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Genetic diversity in yard-long-bean (*Vigna unguiculata* subspecies unguiculata cv-gr *sesquipedalis*) as revealed by simple sequence repeat (SSR) markers

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ABSTRACT

Genetic diversity was investigated among fifty accessions of yard-long-bean (*Vigna unguiculata* subspecies *unguiculata* cultivar group sesquipedalis) using simple sequence repeat (SSR) markers. Sixteen SSR primers were used to amplify DNA of the 50 accessions. A total of 63 polymorphic bands were detected and these were used for cluster analysis. A dendrogram generated following unweighted pair group using mathematical average (UPGMA) cluster analysis isolated one of the accessions from the others at a similarity coefficient of 0.68. However, at 0.76 similarity coefficient, nine clusters were formed with the eighth and ninth clusters containing more than two-thirds of the total accessions used. There were four accessions that paired at 1.0 similarity coefficient. The members of each pair could not be separated from each other on the dendrogram suggesting very close relationships between each pair. The distribution of the yard-long-bean accessions along the axes of the first two principal components showed to a very large extent similar grouping as were identified on the dendrogram. Accessions of yard-long-bean from India showed the highest level of genetic diversity, as revealed by SSR markers, compared with those from other countries thereby providing further evidence in support of the suggestion that India may be the center of diversity for the crop. Thus SSR markers are useful in assessing the genetic diversity among individuals of yard-long-bean.

Key words: Asparagus bean, DNA markers, Genetic variation, Microsatellite markers, UPGMA, vegetable cowpea.

INTRODUCTION

The yard-long-bean also known as asparagus bean belongs to the genus *Vigna* section *Catiang* (VERDCOURT, 1970; MARECHAL *et al.*, 1978). Cultivated cowpea and the yard-long-bean are grouped into subspecies *unguiculata*, which is subdivided into four cultigroups namely *Unguiculata*, *Textilis*, *Biflora* and *Sesquipedalis* with both belonging to the first and last cultigroups respectively (WESTPHAL, 1974; MARECHAL *et al.*, 1978; NG and MARECHAL, 1985). The genus Vigna is a large one and depending on the author the number of species is variable: 170 (FARIS, 1965), between 150 and 170 (SUMMERFIELD *et al.*, 1974), 150 (VERDCOURT, 1970), 154 (STEELE, 1976), and 85 (MARECHAL *et al.*, 1978). MARECHAL *et al.* (1978) subdivided *Vigna* into seven subgenera namely: *Vigna*, *Sigmoidotropis*, *Plectotropis*, *Macrorhyncha*, *Ceratotropis*, *Haydonia* and *Lasiocarpa*.

The yard-long-bean (*V. unguiculata* cultigroup *sesquipedalis*) is the type of cowpea most commonly grown and consumed as vegetable in some countries, especially in Asia. When grown, yard-long-bean plants require staking in order to avoid pods making contact with the soil, which could lead to pods rotting. Both cowpea, (*V. un-*

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TABLE 1
List of yard-long-bean accessions used for the study.
The codes represent the number used in the dendrogram and PCA plot

Codes	TVu No	Origin	Codes	TVu No	Origin
1	21	Philippines	26	2449	Iran
2	22	Philippines	27	2822	Iran
3	522	USA	28	2844	Iran
4	566	USA	29	2852	Iran
5	8381	USA	30	3107	Iran
6	594	Nigeria	31	3238	Iran
7	8429	Nigeria	32	3369	Iran
8	14176	Nigeria	33	3656	New Caledonia
9	14204	Nigeria	34	6644	Liberia
10	16646	Nigeria	35	7270	China
11	992	India	36	14858	China
12	9070	India	37	14862	China
13	9078	India	38	14866	China
14	9081	India	39	14867	China
15	9092	India	40	14868	China
16	10318	India	41	14870	China
17	10322	India	42	14878	China
18	12000	India	43	7275	Sri.Lanka
19	12657	India	44	7798	Papua New Guinea
20	13167	India	45	8390	Papua New Guinea
21	1216	Russia	46	9215	Papua New Guinea
22	1366	Uganda	47	10407	Bangladesh
23	1411	Brazil	48	10811	Tanzania
24	1877	Dem. Rep. of Congo	49	11381	Cameroon
25	2263	Iran	50	13018	Madagascar

