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Insight into Genetic Diversity of Cultivated Lima Bean (*Phaseolus lunatus* L.) in Benin

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Abstract

Lima bean is a tropical and subtropical legume from the genus Phaseolus which is cultivated for its importance in food and in medicine, but which remains a Neglected and Underutilized Crop in Benin. Understanding the genetic diversity of a species' genetic resources is useful for the establishment of appropriate conservation strategies and breeding programs and for sustainable use. We use 6 out of ten SSR markers to analyze the diversity and population structure of 28 Lima bean landraces collected in Benin. A total of 28 alleles with an average of 4.16 alleles per SSR were amplified. The Polymorphic Information Content value ranged from 0.079 to 0.680 with an average of 0.408. The analysis of population structure revealed three subpopulations. PCoA revealed three well-separated clusters among the analyzed accessions in accordance with the population structure results and the clustering based on the Neighbor-Joining tree. AMOVA showed highly significant ($p = 0.001$) diversity among and within populations. Hence, 32% of the genetic variation was distributed among the population and 68% was distributed within populations. A high PhiP value (0.321) was found between the three sub-subpopulations indicating a high genetic differentiation between these sub-subpopulations. By exhibiting the highest average number of alleles, Shannon-Weaver information and Shannon-Weaver diversity indices, and the highest mean number of private alleles, subpopulation 1 is the main gene pool of the analyzed collection. The present study is an important starting point for the establishment

of appropriate conservation strategies and breeding programs for Lima bean genetic resources.

Keywords

Phaseolus lunatus L., Benin, Genetic Diversity, SSR Markets, Conservation

1. Introduction

Lima bean (*Phaseolus lunatus* L.) is a tropical and subtropical legume cultivated for its edible seeds [1] in the home gardens or intercropped with cereals in the field [2]. The species is of considerable importance in food and in medicine and is considered, for this purpose, as the main source of plant protein for human nutrition [3]. It is consumed in various forms (pulp, paste, and dietary supplements). The Lima bean is the second agro-nomically and economically most significant species within the Phaseolus genus behind the Common bean in the world [4] [5]. In Benin, despite its importance, the legume is referenced as a Neglected and Underutilized Crop Species [6]. Given that nowadays, for the reason of research of profits, most producers have opted for industrial crops, on the one hand, and on the other hand, due to the lack of improved varieties. Hence, in the country, there is no research effort on Lima bean improvement.

In general, the lack of sufficient characterization of genetic resources and by ricochet the lack of genetic data constitute for any crop the main brake to any improvement program [7]. Genetic diversity studies by discovering and characterizing novel genes or alleles likely to be introgressed into elite germplasm seem to be the primary basis for successful plant breeding [8]. They offer thereby opportunities for genetic improvement and provide valuable information for effective conservation [9] [10].

Plant genetic diversity can be studied at the phenotypic and molecular level or by using agro-morphological characteristic measurements using phenotypic, molecular, or agro-morphological markers [11]. However, agro-morphological markers such as yield components are known to be affected by environmental factors [12] [13]. Thus, the use of genetic molecular markers has gained prominence in genetic diversity studies. These markers are not regulated through the environment, but their utilization conditions have no effects on which the plants are cultivated [14]. They are one of the powerful tools used in the characterization of genetic resources [15].

The advantage of choosing a given marker lies not only in its accessibility, the cost, in the reproducibility of the results but also in its ability in revealing polymorphisms in the nucleotide sequences [14]. These polymorphisms are revealed by molecular techniques such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), microsatellite or

Simple Sequence Repeat (SSR), Random Amplified Polymorphic DNA (RAPD), Single Nucleotide Polymorphisms (SNPs) and others [15].

