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RESEARCH ARTICLE

ASSESSMENT OF THE GENETIC DIVERSITY AND THE RELATIONSHIP AMONG COMMON BEAN (*PHASEOLUS VULGARIS* L.) ACCESSIONS FROM DR-CONGO GERMPLASM USING SSR MOLECULAR MARKERS

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ABSTRACT

The genetic diversity of Common bean accessions from the germplasm of INERA in DR-Congo was assessed to understand their phylogeny relationship using SSR molecular markers. A set of 91 accessions, comprising 21 from CIAT/Columbia, 36 from Mvuazi, 30 from Mulungu and 4 from Gandajika, were genotyped with 12 SSR markers that generated 89 alleles with an average of 7 alleles per locus. Polymorphism information content (PIC) value was 0.64 indicating a fair diversity with a range of 0.40-0.82. The average heterozygosity level per locus was 0.24. The variability was as low as 1% among the populations, 64% within individual and 35% within population. Clustering analysis based on the genetic similarity grouped the 91 Common bean genotypes into 2 main distinct clusters. Information generated from this study can be driven to select parents for breeding or develop hybrid lines for yield increase, high biomass, resistance against abiotic/biotic stresses and segregating populations to map genes/QTLs for yield in Common bean in the western DR-Congo.

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INTRODUCTION

Common bean is one of the four main legume crop cultivated in DR-Congo. The national agricultural production statistics of the decade 1999-2009 indicates that beans occupy the 8th rank and agricultural production of beans in which Common bean occupy the 1st place of most cultivated beans in DR-Congo (SENSA,2009). Common bean is one of the most important food crops in the country, playing important role in the human food and providing more than 45% of proteins consumed (Allen *et al.,* 1996). It constitutes also a source of indispensable nutrients for food of the rural resource poor (Mbikayi, 2007), and for those having a limited access to meat, fish and milk, and being satisfied to food proteins with high biologic quality (Anonyme, 1991). By its substantial content in micro-elements Iron and Zinc, it is one of the bio fortified foods (Harvest Plus, 2009) classified among food ofadvisable choice to pregnant women, children and vulnerable groups (Mbikayi, 2007) as well as to the HIV/AIDS patients (PABRA/ECABREN/SABRN/CIAT, 2006). It also is one of the most important food crops, as source of incomes to the producers, and because of its ecological diversity. DR-Congo presents various potentialities of production, big potentialities in the high altitude zone of the East of the country and as well as of middle potentialities on low altitude of Kongo Central and Katanga (Wortmann *et al.,* 1998). The potential of varieties distributed in the country of 500 – 750 kg/ha (Matondo *et al.,* 2000) is less to the one of 1,500 kg/ha of the three countries of the under-region of Big Lakes of Africa (CPGL), caused by abiotic/biotic factors (Wortmann and Allen, 1994) of which the weak- fertility of soil, water deficit

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caused by stress of the present climatic changes, insects pests and illnesses (Allen and Edje, 1990; Allen *et al.,* 1989; Allen *et al.,* 1996; ECABREN, 2000). This weak potential yield is also subjugated by the weak level of improved quality of varieties and the weak acceptability varieties released to the users, by poor technical management and lack of coherent policies of production and merchandising. So, INERA (National Institute for Study and Agronomic Research)(NARS) has various Common bean collection maintained in Mvuazi (Bas-Congo) for low altitude, and Mulungu (Southern Kivu) in the high altitude, and the efforts of its National Grain Legume Program (PNL) is to release interesting varieties in relation with objectives assigned in its classic selection. The diversity of the accessions maintained by INERA still remains apparently little known and not much exploited, however the Common bean benefits from a migratory flux releasing varieties trough the country. To get solution, INERA by its breeding program and others structures (PABRA/CIAT, ECABREN, ASARECA, Consortium CIALCA (Bioversity International, in partnership with IITA and CIAT)) make efforts to introduce new genetic materials for improving agricultural livelihoods, and are working towards development of high-yielding varieties and hybrids with better quality, disease resistance, drought tolerance and agronomic traits (Klein et *al*. 2008).

