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Microsatellite analysis of selected *Lablab purpureus* genotypes in Kenya.

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Abstract

*Lablab **(Lablab Purpureus**) is a legume of high nutritional value and broad social acceptance ranging from human food to soil fertility improvement to foarge. Information on genetic worth and diversity of lablab genotypes in Kenya is scanty and this is a great impediment to the improvement of the crop hence its utilization. This research was conducted to determine genetic diversity in lablab germplasm in Kenya using SSR primers. Thirteen genotypes comprising of collections from farmers and gene bank were planted in individual lines in the green house at Chepkoilel University College. CTAB DNA extraction from two weeks old leaves was done followed by PCR amplification using twenty one primer sets from common beans **(Phaseolus vulgaris)** and products separated on agarose gel. Four primers showed diversity in lablab, mapping a total of 14 amplicons with an average of 3.5 bands per primer. Clustering by UPGMA and PCoA showed similarity between genotypes grown by farmers and gene bank accessions. Genetic distance computed using Popgene ranged between 0.000 and 0.620, suggesting narrow variability among the materials. The genetic base of cultivated lablab is relatively narrow and needs to be expanded.*

Key words: *Lablab **(Lablab purpureus),** microsatellites (SSR), genetic diversity.*

INTRODUCTION

Lablab is a food legume with wide usage. Diversity of genotypes grown by farmers in Kenya has not been studied and this is a major hindrance to improvement of the crop in Kenya. The germplasm resource in the country has about 340 accessions at Muguga gene bank (Adebisi and Bosch, 2004) and this untapped gene pool can be exploited in improvement programmes of the crop. Due to advances in molecular biology techniques, large numbers of highly informative DNA markers have been developed for identification of genetic polymorphism (Fevzi, 2001). Assessment of genetic diversity based on

phenotype has limitations, since most morphological characters are greatly influenced by environmental factors and developmental stage of the plant (Konstantinos *et al*., 2008). In contrast, molecular markers based on DNA sequences, are independent of environmental conditions and show a higher level of polymorphism (Rai *et al*., 2010). This necessitates assessment of genetic diversity in selected lablab genotypes using the modern molecular approaches.

Molecular markers, combine many desirable marker properties including, high level of polymorphism and information content, un-ambiguous designation of alleles, even dispersal, selective neutrality, high reproducibility, codominance, rapid and simple genotyping assays (Grisi *et al*., 2007; Cheng-Dao *et al*., 2001). To date, no SSR markers for *Lablab purpureus* have been reported. However, the vast amount of markers available from related legume species can be applied in lablab studies considering the fact that mapping within the legume species provide evidence that these markers can be used in neglected species such as *Lablab purpureus* (Venkatesha *et al*., 2007; Zhu *et al.,* 2005).

Assessment of genetic variation in available germplasm collections forms an integral part of any crop improvement programme (Somta *et al*., 2009; Gnanesh *et al*., 2006). However, information on genetic diversity of lablab bean is very scanty. The bean has been neglected in research and development and consequently our understanding of its diversity is limited (Tefera *et al.,* 2008). According to Maass *et al*. (2010), the available diversity of lablab in Africa might be under threat of genetic erosion due to limited research and development. This study was carried out to determine the extent of genetic diversity among lablab genotypes grown in Kenya. The findings from the study would aid in identification and selection of suitable germplasm for improvement of the crop.

Materials and methods

Thirteen genotypes comprising of six collections from farmers and seven accessions from gene bank (Muguga-Kenya) were selected based on yield, maturity time, resistance to pests and diseases among other agromorphic features. The selections were genotyped for diversity using 21 SSR primers sets for *Phaseolus vulgaris* obtained from Invitrogen Company (Table 1).

Table 1. List of primers and their sequences used in the genotyping of lablab genotypes under study.