Crops can be divided into two main categories which are stapled and underutilized also called Neglected and Underutilized Crop Species (NUS) [16]. The last ones are mostly wild or semi-domesticated species adapted to local environments [17] [18]. NUS is known to have not neglected commercial value and plays an important role in household income improvement. Most of them are cheap, accessible for the local population, and therefore contribute to food security and nutrition, generally more adapted to extreme soil and climatic conditions as they contain the relevant alleles and mechanisms for growth in poor environments and for resilience under [19]. NUS also have been recognized as potential sources of resilience traits) which can be used to improve major crops [20] [21].

In the current context of climate change and the fight against hunger and malnutrition, attention is turned to neglected and underutilized crops [22] like Pigeonpea, Kersting groundnut, or Lima bean [6]. In Benin, there has been some research directed towards the genetic diversity of NUS such as pigeon pea [23] [7] and Kersting groundnut [24], unlike the Lima bean which has not received any attention in terms of scientific research.

For all crops, understanding genetic diversity is useful for the establishment of appropriate conservation strategies and breeding programs and for sustainable use [2] [7]. This research was designed to analyze the genetic diversity and population structure of 28 Lima bean landraces collected in Benin using microsatellite markers, for the implementation of effective breeding and conservation programs.

2. Materials and Methods

2.1. Plant Material, DNA Isolation, and Quantification

A total of 28 accessions of Lima beans collected in the republic (**Table 1**) were analyzed. The study was carried out in the Laboratory of Molecular Biology and Bioinformatics Applied to Genomics at the National University of Sciences, Technologies Engineering and Mathematics of Abomey in Benin.

DNA was extracted from young leaves using the SDS method in accordance with [25] with minor modifications. Briefly, 500 mg of young leaves of *Phaseolus lunatus* were ground in liquid nitrogen in a mortar with 1000 µl of SDS buffer (200 mM Tris-HCl, 25 mM EDTA, 250 mM NaCl, 0.5% SDS). The ground material was incubated at 37˚C in a water bath for 1 hour. After cooling at room temperature, 800 µl of the phenol/chloroform/isoamyl alcohol (25:24:1) mixture was added to each sample and the whole was mixed before then being centrifuged at 15,000 rpm for 10 min at 4˚C. The supernatant was collected and mixed in equal volumes with a solution of Chloroform/isoamyl alcohol (24:1). The mixture was then centrifuged at 15,000 rpm for 10 min at 4˚C. The supernatant was once again collected in a new tube and the DNA precipitated with 0.1 volume

Table 1. Geographical distribution of the 28 accessions collected.

Accession	Village	District	Municipality
V1	Clèdji	Ouèssè	Ouèssè
V2	Azéhounholi	Adogbé	Covè
V3	Yawa	Kpingni	Dassa-Zoumè
V4	Voli	Adogbé	Covè
V5	Kenouhoué	Tota	Dogbo
V6	Agnivedji	Lokossa	Lokossa
V7	Adjoya	Tchetti	Savalou
V8	Domè	Adogbé	Covè
V9	Fita	Kpingni	Dassa-Zoumè
V10	Malè	Canna 1	Zogbodomey
V11	Sèwacomey	Zoungbonou	Houéyogbé
V12	Pénéssoulou	Pénéssoulou	Bassila
V13	Adjoya	Tchetti	Savalou
V14	Akpassi	Akpassi	Bantè
V15	Kpodavè	Tota	Dogbo
V16	Agnivedji	Lokossa	Lokossa
V20	Adjoya	Tchetti	Savalou
V21	Zounta	Zounguè	Dangbo
V22	Okeola	Pobè	Pobè
V23	Pénélan	Pénéssoulou	Bassila
V24	Dogoudo	Canna 1	Zogbodomey
V25	Dogoudo	Canna 1	Zogbodomey
V26	Kajoca	Kpankou	Kétou
V27	Yawa	Kpingni	Dassa-Zoumè
V28	Pénélan	Pénéssoulou	Bassila
V29	Goutin	Adjohoun	Adjohoun
V30	chouchoubou	Tanguiéta	Tanguiéta
V33	Dogoudo	Canna 1	Zogbodomey

of sodium acetate (3M, pH 7.0) and 0.7 volume of cold isopropanol and left at −20˚C for 20 min. Final centrifugation of 15 min at 15,000 rpm 4˚C will be done to recover the pellet of ADN. The pellet of ADN was washed 3 times with ethanol at 70% and once with ethanol at 100% and dried at room temperature for about 2 hours and then suspended in 1X TE storage buffer.