Important efforts (breakthrough) are made in developing and releasing common bean varieties for food production and food security, commercial cultivation and guarantee of incomes**.** Butto date, there is insufficient data on genetic diversity in Common bean germplasm collections in DR-Congo. A research program aimed to enhance the capacity to use and develop participatory approaches to managing the agricultural biodiversity is being implemented. A more comprehensive assessment of genetic diversity would allow curators and users to manage and access ex situ collections more efficiently. Moreover, the knowledge of the genetic variation within accessions from germplasm collection is essential to the choice of strategy to incorporate useful diversity into the program and to facilitate the introgression of genes of interest into commercial cultivars. It will be also important to understand the evolutionary relations among accessions; to better sample germplasm diversity and to increase conservation efficiency using acquired knowledge (Fu, 2003, Mudibu *et al.,* 2011). The methods for detecting and assessing genetic diversity have extended from analysis of discrete morphological and molecular traits. Several DNA marker systems are now in common use with success in diversity studies of plants, like RAPD (Randomly Amplified Polymorphic DNA), ISSR (Inter Single Sequence repeats), SSR (Simple Sequence Repeat), etc (Nkongolo *et al.,* 2011).

The simple sequence repeat (SSR) markers are useful tools for genetic assessment of variation. Polymorphism generated by SSR assays have been used for assessment of variation germplasm identification and genetic mapping in different species.Many studies have recommended the use of SSR markers in analyses of genetic diversity due to their high degree of polymorphism (Gele*ta et al.,* 2006; Ali *et al.,* 2008; Shehzad *et al.,* 2009). They are codominant markers dispersed throughout the genome, and have multiple alleles that often have conserved loci between related species (Brown *et al.,* 1996; Schulman, 2006). Powell et *al*. (1996) stated that SSRs are able to discriminate among closely related individuals, and have advantage over other markers in their ability to trace pedigrees in plants. Genetic similarity estimates among genotypes are important in selecting parental combinations for creating segregating populations so as to maintain genetic diversity in a breeding program (Becelaere et *al*. 2005),to develop mapping populations for detecting quantitative trait loci (QTL) (Varshney, 2011) and to categorize lines into heterotic groups for hybrid crop breeding (Menz et al.,2004). Simple sequence repeats (SSR), also known as microsatellites, are based on tandem repeats of one to six core nucleotide elements, and are multiallelic and codominant and contain considerable genetic variation (Blair *et al.,* 2003; Song *et al.,* 2004; Cabral *et al.,* 2011).Microsatellites are short tandem repeats of DNA, generally distributed throughout the genome of an organism. Microsatellites markers (SSRs) are becoming the markers of choice in plant breeding programs. They are polymerase chain reaction (PCR)-based, codominants, easily reproducible, highly multiallelic and can be genotyped on semi-automated DNA sequences using multiplex assays, allowing for high throughput DNA typing (Buso *et al.,* 2006).

The aim of this study is to assess the genetic variation among the main accessions of the Common bean germplasm maintained by INERA in DR-Congo. To achieve this aim, the study sought to address the following objectives:

- To identify the level of existing polymorphism among the principal common bean accessions maintained by INERA;
- To evaluate the diversity and the genetic relations of the bean accessions common using molecular markers SSR.

This study is focused on the following assumptions:

- Existence of the same level of variation between the morphological and agronomic characteristics with the genetic variability of the common bean accessions maintained by the INERA (RD-Congo),
- Existence of several phylogenic groups among the accessions in relation to their morphological and agronomic characteristics, and their origins.

MATERIALS AND METHODS

Germplasm collection

The plant material was constituted by a set of 91 accessions coming from INERA's research centre of Mvuazi (Kongo Central), selected as: 36 from Mvuazi, 4 from Gandajika, 30 from Mulungu and 21 from CIAT/Columbia. The main accessions targeted the Common bean of various origin (local, exotic, inbred lines) maintained in Mvuazi (Kongo Central), representing the big diversity of accessions and varietiesof INERA. Ten to twenty seeds descended of the progeny of each 91main accessions concerned, harvest during the year, in holds at random, constituted the basis materials for this study.