PRIMER NAME	FORWARD	REVERSE
BM143	GGGAAATGAACAGAGGAAA	ATGTTGGGAACTTTTAGTGTG
PV-atgc001	TGCCACCACAGCTTTCTCCTC	TATGAGAGAAGCGGTTGGCACG
PV-cct001	GAGGGTGTTTCACTATTGTCACTGC	TTCATGGATGGTGGAGGAACAG
VA-ag001	GGGTAGTAAAGGAAAGAGAAGAAAGAG	CCACCTTCTGTACTCGTTCCATG
PV-gaat002	AAACACACAAAAAGTTGGACGCAC	TTCGTGAGGTAGGAGTTTGGTGG
PV-ag004	TTGATGACGTGGATGCATTGC	AAAGGGCTAGGGAGAGTAAGTTGG
PV-at006	CCGTTGCCTGTATTTCCCCAT	CGTGTGAAGTCATCTGGAGTGGTC
BMd-1	CAAATCGCAACACCTCACAA	GTCGGAGCCATCATCTGTTT
BMd-12	CATCAACAAGGACAGCCTCA	GCAGCTGGCGGGTAAAACAG
BMd-20	GTTGCCACCGGTGATAATCT	GTGAGGCAAGAAGCCTTCAA
BMd-22	GGTCACTTCCGGAGCATTC	CGGGAAATGGAAGTCACAGT
BMd-44	GGCAGCTTACTAACCCGAAA	TTCCTTCCCCTTTCTTCTCC
BMd-45	GGTTGGGAAGCCTCATACAG	ATCTTCGACCCACCTTGCT
BMd-53	TGCTGACCAAGGAAATTCAG	GGAGGAGGCTTAAGCACAAA
BMd-46	GGCTGACAACAACTCTGCAC	CTGGCATAGGTTGCTCCTTC
GATS54	GAACCTGCAAAGCAAAGAGC	TCACTCTCCAACCAGATCGAA
GATS11	CACATTGGTGCTAGTGTCGG	GAACCTGCAAAGCAAAGAGC
BM114	AGCCTGGTGAAATGCTCATAG	CATGCTTGTTGCCTAACTCTCT
BM139	TTAGCAATACCGCCATGAGAG	ACTGTAGCTCAAACAGGGCAC
BM141	TGAGGAGGAACAATGGTGGC	CTCACAAACCACAACGCACC
PV-ag001	CAATCCTCTCTCTCTCATTTCCAATC	GACCTTGAAGTCGGTGTCGTTT

DNA Extraction

Three seeds per genotype were singly planted in a pot in the green house at Chepkoilel University College, Moi University. Genomic DNA was extracted using the CTAB procedure described by Ellis *et al*., (2005), with some modifications. Young leaves from two-week old plants were ground into fine powder under liquid nitrogen using a pestle and motor. The extracts were transferred into labeled eppendorf tubes each containing 500 µl of 2 x CTAB/ mercaptoethanol extraction buffer and samples placed on ice. The contents were subsequently incubated in a water bath at 65 ° C for 1 hour with inversions after every fifteen minutes. This was followed by addition of 500 µL of chloroform-isoamyl alcohol (24:1) and inversion for 5 minutes at room

temperature. The mixture was then centrifuged at 14000 rpm for 10 minutes. Without disturbing the bottom layer, 400 µL of top clear layer was pipetted into fresh eppendorf tubes and 250 µL of isopropanol added. The contents were gently mixed by a few inversions and then incubated at room temperature for 10 minutes.

The mixture was centrifuged at 14000 rpm for 10 minutes to pellet the DNA and supernatant discarded gently using yellow tips before adding 320 µL of 1 x TE. The samples were then placed on ice. A further 40 µL of magnesium chloride was added and the contents incubated on ice for 10 minutes followed by centrifugation at 14000 rpm for 10 minutes and the supernatant discarded. The pellet was vacuum-dried for 5 minutes before adding 5 µL of R-nase enzyme and placing in a water bath (37 ° C) for two hours. Then 40 µL of sodium acetate was added followed by 250 µL of isopropanol and the contents incubated for 15 minutes at room temperature. This was followed by a ten minutes centrifuge of 14000 rpm so as to re-pellet the DNA and the supernatant discarded.