DNA quality was checked on agarose gel at 0.8%. Its concentration was assessed with NanoDrop Lite (Thermo Fisher Scientific) and DNA purity was accessed using the absorbance ratio (A260/A280). Based on the obtained DNA

concentration, different dilution rates were applied to each sample to obtain a concentration of 5 ng/µl necessary for a PCR amplification reaction.

2.2. SSRs Amplification and Gel Electrophoresis

A total of 10 microsatellite markers isolated and optimized for Common beans were used [26] (**Table 2**). PCR reactions were induced with 20 ng of DNA, 1 U of Taq DNA polymerase, 2.0 mM of magnesium chloride ($MgCl_2$), 0.2 mM of each dNTP, 0.1 µM of each primer, and 1X PCR reaction buffer in a final volume of 20 ml [26]. The program consisted of an initial denaturation at 94˚C for 2 min, followed by 45 cycles at 94˚C for 30 s, 49˚C for, 30 s and 72˚C for 30 s and a final extension at 72˚C for 10 min [11]. Amplification products were migrated on agarose gel at 2%, revealed with Ethidium Bromide, and then visualized on a UV transilluminator.

2.3. Data Analysis

At each locus, different bands recorded an allelic composition. Thus, SSR alleles were coded as individual markers with 1 for the presence and 0 for the absence of the allele as binary data [27]. Genetic diversity parameters such as polymorphism rate (P), allelic diversity, and Polymorphism Information Content (PIC) were estimated. PIC value was calculated as followed: $PIC = 1 - \sum fi^2$: where fi is the frequency of each allele.

To assess genetic relationships between accessions, the dissimilarity between paired accessions was assessed using Darwin 6.0.21 software [28]. The generated dissimilarity matrix was used to infer a dendrogram using the Neighbor-Joining method.

The determination of the genetic groups was supported by population structure

Table 2. Characteristics of the 6 SSR markers.

Name	Sequences (5'-3')	Motif	TA
AG1	F: CATGCAGAGGAAGCAGAGTG R: GAGCGTCGTCGTTTCGAT	$(GA)_8$GGTA$(GA)_5$GGG GACG(AG)4	55˚C
BM211	F: ATACCCACATGCACAAGTTTGG R: CCACCATGTGCTCATGAAGAT	$(CA)_{13}$	54˚C
BM160	F: CGTGCTTGGCGAATAGCTTTG R: CGCGGTTCTGATCGTGACTTC	$(GA)_{15}$ $(GAA)_5$	55˚C
GATS91	F: GAGTGCGGAAGCGAGTAGAG R: TCCGTGTTCCTCTGTCTGTG	$(GA)_{17}$	55˚C
BM141	F: TGAGGAGGAACAATGGTGGC R: CTCACAAACCACAACGCACC	$(GA)_{29}$	55˚C
BM164	F: TCTTGCGACCGAGCTTCTCC R: CTGAATCTGAGGAACGATGACCAG	$(CT)_{17}$	55˚C

analysis using the Bayesian model-based analyses using the software package Structure v2.3.4 [29]. The membership of each accession was run for the value of K = 2 to K = 10 with the admixture model and correlated allele frequency. Accessions with membership probabilities greater than 0.8 were grouped together and accessions with membership probabilities below 0.8 were assigned to the admixed group. For each K, it was replicated 3 times. Each run was implemented with a length of the burn-in period of 1000 followed by 10,000 Markov Chain Monte Carlo (MCMC) replicates. The ΔK method was used to identify the optimal value of K [30] using the online Structure Harvester program [31].