guiculata) and yard-long-bean (cultigroup *sesquipedalis*) are products of a post domestication evolution of *V. unguiculata* in different parts of the world (SMARTT, 1985). In the African context the role of *V. unguiculata* is predominantly that of pulse, though they may be exploited to a minor degree as vegetable or spinach (cultigroup *sesquipedalis*), as are *Phaseolus* beans (SMARTT, 1985). According to NG and MARECHAL, (1985) the yard-long-bean evolved in South-East Asia from *V. unguiculata* under intense human selection. The pod morphology in yard-long-bean having been impacted by human preference for succulent and fleshy pods has had repercussions on number of seeds produced per pod. The trend usually is that the parts of a crop plant actually exploited by man for food show gigantism and this is attested to by the long and pendulous pods of yard-long-bean which do not contain significantly higher number of seeds than non-pendulous grain type cowpea (SMARTT, 1985). Pod length in yard-long-bean ranges between 20 and 100 cm among the varieties grown and consumed in countries such as Nepal, Sri Lanka, Philippines, China, India and some countries in the West Indies (TIMSINA, 1989; TIAN and XU, 1993). Genetic studies have shown that pod length is heritable with at least two genes controlling the trait following a cross between cultigroup *sesquipedalis* and cultigroup *textilis* (KRISHNASWAMY *et al.*, 1945). However, JINDLA and SINGH (1970) reported that pod length is governed by multiple factors.

Different types of molecular markers have recently been developed and these are being used to measure genetic diversity among accessions of various crops. LI *et al.* (2001) used SSR markers to study genetic diversity among cowpea germplasm and breeding lines. Their report showed that SSR markers detected variations among the lines tested which were also well differentiated from one another. A number of yard-long-bean lines were included in assessment of genetic diversity involving accessions of different *Vigna* species based on RFLP (FATOKUN *et al.*, 1993) and inter simple sequence repeats (ISSRs) (AJIBADE *et al.*, 2000). The results showed very close relationship between cowpea and yard-long-bean. In addition, molecular markers were found useful in determining genetic variation in

the genus *Vigna*. There is very limited report on the extent of genetic diversity among yard-long-bean accessions from different regions, and where available they were based on morphological parameters. REIS and FREDERICO (2001) tested eight isozymes on *Vigna unguiculata* which included some accessions of yard-long-bean and found that only one isozyme, esterase, detected polymorphism among the genotypes. We report here the results of a study using a subset of the SSR markers developed and tested by LI *et al.* (2001) to estimate the extent of genetic diversity among accessions of yard-long-bean (*Vigna unguiculata* cv-gr *sesquipedalis*).

MATERIALS AND METHOD

Plant material and DNA extraction

Fifty accessions of yard-long-bean (*V. unguiculata* cultigroup sesquipedalis) from diverse geographical locations (Table 1) were selected from the gene bank maintained at the International Institute of Tropical Agriculture (IITA) Ibadan. Nigeria.

Efforts were made to ensure that yard-long-bean accessions included in this study were selected from a wide range of countries. The number of accessions selected for the study was based on the proportion of accessions available from each of the countries, the geographical distribution and variations in seed coat colour, shape and size. Three seeds from each accession were sown in pots containing 25 kg top soil and

TABLE 2
List of SSR primers used: primers sequences, alleles sizes, number of alleles and polymorphism information content (PIC) scores

SSR	Primer sequence	Allele size (bp)	No. of alleles	PIC scores
VM 9	5'accgcacccgattatttcat 5'atcagcagacaggcaagacca	271	2	0.207
VM 27	5'gtccaaagcaaatgagtcaa 5'tgaatgacaatgagggtgc	207	2	0.340
VM 34	5'agctcccctaacctgaat 5'taacccaataataagacacata	216	2	0.207
VM 35	5'ggtcaatagaataatggaaagtgt 5'atggctgaaataggtgtctga	127	4	0.414
VM 36	5'actttctgttttactcgacaactc 5'gtcgctgggggtggcttatt	160	2	0.438
VM 37	5'tgtccgcgttctataaatcagc 5'cgaggatgaagtaacagatgatc	289	3	0.438
VM 39	5'gatggttgtaatgggagagtc 5'aaaaggatgaaattaggagagca	212	5	0.500
VM 53	5'tcaacaacacctaggagccaa 5'atcgtgacctagtgcccacc	144	3	0.553
VM 62	5'gcgggtagttacaatttg 5'gtactgttccatggaagatct	217	5	0.207
VM 67	5'ggatacccatacccgctaaac 5'acatcaatgcctccacagtatct	160	2	0.340
VM 68	5'gaatgagagaagttacggtg 5'gagcacgataatatttggag	250	3	0.340
VM 70	5'gatggtcctttagcttcatgctt 5'acagtcggtttccatatctcgcc	223	4	0.438
VM 71	5'tcgtggcagagaatcaaagacac 5'tgggtggaggcaaaaacaaaac	225	2	0.207
VM 74	5'ctctacaccttccatcattc 5'cctttgctgtgtggtggttt	134	3	0.553
VM 98	5'ggaagcctttggaaattgatg 5'ccctacattgaaggtcaacaa	168	3	0.207
VM 103	5'ctcttgggtacttgactagtc 5'ggcttgaatttccgtatgctg	176	2	0.438
	Total		47	2.94
	Mean		5.83	0.364

