



Genetic diversity assessment of winged bean (*psophocarpus tetragonolobus*) accessions revealed by Inter-Simple Sequence Repeat (ISSR) markers

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Abstract

Winged bean (*Psophocarpus tetragonolobus* (L.) DC.) is a tuberous underutilised legume grown in tropical regions with great potentials as a protein rich and food security crop for the tropics. The genetic diversity in 20 winged bean accessions from diverse locations were determined using Inter-Simple Sequence Repeat (ISSR) markers. ISSR primers: UBC810, UBC811, UBC827 and UBC855 generated a total of 127 amplified bands which were all polymorphic (100%). The number of polymorphic bands within the 20 winged bean accessions identified per primer ranged from 14 (UBC811) to 53 (UBC855) and 9 (UBC811) to 18 (UBC855) accessions were identified. The polymorphic information content (PIC) ranged from 0.500 (UBC 811) to 0.874 (UBC 855) with an average of 0.718. The high percent polymorphism (100%) obtained indicates high genetic diversity across the genomic loci of the accessions studied and revealed the usefulness of ISSR markers in determining the extent of genetic variability in *Psophocarpus tetragonolobus*. Primer UBC855 was most informative for genetic diversity studies in winged bean with a PIC of 0.874. ISSR markers are suitable for genetic diversity assessment in winged bean and could enhance the efficient management of germplasm for winged bean breeding and conservation.

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Introduction

Winged bean (*Psophocarpus tetragonolobus* (L.) DC.) from the *fabaceae* family, is an underutilised leguminous crop grown in tropical regions. It is often called Winged bean or Goa bean [1]. *Psophocarpus tetragonolobus* is grown in many parts of the humid tropics, including Central and South America, the Caribbean, Africa, Oceania and Asia grown abundantly in hot, humid

equatorial countries, like India, Burma, Sri Lanka, Thailand and Philippines [1]. Underutilised crops have attracted relatively little attention from researchers and funding organisations across the world, and as a consequence, the potential for these crops to become part of sustainable agricultural systems has not been fully exploited. Underutilised crops supply essential micro-nu-



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trients and thus complement staple foods [2]. Winged bean has great potentials as a major multi-purpose food crop in tropics of Asia, Africa and Latin America [3]. *Psophocarpus* species are characterized by their tuberous roots and winged pods [4]. It is mainly self-pollinated and possesses a twinning pattern, tuberous roots, longitudinally winged pods, and both annual and perennial growth forms [5,6]. Since the species is self-pollinated, homozygosity will be higher than heterozygosity making it possible for molecular breeding to be used to facilitate utilization of winged bean genetic resources, especially among accessions, through hybridization of beneficial traits [7]. Winged bean has a lot of nutrients and all parts of the plant are edible. Leaves can be eaten like spinach, flowers can be used in salads, tubers can be eaten raw or cooked, and seeds can be used in similar ways as soybean and each of these parts contains Vitamin A, Vitamin C, Calcium and Iron, among other nutrients [8]. The nutritious tuberous roots are about 20% protein, the leaves and flowers are also high in protein (10–15%) [6]. Its protein content and oil quality are comparable to those of soybean and higher than those of other pulses [9]. However, in areas where protein deficiency is high, the winged bean with its high protein content can play a major role in meeting the nutritional needs of the people. In spite of its various uses and its potential as a crop to help overcome malnutrition, winged bean is yet to be fully exploited. Assessment of genetic variations and relationships among leguminous crops could for this reason, play a critical role in breeding programmes to improve grain yield, oil and protein content. Genetic improvement of winged bean varieties which targets production traits such as increased harvest index, increased yield and controlled growth habit, could be significantly enhanced via the application of biotechnological tools. Morphological characterization is often used for genetic diversity studies in crops based on phenotypic traits but these are affected by environmental factors. Several molecular markers exist that can be used as tools to determine genetic variability within/among populations. Moreover, molecular markers permit comprehensive assessment of genetic variability present within a species leading to the identification of novel alleles for traits of interest [10]. Several genetic diversity studies have been carried out on winged bean using molecular markers including; RAPD and ISSR [1,7]; ISSR [11]; SSR and SNP [6] and expressed sequence tags (EST) [7]. The information obtained from these markers could facilitate the development of improved cultivars of winged bean and evaluate its importance for nutritional security. Inter-simple sequence repeat (ISSR) is the amplification of segments of DNA that occur at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite directions [12,13]. Inter simple sequence repeat marker in addition to its suitability for genetic diversity