Principal Coordinate Analysis (PCoA) and Molecular Variance Analysis (AMOVA) were performed using GenAlEx 6.503 software [32] to estimate the genetic differentiation within and among the different subpopulations. Population genetic parameters such as the average number of total alleles (Na), Shannon-Weaver information index (I), the average number of private alleles (Pa), Shannon-Weaver diversity index (h), percentage of polymorphic loci, PhiPT and allele across the different subpopulations were calculated. Data were coded as suggested in the GenAlEx 6.503 software user manual [32].

3. Results and Discussion

3.1. SSR Genotyping and Genetic Diversity Analysis

SSR markers are nuclear markers that are widely used in population genetic studies and provide important information for the preservation of genetic diversity [33]. In the present study, of the 10 SSR markers, 6 were amplified in all accessions and were therefore retained for the various analyses. The 6 SSR markers analyzed generated a total of 28 alleles with an average of 4.16 alleles per SSR. The number of alleles ranged from 2 (BM160 and GATS91) to 8 (BM141). As repeat polymorphisms revealed by SSRs or RAPD and over DNA markers result from the addition or deletion of the entire repeat units or motifs [34], the polymorphism revealed by these types of markers depends both on the repeat pattern, its location in the genome of the species in connection with the speed of evolution of the species and on the size of the analyzed collection [35]. Thus, the relatively lower number of alleles reported in this study can be explained by the size of the analyzed collection, the location of the markers, or also, the number of markers used. The polymorphic information content value which indicated the informativeness of the loci and their ability to detect differences between the genotypes [36] ranged from 0.079 for the GATS91 marker to 0.680 for the BM 140 marker with an average of 0.408 (**Table 3**). This average PIC value is relatively low and supported the low level of discrimination of the SSR markers used in the present study however BM 140 marker was found to be the most appropriate for testing genetic diversity given its high level of polymorphism. These diversity indices which are the average number of alleles per locus and the PIC reported in the present study were slightly lower than the previously reported by Gomes *et al*. [26] who reported 10.27 alleles per locus and an average PIC value

Table 3. Genetic diversity parameters of the 6 SSR markers across analyzed accessions.

SSR markers	Na	PIC	P
BM211	4	0.451	100
BM160	2	0.437	100
GATS91	2	0.079	100
AG1	6	0.605	100
BM164	3	0.197	100
BM141	8	0.680	100
Moyenne	4.16	0.408	100

of 0.675 studying a set of 153 lima bean accessions. These findings reinforced the fact that the size of the collection influences the number of total alleles.

However, these markers, although having presented a relatively low level of discrimination, made it possible to reveal using the Neighbor-Joining tree based on the genetic distance, 3 clusters within all the 28 analyzed accessions. Cluster 1 labeled in red grouped 8 accessions, cluster 2 labeled in black grouped 6 accessions, and cluster 3, labeled in blue grouped 14 accessions (**Figure 1**). This grouping of accessions revealed potential duplicates in the analyzed collection. Indeed, an analysis of the inferred tree showed 5 pairs of duplicates (V8V9, V3V20, V27V7, V5V23, and V26V24) which exhibited the same evolutionary distance. This may be due to usual genotype exchanges among farmers.

3.2. Population Structure and Genetic Differentiation Analysis

Revealing Lima bean population structure and genetic diversity is important for breeding efforts which is an essential step in any genetic improvement process through the identification of promising genotypes [37]. Data generated from SSR genotyping was used to assess the population structure of the Lima bean collection. The estimation of the delta K value, using Evanno's method, showed the highest peak at K = 3 (**Figure 2** and **Figure 3**), indicating that the 28 accessions could be grouped into three sub-populations based on differences in their genetic traits. The first one labeled in red grouped 9 accessions, the second labeled in green grouped 7 accessions, and the third labeled in blue groups 12 accessions. Our results contradicted Gomes *et al*.'s [26] study in the determination of the genetic diversity and population structure from a core collection of 153 Lima beans. Maybe to our SSR markers' low level of discrimination. According to the probability of membership, the accessions of the different subpopulations were classified as pure or admixed. To this end, 25 accessions presented a certain belonging to one of the three sub-populations. Thus, the results of the structure revealed a low level of admixture of 10.71% suggesting a non-negligible differentiation between the different sub-populations [38].