TABLE 3
Polymorphism Information Content (PIC) among accessions of yard-long-bean selected from India, Iran and China

	India (8)		Iran (8)		China (8)	
SSR Primers	No. of alleles	PIC	No. of alleles	PIC	No. of alleles	PIC
VM 34	2	0.2068	2	0.3398	2	0.2068
VM 35	2	0.4138	2	0.4138	2	0.3398
VM 36	2	0.4375	0	0.0000	0	0.0000
VM 37	2	0.4375	2	0.4375	0	0.0000
VM 39	3	0.4998	3	0.4998	3	0.4998
VM 62	2	0.2068	2	0.2068	0	0.0000
VM 67	2	0.3398	0	0.0000	0	0.0000
VM 68	2	0.3398	2	0.3398	2	0.2068
VM 70	2	0.4375	2	0.4375	0	0.0000
VM 71	0	0.0000	2	0.2068	0	0.0000
VM 74	3	0.5525	2	0.4138	0	0.0000
VM 78	3	0.5525	3	0.4998	2	0.4138
VM 98	2	0.2068	2	0.2068	0	0.0000
VM 9	2	0.2068	0	0.0000	0	0.0000
Total	29	2.23	4.8379	0.371	24	2.18
Mean	4.0022	0.364	11	2.20	1.667	0.333

[a]0 = Monomorphic primers.

placed on the floor in a screen house at IITA, Ibadan, Nigeria. Newly opened fresh young leaves were picked from each accession two weeks after seedling emergence for DNA extraction. Extra leaves from the same plants were collected in polyethylene bags and stored in –80°C freezer as backup. Each leaf sample was placed in 1.5ml Eppendorf tube, quickly frozen in liquid nitrogen and ground with konte pestles into fine powder. DNA was extracted from the ground leaves according to the procedure described by DELLAPORTA *et al.* (1983). The DNA was diluted in 0.1 × TE (1mM Tris 0.1mM EDTA, pH 8.0) to 10ng/µl concentration.

Primer Screening

Seventy of 121 SSR primers (LI *et al.*, 2001) were screened and optimized for polymorphism and annealing temperature (T_{ann}) using ten randomly selected accessions of yard-long-bean to ensure optimal primer performance across accessions.

Optimal PCR amplification across the ten accessions was achieved with the range between 54°C and 64°C annealing temperature. Sixteen primers that showed good and clear polymorphism with the PCR products were therefore used for the study.

PCR amplification

A 10µl reaction volume containing 1.0µl of 10x buffer, 2.0µl of 10ng/µl template DNA, 1.0µl $MgCl_2$, 0.8µl mixture of 10mM dNTPs (dATP, dCTP, dGTP and dTTP), 4.6µl of ultra pure water, 0.5µl of SSR primers and 0.1µl red hot *Taq* (promega) was loaded in Perkin Elmer Mj cycler for DNA amplification. The PCR reaction was carried out with a profile of 18 cycles at 94°C for 1 minute initial denaturing and extension at 72°C for 1-minute. Annealing temperatures were progressively decreased by 0.5°C every cycle from 64°C to 54°C. The reactions continued for 30 additional cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute and ended with a 10-minute extension at 72°C after about 3 hours. 2.0µl of PCR products were loaded in 3% agarose gel to check for polymorphism before running those that showed polymorphism on polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis of PCR products and data analysis

PCR products were separated on a sequencing gel containing 70ml freshly prepared 6% polyacrylamide solution, 350µl ammonium persulphite (APS) and 35µl TEMED. The gel was run at constant power of 50W, 2500V and 60mA for 3hours. The gel was later fixed, stained, and developed using silver staining kit (Promega corp. Madison WI). Fragments that were clearly resolved on gels were scored as 1 or 0 i.e., present or absent respectively on all the fifty accessions of yard-long-bean. The bands that could not be confidently scored were regarded as missing data. Sixty-three clearly resolved DNA bands amplified by sixteen SSR primers were used for the cluster analysis.