A, 1.0 ml aliquot of 70 % ethanol was then added to wash the pellet followed by another centrifuge of 14000 rpm for 5 minutes. The supernatant was discarded followed by a quick spin of 10000 rpm for 2 minutes. The pellet was then vacuum-dried for 3 minutes to remove any remaining liquid and the DNA pellet re-suspended in 50 µL of 1 x TE. It was left to stand for 10 minutes at room temperature before storing at 4° C.

DNA Concentration and Quality Assessment

The quality and quantity of DNA extracted was determined by comparing against known standards of lambda (λ) DNA on 1% agarose gel in 1 x TBE buffer (44.5mM Tris HCL pH 8.3, 44.5 mM boric acid, 1.25mM EDTA). Seventy grams of agarose were added to 70 ml of TBE in a conical flask. The contents were heated in a microwave for 1½ minutes and cooled gently before being cast in a gel electrophoresis tank which had a comb. It was left to set for about ten minutes. The comb was then removed from the casting tray and 1 x TBE buffer poured to cover the fill line mark.

DNA samples were prepared by mixing 5 µL of DNA extract with 1 µL of loading dye in labeled PCR tubes and the contents homogenized by a gentle tap from which a 5 µL aliquot taken for loading on the gel. The samples were then

loaded alongside standards (3 µL, 5 µL and 10 µL) of lambda (λ) DNA and run at 70 V for 45 minutes. This was followed by staining with ethidium bromide for 30 minutes and a brief rinse of twenty minutes in distilled water. Bands were visualized under UV light and photographed (Figure 1). Concentration of DNA from each sample was assessed against the standards. The DNA extracts were stored at –20 ° C throughout the entire study period.

Amplification by Polymerase Chain Reaction (PCR)

Twenty one microsatellite primer pairs from common beans (*Phaseolus vulgaris*) were used to investigate genetic diversity in the 13 genotypes. PCR amplification was conducted in PCR premix tubes that had dNTP's, KCl, MgCl, taq polymerase and dye. Quantities for each PCR reaction are shown in (Table 2). Total PCR volume was 20 µL.

Table 2. PCR components and their volumes

Components	x µL	Master Mix x 14 µL
0.5 µL forward primer (0.25 µM)	0.5	7.0
0.5 µL reverse primer (0.25 µM	0.5	7.0
PCR premix (5 µL)	5.0	70.0
Molecular water	11.5	161.0
Genomic DNA (10 ng/ µL)	2.5	(245=14x17.5 µL)
Total PCR volume	20.0	

PCR amplification was conducted in a 96-well plate thermocyler according to Blair *et al*., (2006), with some modifications using a programme as outlined in (Table 3).

Table 3. PCR primer amplification regimes

Step	Temperature	Time
1 Initial denaturation	92 º C	3 min
2 Denaturing	92 º C	30 sec
3Annealing	*	30 sec
4 Extension	72 º C	45 sec
5 Cycling	repeat step 2- 4 for	34 cycles
6Final extension	72 º C	5 min
7Hold	4º C	

* Annealing temperature for each primer

Amplification products were separated on 2 % agarose gel in 1 x TBE buffer for 135 minutes according to allele sizes based on 1kb ladder and visualized by staining with ethidium bromide. Band sizing was done by comparing them to standard ladder that was loaded on every gel. Allele size and intensity were considered during the scoring to distinguish homozygotes from heterozygotes.

Data Analysis

SSR band data was scored as [AA, BB (homozygote) and AB (heterozygote)] matrix and resulting data analyzed using the Popgene programme version 1.31. Percentage polymorphism was calculated as a proportion of polymorphic bands over total number of bands. Relationship among genotypes was estimated based on Nei (1978) genetic distance using UPGMA clustering method. The UPGMA tree was constructed using the same software. The analysis was repeated twice to verify the results and check on reproducibility of allele scoring. Only reproducible alleles were scored. Principal coordinate analysis (PCoA) was constructed using GenAlex 6.3 version based on Nei (1978) genetic distances.

RESULTS

DNA quantification

Line (L1) contains 5 µL of lambda (λ) DNA (10 ng /µL), while lanes S1 to S9 contain genomic DNA from some genotypes (Figure 1). DNA yield from the extracts was obtained by comparing band size and intensity to that of lambda DNA, whose concentration was 10 ng/µL. The yields ranged from 25 to 50 nanograms.