study, it is highly polymorphic, reproducible, cost effective and requires no prior sequence information [14]. This suggests that ISSR could be an equitable tool to assess the changes of diversity in agronomically important crops as well as underutilised crops with unknown sequences [15]. Information provided by molecular characterization can be useful in identifying promising winged bean genotypes that could be incorporated into breeding programmes for genetic improvement. Therefore, this study was conducted to assess the genetic diversity and relationship among 20 accessions of winged bean using ISSR markers as well as to identify the most informative and polymorphic markers suitable for future phylogenetic studies and genetic improvement of winged bean.

Materials and methods

Plant material

A total of 20 winged bean accessions (*Psophocarpus tetragonolobus* (L.) DC.) were obtained from the Genetic Resources Centre of the International Institute of Tropical Agriculture (IITA) Ibadan (Table 1).

Genomic DNA Extraction

Total genomic DNA was isolated from fresh leaves of two weeks old seedlings using a modified Sodium dodecyl sulfate (SDS) extraction protocol [16]. Yield and purity of the genomic DNA was estimated on a Nanodrop spectrophotometer (Thermo Scientific USA).

ISSR-PCR Amplification

Inter simple sequence repeat (ISSR) primers were screened for consistent, well banded, reproducible profiles using winged bean DNA. The annealing temperatures for the primers were optimized using different temperatures (45°C, 49°C, 50°C, 53°C, 54°C, 55°C, 57°C, 58°C and 60°C) and the final reactions were carried out at these optimized temperatures that gave optimum polymorphism (Table 2). Five (5) ISSR primers were used for PCR amplification. These primers were synthesized by Inqaba Biotech South Africa.

The total volume of 10 µl contained 2 µl of template DNA (50 ng), 2 µl of primer (10 pm), 1 µl each dNTPs (200 µm), 1 µl of MgCl₂ (25 mM) ion concentration in suitable 10X buffer, 0.2 µl of 0.5 unit of the thermostable *Taq* DNA polymerase and 3.8 µl of sterile distilled water. The thermocycler was programmed with an initial denaturation for 5 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 secs, annealing at optimized temperature (45/53°C) for 1 min and extension at 72°C for 2 min followed by 7 min of extension at 72°C

Table 1: List of Winged bean accessions used for this study

TPt-2	NGA	Ibadan Local-2	B.T. Kang	Light Brown
TPt-5	NGA	-	-	Black
TPt-9	N/A	DASF1(Ups32)	Dr. T.N. Kahn.	Brown
TPt-10	PNG	New Guinea Bulk	Dr. T.N. Kahn.	Brown
TPt-11	CRI	PI.338610	-	Brown
TPt-12	LIB	-	Dr. Okigbo	Brown
TPt-13	NGA	-	Dr. Okigbo	Brown
TPt-14	IDN	Indonesia 799(2)B	Dr.S. Sastraprada & B.N Bog	Brown
TPt-16	IDN	Indonesia 1009(A)	Dr. S. Sastraprada & B.N. Bog	Brown

TPt-17	IDN	Indonesia 1016	Dr.S. Sastraprada& B.N. Bog	Brown
TPt-18	N/A	-	Dr D. Nangju	Brown
TPt-19	NGA	Ups-31	Dr. Okigbo	Black
TPt-21	PNG	Ups-38	Dr. Okigbo	Brown
TPt-22	PNG	Ups-45	Dr. Okigbo	Brown
TPt-26	NGA	Ups-71	Dr. Okigbo	Brown
TPt-31	IDN	-	-	Brown
TPt-33	N/A	-	-	Brown
TPt-51	BGD	BARI-0026	-	Dark Brown
TPt-53	BGD	BARI-0032	-	Dark Brown
TPt-154	N/A	-	-	Light Brown