Pairwise distance (similarity) matrices were computed using sequential, hierarchical and nested (SAHN) clustering option of the NTSYS-pc version 2.02j software package (ROHLF, 1993). The program generated a dendrogram, which grouped the test lines on the basis of Nei genetic distance (NEI, 1972) using unweighted pair group using mathematical average (UPGMA) cluster analysis (SNEATH and SOKAL, 1973).

The polymorphism information content (PIC) provides an estimate of the discriminatory power of locus or loci, by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles. PIC values were calculated by the algorithm: PIC = $1-\Sigma^{n}Pi^{2}$ where i starts from 1, Pi^{2} = frequency of the ith alleles. (OTT, 1999; BROWN *et al.*, 1996). PIC values range from 0 (monomorphic) to 1 (highly discriminative, with many alleles each in equal and low frequency). The two dimension Principal Component Analysis (PCA) was also used in the analysis. Only the distribution of the accessions along the first two principal components is considered in this paper.

RESULTS

Data generated from the polymorphic bands resulting from 16 SSR primers that amplified the DNA of 50 yard-long-bean lines were subjected to the computer program (NTSYS-pc version) for analysis. The analysis generated polymorphism information content, a dendrogram and principal component plots.

Polymorphism information content (PIC)

Allele number per primer varied from 2 to 5 with an average of 2.94 alleles per primer (Table 2). Primers VM39 and VM62 generated the highest number of bands among the accessions used with 5 alleles each while primers VM9, VM34, VM36, VM27, VM71, VM67 and VM103 had the least with 2 alleles each. The polymorphism information content (PIC) varied from 0.207 to 0.553 with an average of 0.364. Because of the low number of accessions available from most countries, the PIC values from only three countries (India, China and Iran) were analyzed for diversity index (Table 3).

Thirteen of the sixteen SSR primers showed polymorphism among the eight accessions from India (Table 3). There were a total of 29 polymorphic bands (alleles) among the eight accessions and PIC ranged from 0.21 to 0.55 with a total of 4.84, which was the highest for accessions from any one country. Eleven primers detected 24 polymorphic bands among the eight accessions from Iran. The polymorphism information content (PIC) was 4.00 and ranged from 0.21 to 0.49 with an average of 0.36. The diversity within accessions from China was the least in comparison with those from India and Iran. Only five primers detected 11 polymorphic bands among the eight accessions from China with a PIC value of 1.66.

The Dendrogram

The 50 yard-long-bean accessions used in this study were displayed on a dendrogram (Fig. 1) on which all accessions belonged to a single cluster at 0.68 similarity coefficient. However, at 0.70 similarity coefficient the accession TVu 9078 of Indian origin has been isolated from the other 49 accessions. When the dendrogram is truncated at 0.76 similarity coefficient, nine clusters each with variable number of accessions have formed. The accession TVu 2844 from Iran was next to separate from others at 0.76 similarity coefficient. The third cluster is made up of three accessions one each from Cameroon, India and Nigeria while cluster four has two members one from Nigeria and the second from Madagascar. The fifth cluster has three members one each from India, Nigeria and Democratic Republic of Congo. Two of them TVu 1877 and TVu 8429 from Democratic Republic of Congo and Nigeria respectively, were identical since