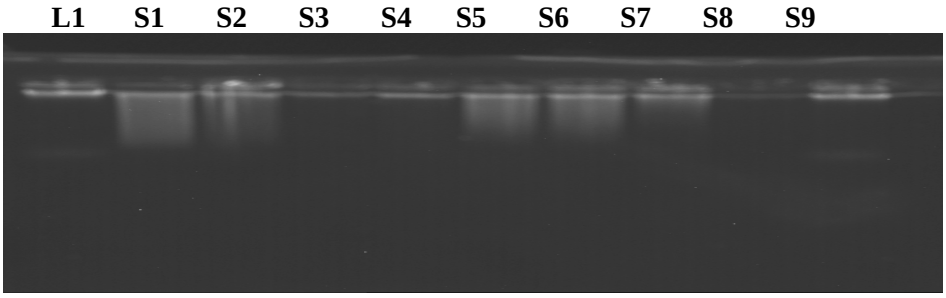

Figure 1. DNA quantification on 1% agarose gel

Microsatellite (SSR) Analysis

Twenty one primer sets were used to amplify DNA extracts from the 13 genotypes. There was 76 % amplification success with 16 out of the 21 primer sets tested amplifying. A total of 122 bands were scored ranging from 100 bp to 1400 bp. Figure 2 shows a PCR product run on 2 % agarose where L is 1 kb ladder while lanes S1 to S6 are amplification products from some genotypes. Four primer sets showed diversity. The total number of bands detected for each polymorphic primer ranged from 24 (PV-Atgc001) to 48 (PV-Ag004), with an average of 30.5 bands per primer. Excluding monomorphic bands, 14 polymorphic bands were scored for analysis, with an average of 3.5 bands per primer (Table 4).

The polymorphic primers enabled differentiation of the genotypes into two main branches using UPGMA clustering method based on Nei's (1978) genetic distance (Figure 3). The first branch had only one genotype 12158, a gene bank accession. The other 12 genotypes were found in the second branch which had four sub clusters. In the first sub cluster, Mbeere 1 and Mbeere 2 had the least distance between them suggesting that the two genotypes had a common source and were similar morphologically and genetically.

Genetic distance based on Nei's (1978) suggestion, ranged from 0.000 to 0.620. However, most genotypes had genetic distances of less than 0.400 implying that gene bank materials and those grown by farmers were similar. Like the UPGMA tree, the principal coordinate analysis (PCoA) showed similarity among the genotypes. Those from farmers scattered on coordinate one of the plot while those from gene bank scattered on the upper part (Figure

4). From the scatter plot, accession 11723 from gene bank clustered together with farmers collections. A similar scenario was observed in the UPMA tree.

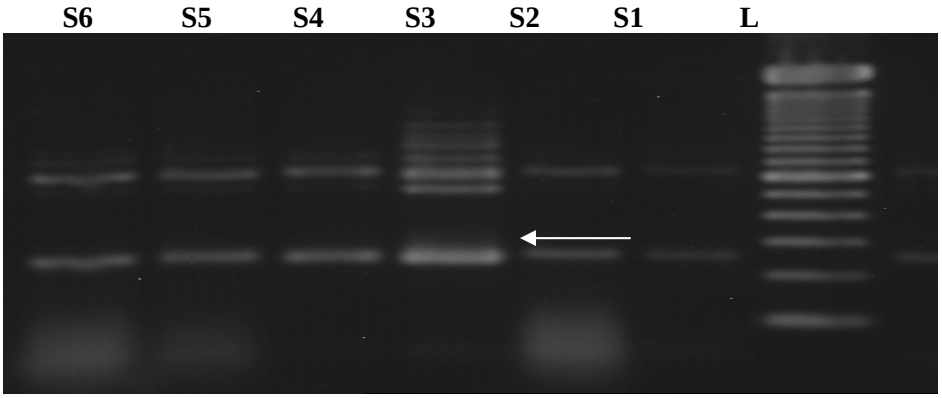

Figure 2. PCR amplification products as visualized on 2% agarose gel

Table 4. Number of polymorphic bands scored per polymorphic primer

Primer code	No.of amplified products	No of polymorphic bands	Proportion of polymorphic bands
PV-Ag004	48	4	0.083
PV-At006	32	5	0.156
PV-Atgc001	24	3	0.125
BMd- 46	18	2	0.111
Total	122	14	0.115