TPT: Tropical Psophocarpus Tetragonolobus; NGA: Nigeria; BGD: Bangladesh; IDN: Indonesia; CRI: Costa Rica; PNG: Papua New Guinea; LIB: Liberia; Source: Genetic Resources Centre Passport data IITA, Ibadan

Table 2: ISSR markers used for the amplification of DNA samples of Winged bean

S/No	ISSR primer	Primer sequence (5'-3')	Melting temp (Tm°C)
1	UBC 841	GAGAGAGAGAGAGACTC	53
2	UBC 810	GAGAGAGAGAGAGAT	45
3	UBC 827	ACACACACACACAC-G	53
4	UBC 811	GAGAGAGAGAGAGAC	45
5	UBC 855	ACACACACACACACCTT	53

Source: Inqaba Biotech, South Africa.

Agarose Gel Electrophoresis

The amplified products were electrophoresed on 0.8% agarose gel containing 1X TBE buffer, stained with 1.5 µl ethidium bromide (1 mg/ml). Three micro litres (3 µl) of each amplified products were loaded with 1.5 µl of loading dye and the PCR product was electrophoresed at 100 V for 1 hr. 1 Kb DNA ladder was used to compare the band sizes of the amplified products. The gel was visualized in a Transilluminator (Model-2, Upland, CA, USA) after electrophoresis. Banding patterns of the 20 accessions were examined and documented. Each band was considered as a character and absence or presence was coded for analysis. Data matrix generated from the ISSR profiles for fragments of similar molecular weight from each individual were scored as present 1 or absent (0). The data obtained from scoring the ISSR bands were used for genetic dissimilarity matrix using Jaccard's similarity coefficient [17]. Phylogenetic relations were determined by cluster analysis using unweighted pair-group method with arithmetic averages (UPGMA) with the Numerical Taxonomic and Multivariate Analysis System (NTSYS-pc) software version 2.02 [18]. Multivariate grouping was carried out using factorial coordinate analysis (FCoA) with Darwin software version 6.0 [19] while polymorphic information content (PIC) was calculated using the method of Ojuederie et al. [17].

Results

Polymorphism Revealed by Inter-Simple Sequence Repeat (ISSR) Markers

A total of five ISSR primers were tested initially and only four primers gave distinct polymorphic bands. A total of 127 alleles with an average of 31.75 per primer were amplified (Table

3). Primer UBC855 amplified a maximum of 53 alleles while UBC811 amplified a minimum of 14 alleles. All of the 127 alleles were polymorphic, revealing 100% polymorphism. The PIC values obtained ranged from 0.500-0.874 with an average of 0.718. Allele frequency ranged from 0.236 (UBC810) to 0.450 (UBC827). Primer UBC855 had the highest PIC and amplified the highest number of accessions (18). The ISSR banding profiles of the 20 winged bean accessions using UBC810, UBC827 and UBC855 are shown in Figures 1 to 3. For the three primers, the band sizes ranged from 100bp (UBC810) to 400p (UBC855).

Unweighted pair group method with arithmetic averages (UPGMA) cluster analysis

The dendrogram grouped the 20 accessions of winged bean into four major clusters (Figure 4). The dissimilarity distance ranged from 0.515 to 0.929. Cluster I consisted of three accessions (TPt14, TPt16 and TPt17) from Indonesia, TPt13 (Nigeria), TPt12 (Liberia) and TPt18 with unknown origin, which was genetically isolated from the other accessions in the group. Most of the accessions fell into the second cluster (8 accessions) which was subdivided into 2 subclusters (2.1 and 2.2). Subcluster 2.1 had 2 accessions (TPt21 and TPt22) from Papua New Guinea, TPt19 (Nigeria) and TPt51 (Bangladesh). Subcluster 2.2 had accessions TPt26 (Nigeria), TPt53 (Bangladesh), TPt31 (Indonesia) and accession TPt33 with unknown origin. Cluster III consisted of two accessions (TPt2 and TPt5) from Nigeria, TPt10 (Papua New Guinea) and accession TPt9 with unknown origin. The fourth cluster consisted of just 2 accessions, TPt11 from Costa Rica and TPt154 with unknown origin

