0,67	0,78	0,70	0,53	0,70	0,73	0,70	0,58	0,73	0,75	0,77	1,00																				
R20-9	0,63	0,63	0,65	0,60	0,62	0,57	0,65	0,67	0,57	0,77	0,73	0,67	0,62	0,77	0,75	0,73	0,73	1,00																			
R20-1	0,60	0,63	0,55	0,53	0,52	0,50	0,55	0,60	0,53	0,67	0,57	0,57	0,65	0,80	0,72	0,70	0,67	0,77	1,00																		
20-3	0,63	0,57	0,58	0,60	0,58	0,53	0,58	0,67	0,63	0,77	0,63	0,63	0,65	0,77	0,68	0,67	0,60	0,87	0,90	1,00																	
20-2	0,70	0,57	0,58	0,63	0,58	0,60	0,65	0,70	0,63	0,63	0,63	0,60	0,58	0,73	0,68	0,63	0,63	0,80	0,77	0,87	1,00																
R40-5	0,75	0,68	0,53	0,65	0,50	0,62	0,63	0,75	0,58	0,62	0,62	0,62	0,57	0,85	0,73	0,68	0,82	0,72	0,82	0,72	0,75	1,00															
R20-8	0,60	0,60	0,55	0,57	0,55	0,63	0,55	0,67	0,63	0,73	0,57	0,63	0,68	0,73	0,72	0,63	0,63	0,67	0,70	0,73	0,70	0,72	1,00														
R20	0,63	0,70	0,62	0,50	0,55	0,53	0,55	0,60	0,57	0,63	0,53	0,53	0,62	0,80	0,75	0,70	0,67	0,73	0,93	0,83	0,77	0,82	0,70	1,00													
R20-1	0,60	0,73	0,68	0,57	0,55	0,57	0,62	0,67	0,53	0,67	0,60	0,63	0,65	0,80	0,75	0,77	0,77	0,77	0,80	0,70	0,67	0,78	0,70	0,80	1,00												
R100-4	0,60	0,57	0,55	0,53	0,48	0,60	0,55	0,57	0,53	0,57	0,57	0,57	0,62	0,73	0,72	0,63	0,57	0,73	0,77	0,80	0,87	0,68	0,77	0,77	0,67	1,00											
R20-4	0,53	0,63	0,55	0,40	0,55	0,47	0,55	0,47	0,43	0,67	0,60	0,53	0,58	0,60	0,58	0,63	0,67	0,70	0,73	0,63	0,57	0,62	0,57	0,70	0,67	0,63	1,00										
R20-2	0,73	0,67	0,52	0,50	0,55	0,60	0,62	0,60	0,60	0,57	0,60	0,47	0,52	0,57	0,55	0,50	0,63	0,50	0,67	0,57	0,63	0,72	0,57	0,70	0,53	0,63	0,80	1,00									
R20-3	0,63	0,63	0,60	0,46	0,48	0,46	0,52	0,58	0,54	0,54	0,42	0,46	0,69	0,63	0,48	0,50	0,50	0,50	0,75	0,63	0,58	0,60	0,63	0,71	0,67	0,67	0,79	0,79	1,00								
R20-20-4	0,58	0,62	0,47	0,55	0,53	0,58	0,57	0,58	0,58	0,55	0,55	0,58	0,63	0,58	0,50	0,55	0,55	0,45	0,65	0,55	0,55	0,63	0,58	0,62	0,52	0,58	0,75	0,85	0,81	1,00							
R100-5	0,67	0,53	0,52	0,63	0,62	0,70	0,55	0,60	0,63	0,57	0,57	0,53	0,58	0,60	0,45	0,50	0,53	0,50	0,53	0,57	0,60	0,62	0,60	0,53	0,40	0,60	0,67	0,83	0,63	0,82	1,00						
R0-2	0,67	0,57	0,52	0,63	0,68	0,67	0,68	0,67	0,70	0,67	0,67	0,63	0,62	0,50	0,42	0,47	0,57	0,53	0,57	0,60	0,60	0,55	0,57	0,50	0,47	0,53	0,70	0,77	0,75	0,82	0,77	1,00					
R20-20-5	0,63	0,60	0,48	0,57	0,62	0,63	0,55	0,60	0,63	0,57	0,53	0,57	0,62	0,60	0,48	0,53	0,57	0,37	0,57	0,47	0,50	0,65	0,63	0,57	0,50	0,53	0,67	0,83	0,75	0,85	0,80	0,80	1,00				
R20-20-1	0,57	0,57	0,48	0,53	0,48	0,57	0,55	0,57	0,47	0,50	0,53	0,50	0,48	0,50	0,48	0,47	0,57	0,43	0,60	0,50	0,57	0,65	0,53	0,57	0,50	0,57	0,70	0,80	0,71	0,85	0,77	0,70	0,73	1,00			
R100-2	0,63	0,57	0,48	0,60	0,62	0,63	0,62	0,67	0,73	0,70	0,57	0,67	0,62	0,53	0,45	0,53	0,53	0,50	0,50	0,60	0,63	0,52	0,60	0,47	0,43	0,57	0,60	0,70	0,67	0,75	0,77	0,87	0,73	0,70	1,00		
R100-4	0,60	0,50	0,52	0,63	0,65	0,67	0,58	0,60	0,67	0,63	0,53	0,60	0,55	0,50	0,38	0,47	0,50	0,47	0,47	0,57	0,60	0,48	0,57	0,43	0,40	0,53	0,57	0,67	0,63	0,72	0,80	0,83	0,70	0,73	0,93	1,00	
R100-3	0,63	0,63	0,55	0,53	0,58	0,60	0,58	0,60	0,50	0,57	0,53	0,50	0,48	0,50	0,52	0,50	0,63	0,40	0,50	0,43	0,50	0,58	0,57	0,50	0,50	0,50	0,63	0,73	0,67	0,72	0,70	0,67	0,73	0,87	0,73	0,77	1,00

