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Phenotypic Evaluation of Traits and Barcode Identification using Plastid-specific Ribulose Bisphosphate Carboxylase (Rbcl) Analysis on Lablab Beans [*Lablab purpureus* (L.) Sweet]

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ABSTRACT

Background: The importance of grain legumes in world food sustainability cannot be overemphasized. Minor legumes on the other hand, contribute to agricultural strengthening, especially in the context of sustainable crop and livestock production systems. In the context of assessing different legume species for crop improvement and identification, twenty (20) accessions of *Lablab purpureus* (L.) Sweet collected to determine the degree of trait variability and to identify accessions with potentials, which can contribute to grain and forage production.

Methods: The present study involved the evaluation of twenty (20) accessions of *Lablab purpureus* (L.) Sweet from IITA, Ibadan. The accessions were grown in the experimental plot and DNA from young and healthy leaves were isolated for determination of genetic diversity using ribulose bisphosphate carboxylase (rbcL). The accessions were also categorized into groups based on the performances and the highest discriminating traits that accounted for greater variability using principal component analysis (PCA) and cluster analysis (CA) respectively.

Result: Cluster analysis grouped all the twenty accessions into three clusters, where cluster I, cluster II and cluster III comprised of eight, seven and five accessions respectively. The PCA revealed hundred seed-weights, number of pod per plant and seed length as the most discriminating trait that accounted for greater variability in the Lablab accessions considered and they should be considered in the hyacinth bean improvement programs. Based on diverse level of phenotypic traits, accessions from each cluster were selected for barcode identification and comparison with other cultivars from other region. Results of the molecular analysis showed two clusters and authenticated the use of universal primers, ribulose bisphosphate carboxylase (rbcL) for DNA barcoding for successful amplification, identification and discrimination of *Lablab purpureus* (L). This revealed diversity of the considered accessions and provided information on relatedness to other accessions from other region of the world.

Key word: Cluster analysis, DNA barcoding, Hyacinth bean, Ribulose bisphosphate carboxylase (rbcL).

INTRODUCTION

Legumes are plants belonging to the family Fabaceae and are known to produce seeds within a pod (Agbolade *et al.,* 2019). On the basis of usefulness and economy of plant, legumes are divided into major and minor species (Assogba *et al.,* 2015). Major legumes are well-liked and widespread with well-established domestication and cultivation, agronomic practices, utilization and conservation (Popoola *et al*., 2019). Minor legumes on the other hand are underutilized species commonly identified as a result of their local worth, poor breeding, variation together with general withdrawal by established agricultural researchers, extension services, donors, technology providers, plant breeders, policy and decision makers, as well as consumers (Singh *et al.,* 2018). Nevertheless, minor grain legumes have diverse potentials and promising as the conventional staple crops. Minor or orphan legumes are often linked to the cultural heritage of their places of origin, well adapted to diverse agro-ecological conditions with wide range resilience

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to different stresses. (Narasimhulu *et al.,* 2018). Lablab (*Lablab purpureus)* is one of the minor legumes or orphan legume crops; also known as the hyacinth bean in English speaking and Dolich in French speaking countries. It is an African species, commonly flourishes in wet tropical regions than in dry regions like Sahel, regardless of its consideration to be more drought tolerant plant than cowpea (Patricia *et al*., 2019). Success of any a breeding programme and crop improvement of specific trait through selection in particular, solely rest on the available genetic variability in the available germplasm of a particular crop (Pitil *et al.,* 2017, Patricia *et al*., 2019).). This study was mainly focused on investigating genetic diversity among twenty *Lablab purpureus* accessions and assessment of morphological and molecular studies using vegetative characters, sequence analysis, calculation of genetic distance, molecular weight of bases, amplified polymorphism, barcode development of multiple sequence alignment, use of a neighbor-joining algorithm and tree construction analysis. This result will help to develop an accurate species identification method for the selected five *L. purpureus* genotypes with the desired traits through developing potential DNA barcodes to support conservation efforts of the genus.