Analysis of Molecular Variance results revealed significant differences within and between populations (p = 0.001). 32% of the genetic variation came from

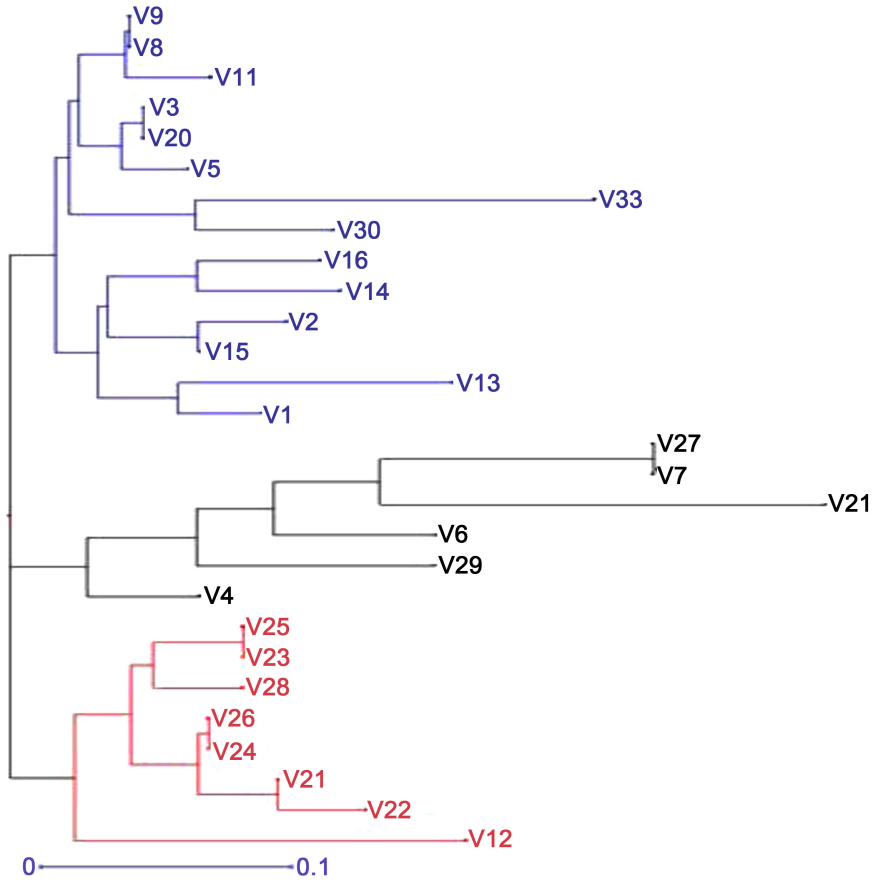

Figure 1. NJ tree based on dissimilarity matrix calculated from the dataset of 6 SSR across the 28 accessions of *Phaseolus lunatus*.