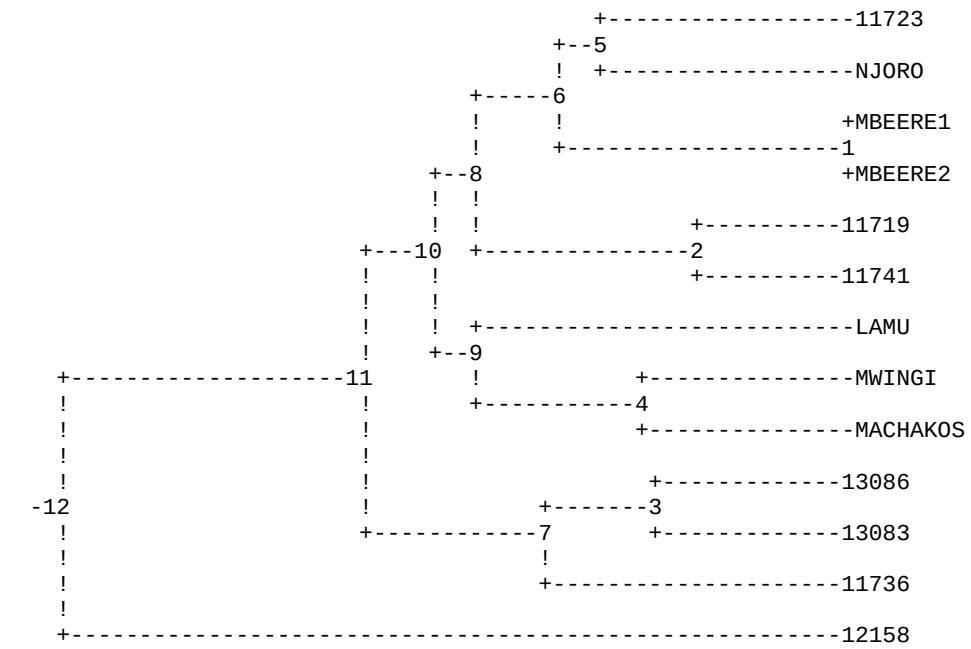

Figure 3. Cluster analysis of 13 Lablab genotypes by UPGMA grouped based on Nei's (1978) genetic distance as estimated by 4 SSR loci.

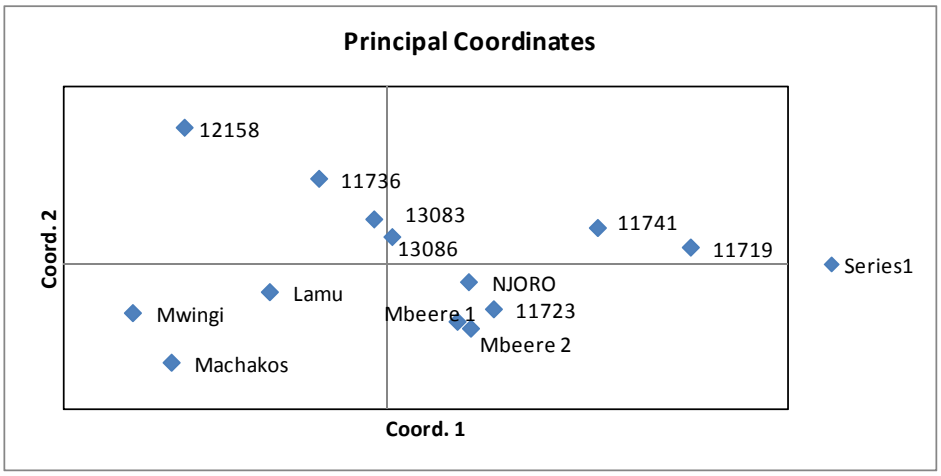

Figure 4. Principal Coordinate Analysi for 13 lablab genotypes as computed using GenAlex