MATERIALS AND METHODS

Sample site

Twenty genotypes of selected Lablab beans [*Lablab purpureus* (L.) Sweet] were collected from International Institute of Tropical Agriculture (IITA), Ibadan and experiment was carried out at Teaching and Research Farm of Federal University, Oye-Ekiti, Ekiti State, Nigeria and at the Bioscience Research Laboratory, IITA, Ibadan, Nigeria; during 2020/2021 academic session/year. The genotypes were evaluated in an Alpha lattice design with two replications using R package "Agricolae" for the randomization. Sowing, spacing, field maintenance and data gathering was in accordance with Popoola *et al*., (2019). Some accessions were selected for the plastid-specific ribulose biphosphate carboxylase (rbcL) study, based on morphological cluster analysis.

Genomic DNA isolation

Total genomic DNA was isolated using a modified CTAB protocol according to Fatima *et al*., (2018b).

DNA quantification

This was carried out in accordance with Jiao *et al*., (2013).

PCR amplification through universal barcode primers (rbcL)

PCR amplification through universal barcode primers (rbcL) was in line with the procedure of CBOL Plant Working Group (2009).

Nucleotide sequencing

The nucleotide sequencing was according to Fatima *et al.,* (2018b).

Statistical analysis

The data collected from the experimental field were subjected to analyses using the R software version 4.2 (R Core Team, 2019). Mean average for all characters were computed for Analysis of variance (ANOVA) to detect the variability present among the twenty-five genotypes of *Lablab purpureus*. Pearson's correlation test was conducted to detect the positive and negative correlation among the characters observed. The variables generated were then subjected to principal component analysis (PCA) which revealed the variables that contributed mainly to the variation observed among accessions used in this study. This was generated using packages such as 'FactoMineR', ggplot2 and FactoExtra to group accessions into different categories. Hierarchical clustering was adopted for the grouping based on the performance of the accessions on the field and Student Newman Keuls (SNK) was adopted for the mean separation of traits across the clusters formed.

Barcodes and phylogenetic analysis

This was in accordance with Hollingsworth *et al*., (2011).

RESULTS AND DISCUSSION

Variability of traits across hyacinths beans accessions

Analysis of variance of variable observed among accessions of *Lablab purpureus* exhibited minor significant differences ($P<0.05$) for different vegetative and yield-related traits. It was found that five (5) of the 16 observed variables showed degree range of variation across the twenty accessions studied (Table 1). The table below showed that stem girth, days to flowering and one hundred seed-weight showed significant variability between the twenty accessions of hyacinth bean ($P<0.05$) Table 1.

Other variables studied like plant height, petiole length, stem girt, number of pods per plants, pod weight and pod length showed no variation across the accessions of hyacinth beans studied (Table 1). Analysis of variance results revealed 20% of variables observed contributed to the diversity across the accessions of lablab in this study. The descriptive statistics of variables observed among *Lablab purpureus* accessions revealed the mean value, standard deviation, minimum and maximum values for all traits.

Correlation matrix between the studied variables of hyacinth bean revealed significant association between many traits (Fig 1). High significant and positive correlation was observed between leaf girth and leaf length. Leaf girth also showed positive and significant correlation with stem girth only. Relationship with other variables were not significantly correlated (Fig 1). Plant height showed no significant correlation with any of the observed variables. Moderate positive correlation was observed between seed weight and 100-seed weight, number of seeds per pod with pod length and pod width. Pod length exhibited positive and significant correlation with hundred-seed weight. This correlation between pod length and hundred-seed weight can be utilized during selection for improvement of yield

traits in *Lablab purpureus*. However, no significant correlation was observed for petiole length with all evaluated variables (Fig 1).

Principal component analysis

The first four Principal Components with Eigen values >1 explained 69% of variation among the accessions of *Lablab purpureus* evaluated in this study. The first PC contributed 26.3% of the variation (Fig 2 and Table 2). PC2 accounted for 17.5%, 14.49% for PC3 and 10.35 for PC 4. The significant variables in each PC was determined using >±0.5 significant correlation (Akohoue *et al.,* 2019). Days to 50% flowering, number of pod per plant, pod length, hundred-seed weight and seed length showed high contribution to first principal component (Table 2). Petiole length, pod length, pod width and Number of pod per plant (NPP) contributed most to PC 2 as in Table 2.