Preparation of the material

Ten seeds descended of the progeny of each 91 accessions concerned, harvested in holds and at random, were sown in the green house. After ten days, two young leaves were harvested per accession, puled into micro tubes with liquid nitrogen at -80°C (Mudibu *et al.,* 2011a) and stocked in a ultra low temperature freezer until AND extraction operation and analysis.

DNA extraction

The total genomic DNA was extracted from individual young leaves (10 days old). DNA was extracted from leave samples, using the method of ZR Plant/seed DNA MiniPrep, Catalog n°D6020 of The Epigenetics Company (USA). DNA from individual seedlings was extracted to access the level of genetic diversity within each accession. The quantity and purity of the DNA were checked using a Nano Drop spectrophotometer ND 8000 and 0,8 % agarose gel was done as for quality check, and this was also necessary for DNA normalisation.

PCR and SSRassay

Twelve SSR primers, formerly adapted for Common bean (directly labelled from Applied Biosystems), were used for their clear polymorphism patterns random their position in the Common bean genome, screening then narrow down to two markers per chromosome for diversity study. Upon dilution of DNA samples to 20 ng µL-1, a 10µL PCR mix consisting of 20 ng of DNA, 10 X reaction buffer, 2 mM $MgCL_2$, 0.2 mMdNTPs, 2 pmols of forward and reverse primers, O.5 U Taq polymerase was prepared for each genotype.Temperature cycling was carried out using the GeneAmp PCR systems 9700 (PE-Applied Biosystems) using a program with an initial denaturation at 95°C for 3 min followed by 30 cycles at 94°C for 45 s, 58°C for 1 min, and 72°C for 2 min with a final hold at 72°C for 20 min, and 15°C for ∞. Aftrer the PCR, a few accessions in each primer were randomly selected and their PCR products (3 µl) run on agarose (2%) gel electrophoresis stained with gel red (2.5 µl) at a voltage of 100 V for 40 minutes.Genotyping was carried out by capillary electrophoresis using the ABI PRISM 3730 (Applied Biosystems). DNA fragments were denatured and size-fractioned using capillary electrophoresis. The peaks were sized and the alleles called using Gene-Mapper software and the internal GS500 (-250) LIZ standard.

Cluster analyses

SSR that gave consistent profiles across populations were selected. Dissimilarity indices was estimated using allelic data by simple allele matching and cluster analysis based on unweighted neighbor-joining (Gascuel, 1997) was carried using DARwin 5.0 dissimilarity analysis software (Perrier *et al.,* 2003). To ascertain the statistical strength of genetic relationships identified through this analysis, bootstrapping of the data (10,000 permutations) was performed. The total number of alleles detected, the number of common alleles with allelic frequencies of at least 5%, the observed size range (in base pairs; bp), the allele size differences (in bp) and the polymorphism information content (PIC) values (Smith *et al.,* 2000), and frequencies of unique alleles was calculated for each SSR marker using PowerMarker V3.25 (Liu and Muse, 2005).

Data analysis

Faint bands were not recorded for analysis; the alleles were called and scored using the Gene-Mapper version 3.7 software and the data was subjected to Allelobin software to check the quality of the SSR markers. The data generated from Allelobin was analyzed using Power-Marker version 3.25 to calculate the Polymorphic Information Content (PIC), heterozygosity, number of alleles for each marker, percentage of polymorphic loci estimates, and genetic diversity among the accessions and their genetic distances. Allele and genotype frequencies were scored using haplotype diversity values with PowerMarker version 3.25 (Schenider et *al*. 2000). Darwin Version 5.0 software was used to calculate the principle coordinate analysis (PoCA) and clustering among the genotypes. To determine the genetic relationships and differentiation; the 91 Common bean accessions were clustered based on the matrix of genetic similarities using the Un-weighted Pair Group Method using the Arithmetic Averages (UPGMA) algorithm. Dissimilarity Index was calculated from allelic data by simple matching. The distances were computed for microsatellite data (11 loci) and trees constructed using the neighbour-joining method with DARwin Version 5.0 software.