Cluster analysis shows that there are variations between mutants and compared to their parents. Variation of diversity in this study is a result of tissue culture processes and the administration of irradiation in callus of napiergrass. This treatment has led to the genotypic diversity of napiergrass. Genetic variation can also occur in tissue culture processes, which are called somaclonal variations, namely variations that arise between individual new cells that are regenerated in the process of cell culture and tissue (Collin and Edward, 1998). The use of growth stimulants in plant cell cultures causes acceleration of the process of cell division to be accelerated and can have an impact on DNA replication errors. This somaclonal variation was found in soybeans (Widoretno et al. 2003), Rice (Lestari et al. 2010), and sugarcane (Suhesti 2015). The irradiation process on callus-shaped plant material can cause a permanent and inherited gene change (van Harten, 1998), because irradiation which can happen in the level of cell, genome, chromosome, and DNA can cause an increase in genetic diversity (Medina et al. 2005). The combination of in vitro selection and irradiation can cause a variety of mutants to be produced, this diversity can occur morphologically, biochemically, and molecularly.

To conclude, in this present study, major differnce were observed in SSR profiles of mutants with different doses of gamma ray follwed by in vitro culture. Some parameters including number of alleles, frequency of the main allele, gene diversity and the PIC value suggested the high variation of these mutants compared to the parent and R0-1 and R0-2, and among mutants. Five SSR markers proved their very informative markers as indicated by the PIC values above 0.7 (ICMP3045, ICMP3018, PSMP2090, PSMP2209, and PSMP2019). Phylogenetic tree was in relevant with the gene diversity and generated the mutant into two main clusters, suggesting their differentiation among them. The gamma ray exposure changed the molecular level of mutants in comparision to the parent, hence, it can be adopted in mutation breeding of .napiergrass. these total mutants need futhers assay on nutrition and agronomical traits that might useful as potential candidate of new varieties of forage.

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