Significant negative correlation was also observed in principal component 1 where leaf girth exhibited high degree of negative contribution. Principal component 2 also showed negative contribution of leaf girth. Principal component revealed the most significant positive correlation for days of germination and leaf girth (Table 2). Negative correlation was also observed for Days to 50% flowering.

Phylogenic pattern

Fig 2 (b) also revealed the phylogenic root of *Lablab purpureus* accessions in this study. This shows discriminating arrangement when morphometric traits are adopted for the grouping. The accessions in this study were grouped into three phylogenic clusters based on the linkage, each cluster containing accessions that are highly similar in terms of the morphological attributes they possess. Cluster 1 consisted of accessions with the accession codes- TLn37, TLn39, TLn43, TLn19, TLn45, TLn36, TLn48 and TLn44; which are further separated into two sub groups. Cluster 2 contains TLn59, TLn 57, TLn6, TLn55, TLn13 TLn57 and TLn70; which are also separated into two subgroups as it happened in cluster 1. Finally, the cluster 3 also consists of TLn12, TLn5, TLn7, TLn49 and TLn46. Accessions in cluster 3 are also further grouped based on the level of similarities between their descriptive variables (Fig 2).

Accessions cluster mean values of the studied traits revealed considerable variation among different groups. Clusters were differentiated using Student Newman Keuls (SNK) test, accessions with the highest days to maturity are embedded in cluster 3 and cluster 2. Cluster with early 50% flowering are found in cluster 1. Performance of most variables showed that all these traits have high degree of similarities in the performance. Therefore, showed low discriminating attributes for all the accessions in this study. Traits such as number of pod per plant (NPP), pod length (PODL), pod width (PODW), seed weight (SW), leaf length (LL) cannot be used to characterize the accessions evaluated in the study due to low diversity attributed to those traits in *Lablab purpureus*. This could attributed to the influence of environmental factors of where the experiment was undertaken. This study suggests more multi-locational

Table 1: Analysis of variance of variables observed among *Lablab purpureus* accessions.

Variables	PH	LG	LL	PL	SG	D50F	DTM	NPP	PODL	PODW	NSPOD	SL	SW	HSW	YPP	DOG
Mean Squ.	17.5^{ns}	0.794^{ns}	0.78^{ns}	0.431^{ns}	0.005***	56.7*	215.66**	123.8^{ns}	2.706^{ns}	0.263^{ns}	0.229^{ns}	0.007^{ns}	0.003^{ns}	38.7**	0.507^{ns}	0.067***
Residual	23.81	1.35	0.72	0.19	4.50e-04	22.17	12.92	2.241	0.315	0.358	0.007	0.002	21.38	0.345	0	0.344

PH- Plant height, LG- Leaf girth, LL- Leaf length, PL- Petiole length, SG-Stem girth, D50F- Days to 50% flowering , DTM- Days to maturity, NPP- Number of pod per plant, PODL- Pod length, PODW-Pod width, NSPOD-Number of pod, SL-Seed length, SW-Seed width, HSW-100 seed weight, YPP- Yield per plant, DOG- Days of germination.

trial of all the considered accession in this region. Additionally, Fig 3 showed a total of five individuals representing the three clusters from the morphological data and the electropherogram results.

Nucleotide sequence statistics

Table 2 shows the sequence characteristics of the studied barcodes. The total sequences obtained from the ribulose bisphosphate carboxylase (rbcL) region of the selected species were aligned using CLC Sequence Viewer 8.The final aligned partial sequences of rbcL in TLn 12 had a length of 153 bp, 158 bp for TLn 13, 162 bp for TLn 45 and TLn 46 and 159 bp for TLn 57 with the variable single stranded weights of 47.654 kDa for TLn 12, 49.208 kDa for TLn 13, 50.483 kDa for TLn 45, 50.134 kDa for TLn 46 and 49.386 kDa for TLn 57. For the double strands, there are variable weights ranging between 94.56kDa for TLn 12, 97.653kDa for TLn 13, 100.12kDa for TLn 45, 100.125kDa for TLn 46 and 98.277kDa for TLn 57 (Table 3).