The genetic distance between accessions was subjected to sequential agglomerative hierarchical nested (SAHN) with un-weighted, pair-group analysis (UPGMA) using Dice's indices as provided in DARwin 5.0. Major clusters were generated from Nei (1987) genetic distance matrices. Analysis of molecular variance (AMOVA, Excoffier et *al*. 1992) was used to partition SSR variation among groups. Significance levels for variance component estimates were computed by a non-parametric permutation procedure using 100 permutations. AMOVA and Fst indices were calculated using the GenAlEx program, version 6.5 (Peakall, R. and Smouse P.E., 2012). Dendrograms was constructed using the neighbour-joining analysis. The method starts with a starlike tree with no hierarchical structure and in a stepwise fashion was find the two operational taxonomic units that minimize the total branch length at each cycle of clustering. The unrooted tree generated by the Neibour joining method was constructed under the principle of minimum evolution. The degree of affinity of similarity based on the data on SSR was be calculated, the significance of the interrelationship was determined.

RESULTS

Marker characterization and allele frequencies

The SSRs used in the study represented di nucleotide repeat units. The allele sizes among the genotypes for all the 12 microsatellites varied from 107 to 229bp (Table 1).

Table 1. The 12 SSR markers used in the study, dyes label, repeat motif, chromosome number and allele size range (http://isa.ciat.cgiar.org/molphas/; Cordoba et *al*., 2010)

Marker	Dye label	Repeat motifs	Chromosome number	Allele size range(bp)
BM 139	NED	$(CT)_{25}$	2	107
BM 140	PET	$(GA)_{30}$	4	190
BM 143	6-FAM	$(GA)_{35}$	3	143
BM 188	VIC	$(CA)_{18}(TA)_{17}$	7	177
BM 151	NED	$(TC)_{14}$	8	153
BMd 1	VIC	$(AT)_{9}$	3	165
BM 172	6-FAM	$(GA)_{23}$	3	107
BM 183	NED	$(TC)_{14}$	7	149
BM 205	6-FAM	$(GT)_{11}$	7	137
GATS 91	VIC	$(GA)_{11}$	2	229
BMd 36	NED	$(TA)_{8}$	3	164
BMd 53	6-FAM	$(GTA)_{5}$	5	105

Number of repeats in the SSR motif had strong correlation with allele number and their polymorphism information content (Table 2).

Table 2. Allele frequency, allele number and diversity indices of 91 Common bean genotypes

Marker name	Major allele frequency	Genotype number	Allele number	Gene diversity	Heterozygosity	PIC
BM 139	0.2747	12	11	0.8418	0.0220	0.8243
BM 172	0.3187	21	11	0.8295	0.1648	0.8119
BM 143	0.3846	13	11	0.7991	0.0220	0.7785
BM 205	0.3132	11	9	0.7544	0.6813	0.7144
GATS 91	0.4066	7	5	0.7141	0.0330	0.6684
BM 183	0.4286	11	7	0.7029	0.0549	0.6574
BM 188	0.4505	6	5	0.6976	0.7253	0.6507
BMd 53	0.4670	7	6	0.6292	0.0110	0.5600
BM 140	0.6154	15	11	0.5854	0.1209	0.5677
BMd 36	0.5110	5	4	0.6145	0.0330	0.5467
BM 151	0.5659	5	4	0.5263	0.0110	0.4277
BMd 1	0.7890	3	5	0.5216	1.0000	0.4070
Mean	0.4354	10	7	0.6845	0.2399	0.6337

Table 3. Genetic diversity for each Common bean populations

		Genetic Diversity				
Population	Sample size	% Polymorphic Loci	Private alleles	Na(SE)	He(SE)	Ho(SE)
Mvuazi	37	100	5	5.000(0.628)	0.632(0.033)	0.267(0.109)
Gandajika	4	75	2	2.417(417)	0.441(0.084)	0.333(0.128)
Mulungu	29	100	5	4.833(588)	0.628(0.028)	0.232(0.112)
CIAT/Colu-mbia	21	100	4	4.750(0.509)	0.629(0.028)	0.267(0.103)
Overall mean	23	93.75	4	4.25(0.5353)	0.582(0.04325)	0.275(0.113)

He = Expected heterozygosity; Na= No. of different alleles; Ho =Observed heterozygosity; SE= Standard Error