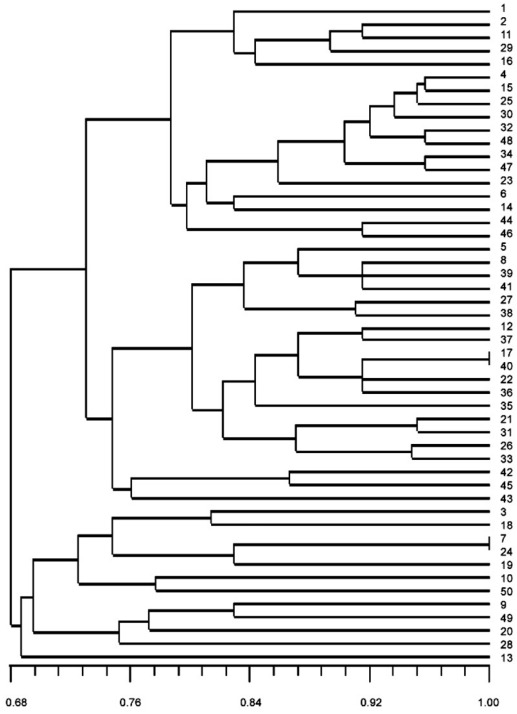

FIGURE 1 - A dendrogram generated from fifty accessions of yard-long-bean following analysis with 16 SSR primers. The numbers 1-50 are the accessions as listed in Table 1.