Multiple sequence alignment

We analyzed the selected *L. purpureus* genotypes from International Institute for Tropical Agriculture, Ibadan and the sequences of the examined region of plastid DNA (rbcL) was utilized. Multiple sequence alignment of the selected three species sequences was carried out by using the online software Clustal W (v1.2.4). We found unique nucleotide sites between TLn 12 and TLn 45, also throughout the selected genotypes. These were the 79th to 80th sites of the rbcL sequences at which A were present throughout the genotypes and at the 53rd and 54th sites, 118 to 119 CC, TA were present respectively (Fig 4).

Bioinformatics analyses of sequences and barcode generation

The rbcL sequences from TLn 46 and TLn 12 were identical (accession number MN966634.1, KF724319.1) with other sequences of the selected species. BLAST search in the NCBI nucleotide database was done using the sequences

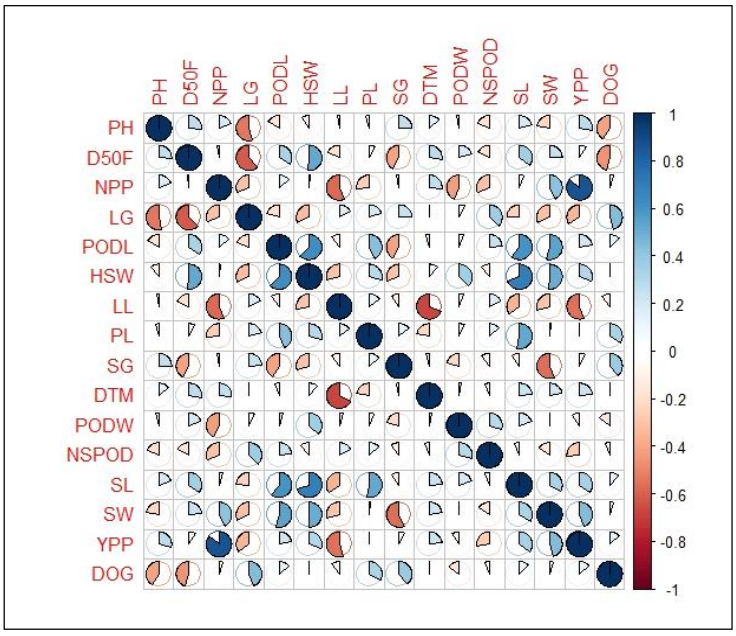

Fig 1: Correlation matrix among traits of *Lablab purpureus.*

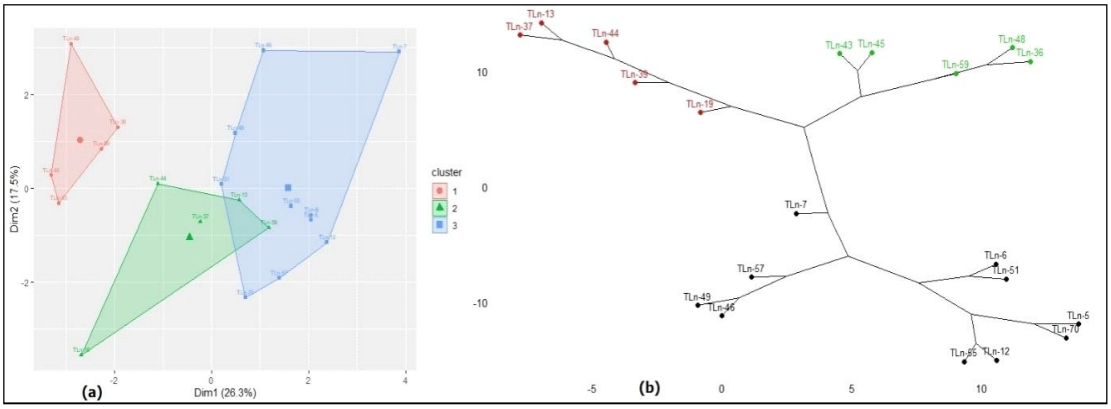

Fig 2: Cluster plot (a) and phylogenetic representation (b) of *Lablab purpureus* accessions using phenotypic traits.