Table 3: Polymorphism revealed by ISSR primers in *Psophocarpus tetragonolobus*

ISSR Primers	Number of amplified bands	Polymorphic bands	Percent Polymorphism	No of Accessions identified	Allele frequency	PIC
UBC 810	33	33	100	15	0.236	0.832
UBC 811	14	14	100	9	0.350	0.500
UBC 827	27	27	100	11	0.450	0.664
UBC 855	53	53	100	18	0.241	0.874
Total	127	127	400	53	1.277	2.870
Average	31.75	31.75	100	13.25	0.319	0.718

PIC: Polymorphic information content

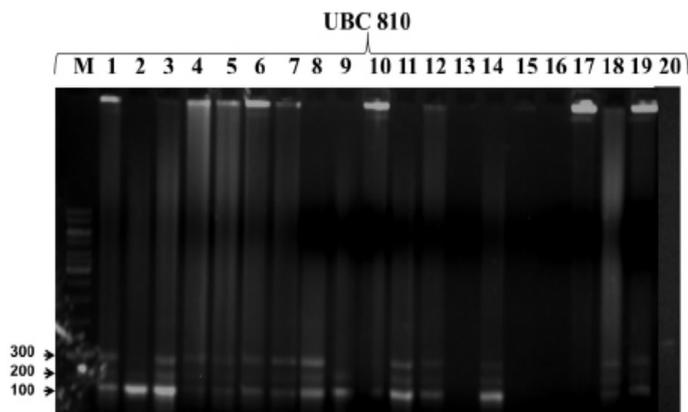


Figure 1: ISSR banding profile of 20 winged bean accessions using primer UBC 810. Lane-m, 100 bp maker, Lanes 1-20 represents UBC 810 DNA profile of 20 winged bean accessions

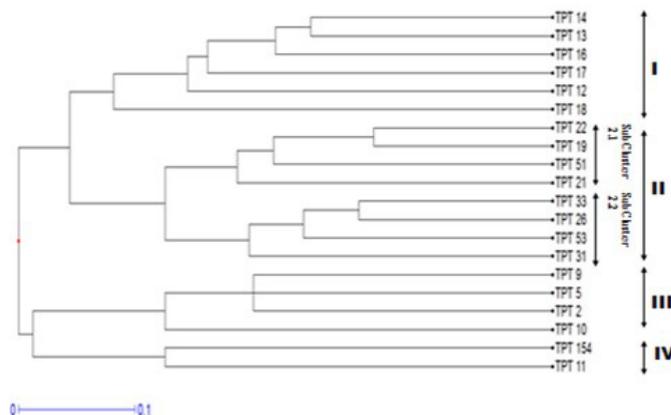


Figure 4: Dendrogram of genetic relationships of 20 accessions of winged bean as revealed by four ISSR markers using UPGMA cluster analysis

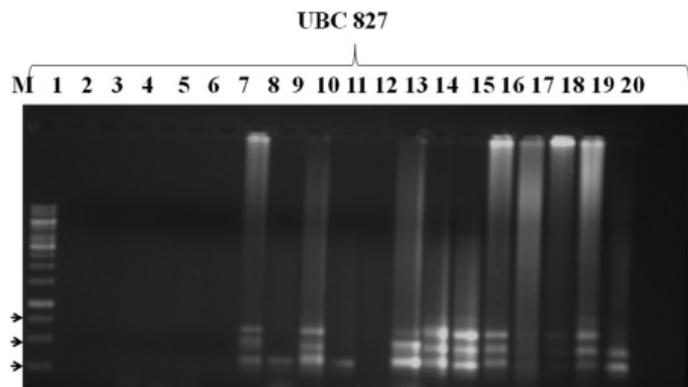


Figure 2: ISSR banding profile of 20 winged bean accessions using primer UBC 827. Lane-m, 100 bp maker, Lanes 1-20 represents UBC 827 DNA profile from 20 winged bean accessions