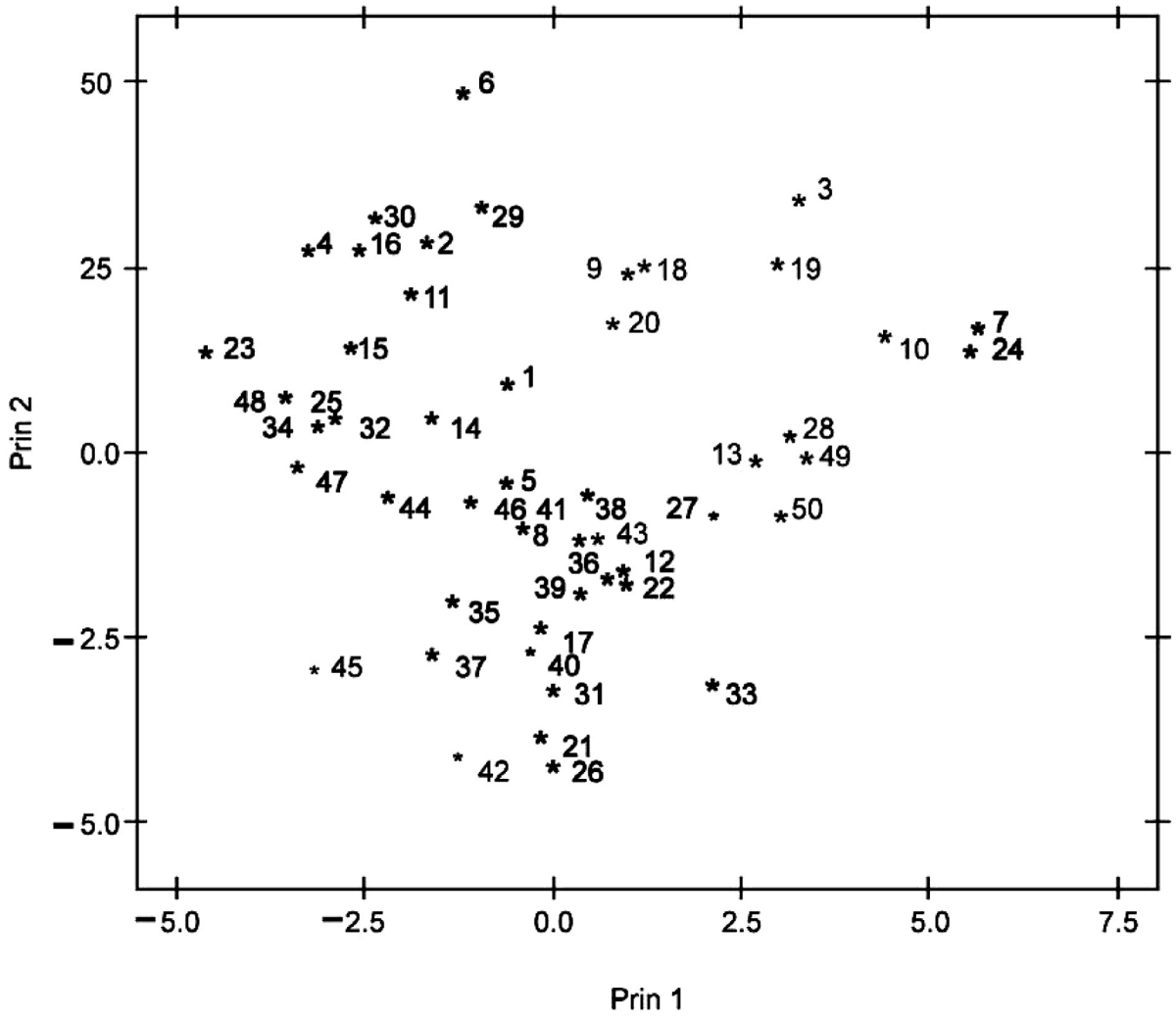

FIGURE 2 - Scatter diagram showing the distribution of the 50 yard-long-bean accessions along the first two principal components. The numbers 1-50 are for the accessions as listed in Table 1.

they remain joined even at 1.00 similarity coefficient. This implies that the two accessions have all bands in common. Cluster six is made up of two accessions one each from India and USA and cluster seven is made up of three accessions one each from China, Papua New Guinea and Sri Lanka. Cluster eight is made up of 17 different accessions from eight countries, with seven of the eight accessions from China belonging to this group. The 17 accessions in this cluster could be sub-divided into two groups at a similarity coefficient of 0.80. One of the two groups has six accessions from USA, Nigeria and Iran with the remaining three from China. Two accessions TVu 14868 and TVu 10322 from India and China respectively also in the group could not be differentiated from one another following SSR analysis of their DNA even at 1.0 similarity coefficient. This suggests that these two lines share same bands as observed among two other accessions in cluster five. The second group is made up of 11 accessions one each from New Caledonia, Russia, Uganda, two from India, two from Iran, and the remaining four from China. Cluster nine has 18 accessions which could be sub-divided to two groups with five and 13 accessions in each. The first group comprises two accessions from Philippines, two from India and one from Iran while the second is made up of three accessions from Iran, two each from India and Papua New Guinea, and one each from Nigeria, USA, Tanzania, Liberia, Bangladesh and Brazil.

Principal Component Analysis (PCA)

The scatter diagram showing the distribution of the accessions along the first two principal components (Prin 1 and Prin 2) (Fig. 2) reveals the relationships among the fifty accessions of yard-long-bean tested. The first principal component (PRIN 1) accounts for most of the variability in the data followed by principal component 2, with each succeeding component accounting for less of the remaining variability. The accessions that belong to the two biggest clusters eight and nine on the dendrogram are also found to be closely associated on the PCA plot. The accessions in cluster nine are found mainly along the upper left section of the PCA plot i.e., the negative part of component 1 and positive part of component 2. However those belonging to cluster eight are found mainly in the lower and central section of the plot i.e., to the negative section of component 2 and positive section of component 1. The two accessions that could not be differentiated from one another in cluster eight, TVu 10322 and TVu 14868 are also located close to one another on the PCA plot as are TVu 8429 from Democratic Republic of Congo and TVu 1877 from Nigeria both in cluster five. Members of the smaller clusters are mostly dispersed towards the positive sections of both components 1 and 2. The exceptions are TVu 14878 (China) and TVu 8390 (Papua New Guinea) which are located towards the bottom left of the PCA plot. The two accessions clustered with TVu 7275 (Sri Lanka) on the dendrogram but the latter is closely located on the PCA plot to members of the large cluster eight.

DISCUSSION

This study revealed that microsatellite markers detect polymorphism among yard-long-bean (*V. unguiculata* ssp. *unguiculata* cv-gr *sesquipedal*is) accessions that were tested and therefore would be useful for assessing genetic diversity in the crop. Published reports from studies carried out in various crop species reveal that microsatellites are markers of choice in measuring genetic diversity among them. In cowpea (*Vigna unguiculata*) Li *et al.* (2001) reported that microsatellite markers were helpful in determining genetic diversity among breeding lines developed at the International Institute of Tropical Agriculture (IITA) and could help to some extent in determining their pedigrees. The 16 microsatellite markers used in this study detected 53 alleles among the fifty yard-long-bean accessions with markers VM39 and VM62 detecting the most number of alleles while marker VM27 detected the smallest number. The latter microsatellite marker, VM27, was also reported to detect the lowest number of alleles among 90 cultivated cowpea lines and one wild cross compatible relative (Li *et al.*, 2001). In their study Li *et al.* (2001) reported that number of alleles range from 2 to 7 in cultivated cowpea lines. This suggests that genetic variation among the yard-long-bean lines based on microsatellite analysis is lower when compared with that observed among cowpea lines. In general, the number of alleles detected among the yard-long-bean is lower when compared with those found among cultivated cowpea and their wild cross compatible relatives (Ogunkanmi, unpublished data). The higher level of genetic variation among the cowpea breeding lines evaluated by Li *et al.* (2001) could be attributed to the diversity among parental lines that were crossed in the process of developing the cowpea breeding lines. Usually crosses are made between many parents from diverse sources because of their desirable traits. Their hybrids are advanced to generate segregating populations from where individuals and families are selected on the basis of their performance. Such selections give rise to the breeding lines.