Table 2: Contribution of principal component axes to the variation among accessions studied.

Variables	PC1	PC 2	PC3	PC4
PH	0.20	-0.40	-0.45	0.64
D50F	0.62	0.20	-0.54	0.13
NPP	0.53	-0.67	0.27	-0.21
LG	-0.61	0.23	0.56	-0.16
PODL	0.61	0.55	0.22	-0.07
HSW	0.74	0.47	0.05	0.10
LL	-0.59	0.41	-0.31	0.05
PL	0.11	0.56	0.40	0.52
SG	-0.39	-0.39	0.38	0.64
DTM	0.40	-0.31	0.08	-0.11
PODW	0.07	0.52	-0.23	0.05
NSPOD	-0.27	0.48	0.04	-0.16
SL	0.69	0.35	0.22	0.44
SW	0.72	0.11	0.16	-0.48
YPP	0.67	-0.45	0.31	0.08
DOG	-0.12	0.05	0.87	0.07
Eigen value	4.20	2.79	2.32	1.65
Percentage variance	26.26	17.47	14.49	10.35
Cumulative percentage	26.26	43.74	58.23	68.59

Legend: Eigen vectors greater than 0.5 are in bold.

of all *L. purpureus* genotypes deposited in the GenBank showing 99.23% homology with the nuclear DNA regions. Comparison of the detected sequences with entries in the database finally provided the plant species identification. The developed barcodes for the *L. purpureus* by using rbcL are shown in Fig 5.

Phylogenetic analysis

Total aligned molecular sequences from the selected *L. purpureus* by rbcL were used to generate a dendrogram which represented the genetic relationship among the selected species in comparison to the NCBI nucleotide sequences. Similar sequences were found to link the accessions considered with the existing accessions of lablab achieved in the NCBI database. This serves as identification insight to the accessions considered as lablab (Fig 6).

Understanding genetic diversity between accessions requires a proper molecular characterization of the germplasm evaluated so as to be able to ascertain the phenotyping findings (Assogba *et al.*, 2015). The primers gave sharp bands that were required for reliable DNA sequencing. This is in line with the observation of Hollingsworth et al., 2009 where the amplification of the rbcl gene using one or two universal primer types had a high success rate. Thus, using DNA barcodes primers systems

Table 3: Nucleotide sequence statistics for the selected accessions of lablab.

Information	TLn 12 (11_RBCL F)	TLn 13 (12_RBCL F)	TLn 45 (13_RBCL F)	TLn 46 (14_RBCL F)	TLn 57 (15_RBCL F)
Sequence type	DNA	DNA	DNA	DNA	DNA
Length	153bp	158bp	162bp	162bp	159bp
Organism	Lablab	Lablab	Lablab	Lablab	Lablab
Name	TLn 12 (11_RBCL F)	TLn 13 (12_RBCL F)	TLn 45 (13_RBCL F)	TLn 46 (14_RBCL F)	TLn 57 (15_RBCL F)
Weight (single-stranded)	47.654 kDa	49.208 kDa	50.483 kDa	50.134 kDa	49.386 kDa
Weight (double-stranded)	94.56 kDa	97.653 kDa	100.12 kDa	100.125 kDa	98.277 kDa