Table 4. AMOVA partitioning SSR variation among populations, within individuals, and within population of 91 Common bean genotypes

Source	d.f	Sum of squares (SS)	Variance components (MS)	P-value	Percentage of variation	Fst
Among Population	3	26.966	8.989	0.054	1	0.013
Within Individuals	87	589.534	6.776	2.668	64	0.650
Within Population	91	131.000	1.440	1.440	35	0.654
Total	181	747.500	5.735	4.162	100	0.316

Table 5. Genetic distance matrices calculated according to Nei (1987) for the 91 Common bean genotypes

Country or Location	CIAT/Columbia	Gandajika	Mulungu	Mvuazi
CIAT/Columbia	0.000			
Gandajika	0.162	0.000		
Mulungu	0.005	0.225	0.000	
Mvuazi	0.060	0.243	0.056	0.000

The 12 SSRs revealed a total of 91 alleles with a mean of 7 alleles per marker (Table 2). This was the same average obtained in Common bean accessions of the bean gene bank (Buso et *al*.,2006; Burle *et al.,* 2010).This was lower than the average of 14 and 10 previously reported in Common bean genepools and in genotypes from the Great Lakes region of Central Africa (Matthew *et al.,* 2012; Matthew *et al.,* 2010) but higher than the average reported fromthree genomic libraries of *Phaseolus vulgaris* L. (Gaitan-Soils *et al.,* 2002) who reported 6.0 alleles per locus, and also higher than the average of 4 alleles (Cabral et *al*, 2011) and 2.4 allele per locus reported in Common bean landraces (Khaidizar et*al*., 2012). The highest number of alleles was 11 in markersBM 139, BM 140, BM 143 and BM 172 with allele size range of between 107-229 base pairs and the lowest number of alleles was two, in markers BM 139 and BM 172 with allele size ranges of 107 base pairs. The PIC value over the 12 SSR markers averaged 0.63, ranging from 0.082 for marker BM 139 to 0.81 for marker BM 172. The mean level of heterozygosity per SSR marker was 0.24. This level of heterozygosity is fairly higher than 0.19 to what was reported by Matthew et al. (2010) (Table 2).

Heterozygosity level ranged from 0.011 for markersBM 151 and BMd 53 to 1.00 for marker BMd 1. Marker BM 139 had the highest gene diversity of 0.84 while marker BMd 1 had the lowest gene diversity of 0.52. The mean gene diversity per SSR marker was 0.68 (Table 2). All the loci were polymorphic with Mvuazi population having the highest number of private alleles (Table 3).

Population structure

There was a clear genetic differentiation among individuals within populations, and within the individuals using significance tests based on 1,000 permutations calculated according to Weir and Cockerham (1984) with the Arlequin software (Table 3). However genetic differentiation among populations was low. The percentage variation among populations was 1% compared to the variation within individuals which was 64%, and that within population was 35%. The F_{ST} value was 0.013 indicating negligible genetic differentiation among the population analyzed, and was 0.65 indicating a very great genetic differentiation within individuals and within population analyzed (Table 4).

F_{ST} values up to 0.05 indicate negligible genetic differentiation whereas>0.25 means very great genetic differentiation within the population analyzed.