The relatively low number of alleles detected among the yard-long-bean accessions is a reflection of the low level of genetic diversity existing in the crop. Based on the results of the present study it will appear that despite that the yard-long-bean accessions were collected from different continents and countries with variable agro-ecologies, the level of diversity among them is low when compared with some other self pollinated crops. For example, microsatellite markers detected between three and 25 alleles in rice (Yang *et al.*, 1994), 11 to 26 in soybean (Rongwen *et al.*, 1995) and three to 16 in wheat (Plaschke *et al.*, 1995). It has been suggested that yard-long-bean evolved from cultivated cowpea introduced from Africa to Asia where the succulent and pendulous pods are eaten as vegetable (Faris, 1965). Ajibade *et al.* (2000) also re-

ported low level of diversity among five yard-long-bean genotypes they tested using inter simple sequence repeat (ISSR) markers. It could be speculated that only a small number of cowpea lines were introduced into India from Africa and the yard-long-bean would have evolved from these which represent a narrow base of cowpea genetic diversity.

The evolution of yard-long-bean from cowpea occurred in Asia (NG and MARECHAL, 1985). The succulent and pendulous pods of yard-long-bean appears to be the major trait that distinguishes it from the cultigroup Unguiculata i.e., cowpea. When compared with cowpea much less genetic variation exists among accessions and varieties of yard-long-bean. Following use of RFLP markers to study diversity among accessions of the genus *Vigna*, FATOKUN *et al.* (1993) opined that the selection which favoured succulent and fleshy pod types among *V. unguiculata* introduced to Asia, especially India gave rise to day yard-long-bean. They stressed that the strong selection pressure that was exerted on this crop in India could have modified it to its present day form

The yard-long-bean accessions from India showed the highest level of variation when compared with those from other countries. This could be explained by the route followed by cowpea the progenitor of yard-long-bean into Asia. According to NG and MARECHAL (1985) cowpea moved from Africa, its center of origin, into Asia through India from where it was later distributed to other parts of the continent. It is conceivable that the selection for plants that would provide succulent and fleshy pods for consumption as vegetable would have commenced in India and this could have been responsible for the higher level of genetic diversity among accessions from the country. VAVILOV (1926) postulated that an area with high level of genetic variation would be one where the crop must have been cultivated for a longer time. Genotypes from such areas accumulate more mutations and gene recombinations following interbreeding among them. All of these contribute to the genetic diversity observed in the various crop species. It is also interesting to note that wild relatives of yard-long-bean have not been reported in Asia rather what are found in north India are intermediates between cultigroups *Unguiculata* and *Sesquipedalis* (NG and MARECHAL, 1985).

There were two pairs of accessions that could not be distinguished by the microsatellite markers. AJIBADE *et al.* (2000) used ISSR to study genetic diversity among *Vigna unguiculata* and found also that two lines of yard-long-bean could not be distinguished from one another. A look at the list of yard-long-bean accessions they used showed that the two that could not be differentiated were both obtained from Nepal even though they had different accession numbers. The high level of similarity among the pairs of accessions may be the result of seed mixture while handling the seeds in the gene bank. It could also be that the similar accessions came from same seed lot and found their way to the gene bank from different sources hence they are given different acquisition numbers. Essentially, they could be duplicates. It is also probable that in one case the two lines one each from Democratic Republic of Congo and Nigeria that could not be differentiated by the microsatellite primers were introduced to Africa through Asian expatriates who may have exchanged seeds for planting in their home gardens where yard-long-bean are most commonly found in these African countries. Like cowpea, yard-long-bean is highly self pollinating which will encourage the plants to maintain their genetic integrity.

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