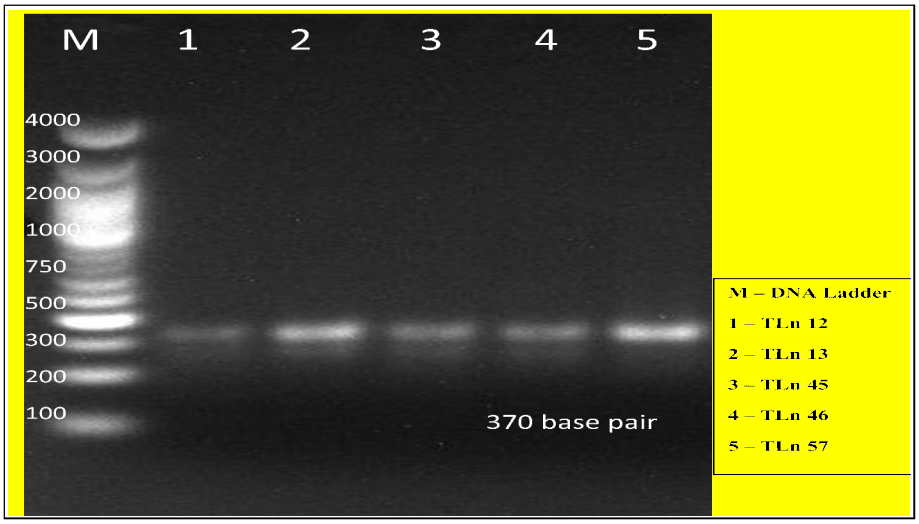

Fig 3: Electropherogram results of *rbcL* gene amplification in Lablab beans.

is reliable, fast and cheap system for amplification, identification and discrimination of *Lablab purpureus* (L). The results revealed that all single barcodes could be easily amplified and sequenced with the selected primers. Besides, rbcL marker is individually able to discriminate species of *Lablab purpureus* accessions investigated and the result corroborates the phylogenic illustration of the accessions using phenotypic traits.

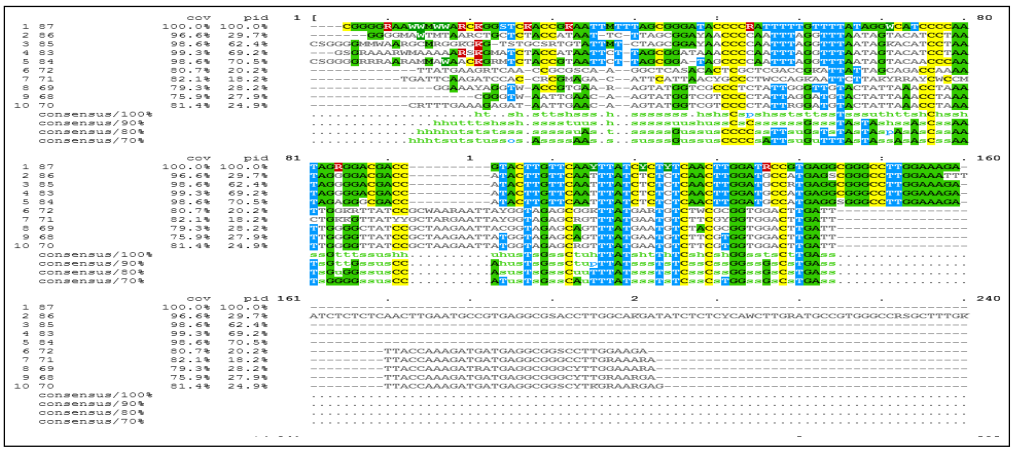

Fig 4: Multiple sequence alignment illustration of the sequences of lablab accessions.