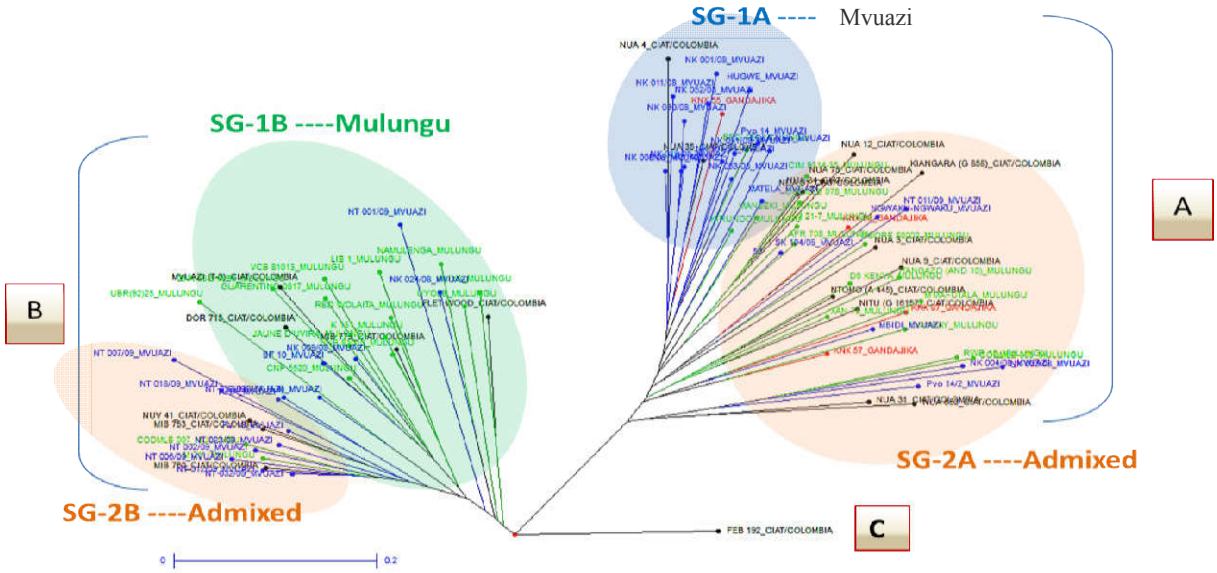

Figure 1. Tree diagram (phenogram) of Common bean genotypes used in the study

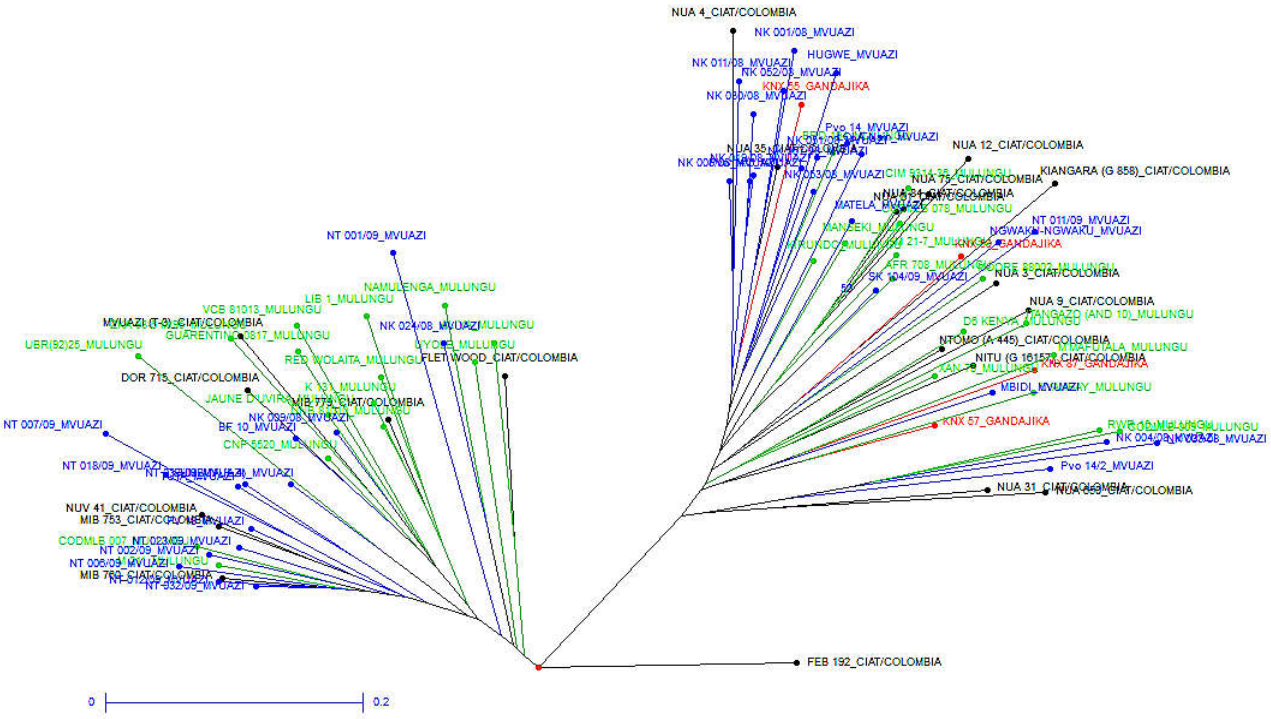

Figure 1 (bis): Tree diagram (phenogram) of Common bean genotypes used in the study