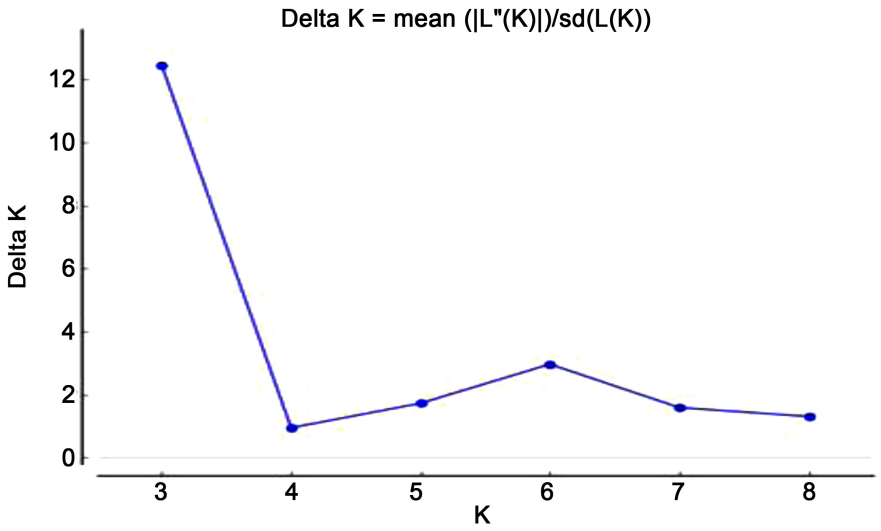

Figure 2. Variation of the probable number of sub-populations (K) in the function of Delta K in the analyzed collection.

inter-population and 68% of the genetic variation came from intra-population suggesting that the genetic variation within populations was larger than that

between populations [39]. However, a high PhiP value (0.321) was found between the three sub-subpopulations indicating a high and significative (p = 0.001) genetic differentiation between these sub-subpopulations. Comparing genetic differentiation between sub-populations showed a low differentiation between sub-populations 1 and 3 (PhiPT = 0.25). The largest index was observed between subpopulation 2 and subpopulation 3 (PhiPT = 0.47). The high level of genetic differentiation found for the Lima bean in the present study may be explained by the reproductive characteristics of this crop. According to Penha *et al*. [11] and Nasir *et al*. [2], Lima bean had a mixed system with a predominance of self-fertilization, with only 38% of natural crossing which limits gene flow and therefore increases diversity among populations. This coincided with the AMOVA results (**Table 4**), where 32% of the total variation was accounted for by among-subpopulation variations. Our results are consistent with Martinez *et al*. [40] who reported low gene flow between populations of Lima beans from the Yucatan Peninsula which could be due to a low rate of seed exchange among farmers which could lead to low intermix among populations [2].

The allelic pattern and genetic diversity indices provided insight into genetic diversity within subpopulations [41]. The average number of alleles (Na) ranged from 3.83 within the accessions in sub-population 1 to 1.67 in sub-population 2. The percentage of polymorphic loci ranged from 50% to 100%. Subpopulation 1 had the highest average number of alleles (Na), Shannon-Weaver information (I), Shannon-Weaver diversity (h) indices (I = 1.18 and h = 0.64) and exhibited the highest mean number of private alleles (Pa = 0.11) (**Table 4**) meaning that subpopulation 1 was more diverse than the two overs. As a result, subpopulation 1 could be considered as the main gene pool and could serve as a source for the

Figure 3. Barplot of sub-populations at K = 3 belonging from the population structure analysis based on 6 SSR markers showing the 3 subpopulations (SP1, SP2, SP3). The vertical lines represented the individuals and the height of each bar represented the probability of each accession belonging to its subpopulation.

Table 4. Analysis of Molecular Variance (AMOVA) of the genetic variation among and within subpopulations of Lima bean using a set of 6 SSR.

Source	df	SS	MS	Est. Var.	%
Among subpopulations	2	25.667	12.833	1.144	32%
Within subpopulations	25	60.369	2.415	2.415	68%
Total	27	86.036		3.559	100%

selection of parents to improve the existing Lima bean landraces. The number of accessions with private alleles ranged from 4 in subpopulation 2 to 9 in subpopulation 1. The number of markers with private alleles ranged from 1 (for a total of 12 accessions) to 5 (for a total of two accessions: V27 and V7) (Table 5 and Table 6).