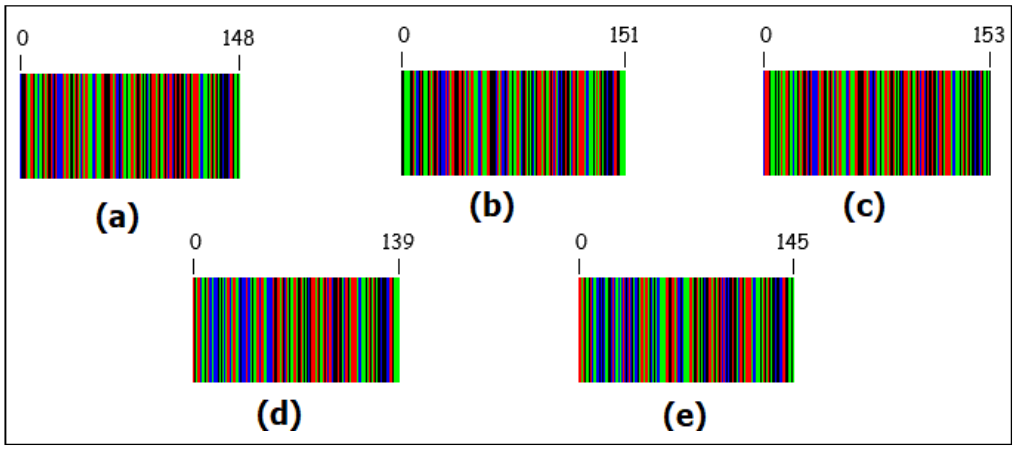

Fig 5: DNA Barcodes representation for Lablab purpureus accessions. a- TLn 12, b- TLn 13, c- TLn 45, d- TLn 46, e- TLn 57.

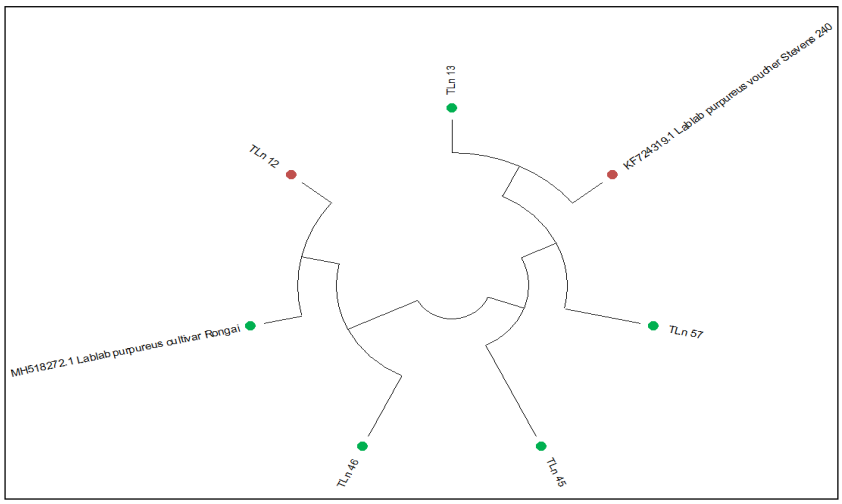

Fig 6: Phylogenetic tree of *Lablab purpureus* accessions compared with known Lablab on NCBI database.

CONCLUSION

Knowledge of phenotyping in variability study is paramount to making a better selection of genotypes during breeding program. This finding revealed the need for molecular improvement of *Lablab purpureus* and the need to breed with cultivars from other developed countries due to low variability observed among the accessions. Of all accessions of Lablab beans evaluated in this study, accessions TLn-12, TLn-13, TLn-36, TLn-45, TLn-46, TLn-57 and TLn-70 showed great potential in the morphological traits. Therefore, genetic compatibility study is required among these accessions and most importantly with other cultivars from other developed countries.

DNA-based barcoding is a vital tool for detecting errors in identifications of minor and underutilized grain legumes such as *Lablab purpureus*. From the sequences, DNA barcodes and distances within the phylogenetic revealed close proximity and indicated their closeness. The present study provides barcodes with DNA sequences for the identification of the selected accessions.

The DNA barcoding established a shared community resource of DNA sequences that can be used for organismal identification and taxonomic clarification of *Lablab purpureus*.

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Author's contributions

Agbolade James Oludare, Idowu Jeremiah Adebare, Isiaka Abiodun Ibrahim designed the research. Isiaka Abiodun Ibrahim and Idowu Jeremiah Adebare performed the field experiments and analyzed the data. IJA, IAI, JOA and IO conducted laboratory tests. JOA, IJA wrote the article. JOA, IJA, IAI and MAA revised the article, JIK edited and proofread the article. All authors read and approved the final manuscript.

Conflict of interest: None.

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