Genetic diversity within regions

The allele frequency based pair-wise genetic distances between the locations calculated using Power- Marker version 3.25 revealed the relatedness of genotypes on a location by location basis (Table 5). Genotypes from Mulungu and Mvuazi were the most distant whereas genotypes from Gandajika and CIAT/Columbia were the closest genetically.

Cluster analysis

The pair-wise dissimilarity indices among the common bean genotypes were estimated using allelic data by simple allele matching followed by cluster analysis using unweighted neighbor-joining algorithm. All the 91 genotypes fell into two (I and II) clusters corresponding mainly to their geographic origin and pedigree. The biggest cluster, cluster I had 52 genotypes with clusters II having 38 genotypes (figure 1 and Figure 2).

DISCUSSION

With the availability of complete Common bean genomic sequence, simple sequence repeats (SSRs) can be used among the preferred markers of choice for studying genetic diversity Common bean (Kumar *et al.,* 2006; Menz *et al.,* 2004; Tams *et al.,* 2005; Shabib *et al.,* 2013; Cordoba *et al.,* 2013) owing to their co-dominance, multi-allelic nature, ease of use and repeatability. The level of allelic diversity observed in the present study was fairly high with SSRs generating 91 alleles. The average number of alleles revealed per SSR locus detected in the present study was 7 and was higher to that detected by Gaitan-Soils *et al.,* 2002, Cabral *et al.,* 2011 and Khaidizar *et al.,* 2012. This could be due to levels of polymorphism of SSR markers, the phenotypic diversity of genotypes that is very