DISCUSSION

Germplasm characterization is an important component in any breeding program for effective and efficient management/utilization of plant genetic resources. Genetic markers are preferable to morphological markers because they correlate variability directly at genetic level providing reliable and enormous data that permit a reproducible estimate of genetic diversity in germplasm (Rai *et al*., 2010). The study thus addressed utility of SSR markers to provide insight into genetic diversity of lablab. Four out of sixteen primer sets showed diversity, generating fourteen polymorphic amplicons with a mean of 3.5 alleles per primer. This was a relatively fair amplification given that the primers had not been pre-tested. In comparable lablab studies using RAPDs and SRRs, Rai *et al*., (2010) and Wang *et al*., (2007), respectively calculated mean amplifications of 5.69 and 3.6 alleles per primer. Similar microsatellites studies in *Phaseolus vulgaris* reported mean amplifications of over 7.0 alleles per primer (Jose *at al.*, 2009; and Maras *et al*., 2008) and 12.2 alleles per primer in soybean (Wang *et al*., 2006). The low amplification of this study could be attributed to use of primers that were not specifically designed from lablab species, therefore primer sequence did not coincide with axon/intron regions from the genome (Venkatesha *et al*., 2007).

Two alleles at a single locus were observed is some genotypes. Such heterozygosity is likely to have come from natural cross pollination (6-10 %) that occurs in lablab (Gnanesh *et al*., 2006). Wang *et al*. (2006) made a similar observation in soybean using SSR primers. However, observed heterozygosity (0.11 %) was very low in all genotypes reflecting inbreeding index characteristic of autogamous species such as lablab. Cardoso *et al*., (2009), also found low heterozygosity (0.16 %) in common bean (*Phaseolus vulgaris*) using microsatellites and attributed it to cross pollination.

The lablab genotypes in this study were genetically similar and this may be partly due to duplication of genotypes among farmers while accessions obtained from the gene bank could be cultivated forms sourced from farmers. Another possibility is that these genotypes were mainly composed of cultivated forms that tend to have low levels of genetic diversity. As a result, the computed genetic distance showed low variability. Most genotypes were in the second cluster, an indicator of similarity among genotypes from the two sources, resulting in low genetic distances (0.000 to 0.620). The PCoA scatter plot also had the genotypes clustering close to each other an indicator of genetic similarity. However, Rai *et al*., (2010) using RAPDs and Maass *et al*., (2005) using AFLP obtained fairly large genetic distances of 0.38 to 0.96 and 0.217 to 0.915 while evaluating lablab germplasm after inclusion of wild relatives for their studies.

A major component of diversity in this study is the differentiation of genotype 12158 from the bulk of the materials. This could be an introduced cultivar from other regions and may carry valuable genes for further improvement of the crop. From the dendogram and scatter plot, farmers' genotypes clustered together except one sub cluster, where gene bank accession 11723 was grouped together with farmers' materials. This accession could have been introduced to the gene bank from farmers at time of collection of these materials. On the other hand, farmers' genotypes could have undergone evolutionary changes in response to selection pressures to suit particular environments (Maass and Usongo, 2007).

We expected to find more genetic variability among gene bank accessions on the basis of it being a conservation centre. Our results suggest that, there is more similarity between cultivated and gene bank materials. Thus, the genetic diversity of cultivated lablab in Kenya is relatively low (Wang *et al*., 2007) for

SSR's, Liu, (1996) for RAPD's, Maass *et al*., (2005) for AFLP and Pengelly and Maass, (2001) for morphological characters and needs to be expanded.

CONCLUSION AND RECOMMENDATION

This study showed that genetic variability of cultivated *Lablab purpureus* is low and needs to be expanded. This could be achieved by incorporating accession 12158 from the gene bank or through new introductions, especially wild genotypes, to broaden the genetic base. Future studies should aim at developing primers for the crop as it has lagged behind in primer library. Results from this study will aid selection and efficient utilization of germplasm in lablab breeding and improvement programmes.

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