The first two axes of the PCoA expressed 43.83% of the total variation in the analyzed collection. The results revealed three well-separated clusters among the analyzed accessions in accordance with the population structure results (Figure 4) and coincided with the clustering based on the Neighbor-Joining tree.

Table 5. Level of genetic diversity within the 3 subpopulations.

Pop	SP1	SP2	SP3
N	9	7	12
Na	3.83	1.67	2.50
I	1.18	0.34	0.53
H	0.64	0.22	0.31
Pa	2.17	0.50	0.67
%P	100.00%	50.00%	83.33%

SP: Subpopulation; N: Number of accessions; Na: Average number of alleles; I: Shannon-Weaver information index; h: Shannon-Weaver diversity index; Pa: Average number of private rare alleles; %P: Percentage of polymorphic loci.

Table 6. List of accessions with 1 or more private alleles.

Accessions	SP	Number of Private alleles	SSR markers
V12	SP1	2	BM211 AG1
V21	SP1	3	BM211 BM164 BM141
V27	SP1	5	BM211 BM160 GATS91 AG1BM141
V29	SP1	3	BM211 BM164 BM141
V30	SP1	2	BM164 BM141
V33	SP1	3	BM211 BM164 BM141
V4	SP1	3	BM211 BM160 BM141
V6	SP1	2	BM211 BM160
V7	SP1	5	BM211 BM160 GATS91 AG1 BM141
V22	SP2	1	BM141
V23	SP2	1	BM141
V25	SP2	1	BM141
V28	SP2	1	GATS91
V1	SP3	1	AG1
V13	SP3	1	BM211
V14	SP3	1	BM211
V15	SP3	1	BM211
V16	SP3	1	AG1
V2	SP3	1	BM211
V20	SP3	1	BM141
V5	SP3	1	BM211

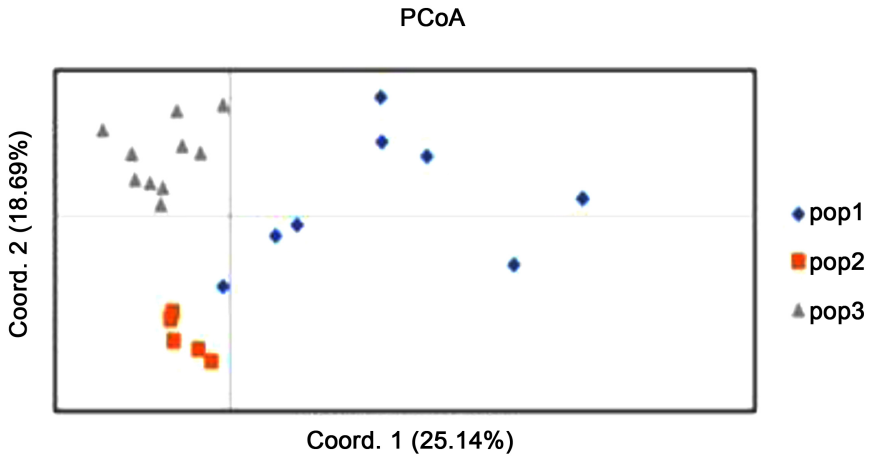

Figure 4. Principal Coordinate Analysis (PCoA) of the 28 *Phaseolus lunatus* accessions using the 6 SSR.

4. Conclusion

In this study, we used SSR markers to assess the genetic diversity and structure of the Lima bean landrace grown in Benin. The polymorphism rate and polymorphic information content values were sufficient to reveal the existence of potential duplicates and 3 highly differentiated subpopulations in the analyzed collection. By exhibiting the highest average number of alleles, Shannon-Weaver information and Shannon-Weaver diversity indices, and the highest mean number of private alleles, subpopulation 1 is the main gene pool of the analyzed collection. The present study is an important starting point for the establishment of appropriate conservation strategies and breeding programs for Lima bean genetic resources. Knowledge of population structure and genome variation is essential for genome-wide association studies of complex traits and for the investigation of functional genes.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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