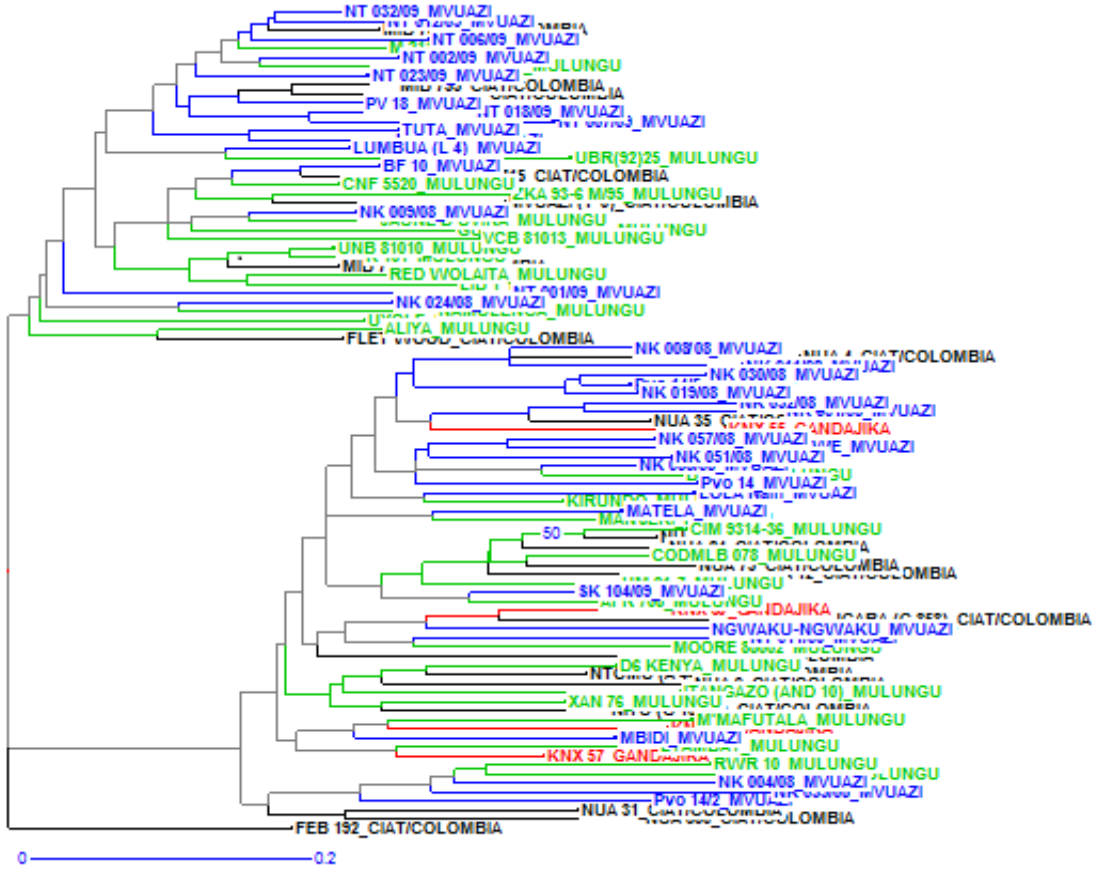

Figure 2. Dendrogram representation of Common bean genotypes used in the study

high in the Grates lakes region (Matthew *et al.,* 2010 and the sensitivity of DNA fragment separation systems. The allele number ranged from 4 (BMd 36 and BM 151) to 11 (BM 139; BM 172; BM 143 and BM 140). All the markers were polymorphic and gave multiple alleles in the range from 4 to 11. Marker BM 139 was highly polymorphic compared to other genomic markers. SSRs with di-nucleotide repeats are the most polymorphic marker class followed by tri-, tetra- and penta-repeat units. A direct relationship exists between marker information content and the number of repeat units (Weber 1990; Innan *et al.,* 1997; Schloss *et al.,* 2002).PIC provides an estimate of the discriminatory power of a locus or loci by the number of alleles expressed and the relative frequencies of those alleles. According to PIC values, no markers were slightly informative (PIC<0.25),2 (BM 151 and BM BMd 1) were reasonably informative (0.25<PIC<0.5, mean PIC = 0.42), 10 (BM 139, BM 172, BM 143, BM 205, GATS 91, BM 183, BM 188, BMd 53, BM 140, BMd 36) were highly informative (0.5<PIC>0.75, mean PIC = 0.68). The overall mean of heterozygosity (0.24) is high than 0.19 observed by Matthew *et al.,* 2010. This high heterozygosity may be also a reflection of the high genetic diversity of the accessions and crossing between accessions though seeds are selected. The gene diversity observed in this study (Mean PIC = 0.63) is similar to the diversity value (0.62) reported by Matthew et al. (2010). The average of observed heterozygosity (0,17) was also high than the one observed by Matthew et al. (2010), reflecting the conditions under the accessions were multiplied and their origin.The SSR loci BM 139, BM 172, BM 143, BM 140 and BM 205were rich in allelic diversity exhibiting (9-11) alleles with highest PIC of 0.82. These primers could be of great use in DNA fingerprinting to characterize Common genetic stocks in view of the emerging needs for Distinctiveness, Uniformity and Stability (DUS) characterization and plant varietal registration, and SSRs are ideal for gene mapping and highly efficient for linkage studies, variety protection, market-assisted selection, studies of diversity, etc (Song *et al.,* 2004; Leal *et al.,* 2010; Cabral *et al.,* 2011).

Conclusion

This study identified a high level of genetic diversity in the accessions. The diversity is valuable considering the high level of Common bean production in the region, which, compared to other regions of the world, and is among the most concentrated and vitally important to its inhabitants. Common bean is an agriculturally complex crop in DR-Congo. It is a source of diversity for breeding programs that may be preserved and prioritized for the crop improvement. This study showed the importance of Common bean SSR markers in the understanding similarities and affiliations among the set of 91 genotypes of Common bean. Based on the above results, the genetic diversity at genetic level exists in Common bean collection. So, pairs of genotypes was identified and can be used to select parents for breeding program to maximize Common bean yield or resistance to abiotic/biotic factors for development of segregating populations in Common bean.

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