Factorial co-ordinate analysis of 20 winged bean accessions

The factorial coordinate analysis (FCoA) placed the 20 winged bean accessions into five groups (Figure 5). Group I had six accessions: TPt12, TPt13, TPt14, TPt16, TPt17 and TPt18. Accessions TPt19, TPt21, TPt22 and TPt51 were placed in group II. Group III had four accessions with TPt2, TPt9 and TPt10 closely related to one another and TPt5 genetically isolated. Accessions TPt11 and TPt154 were the only accessions in group IV while Group V had four accessions including TPt26, TPt31, TPt33 and TPt53.

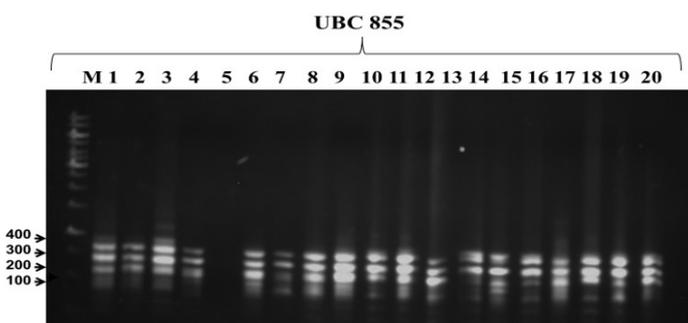


Figure 3: ISSR banding profile of 20 winged bean accessions using ISSR primer UBC 855. Lane-m, 100 bp maker, Lanes 1-20 represents UBC 855 DNA profile of 20 winged bean accessions

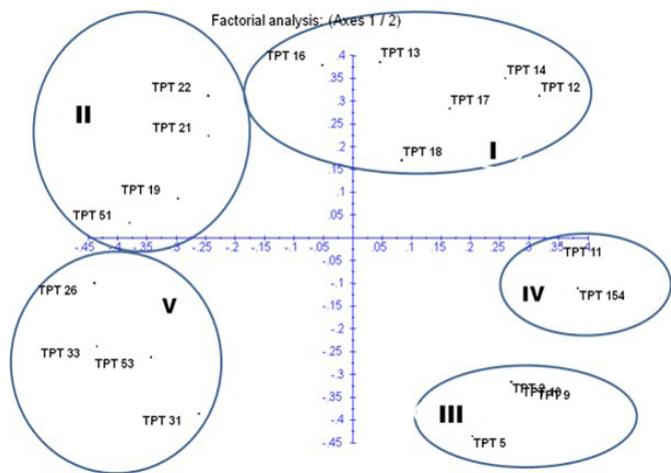


Figure 4: Factorial co-ordinate analysis for winged bean accessions as generated by DARwin software version 6.0 using dissimilarity coefficient matrix

Discussion

This study investigated the genetic diversity and relationship among 20 accessions of winged bean using five ISSR primers. The utilization and efficiency of ISSR markers to determine genetic polymorphisms have been proven to be efficient for diversity studies in other leguminous crops such as chickpea [20], groundnut [21], wheat [22], common bean [23], mungbean [24] and equally in winged bean [1,11]. Four ISSR primers used for genetic diversity assessment in this study produced 14 to 53 polymorphic bands per locus. This is higher than the range of 5 to 15 polymorphic bands reported by Mohanty et al. [1] and 7 to 11 polymorphic bands reported by Chen et al. [11]. Hundred percent polymorphism (100%) obtained in this study for four ISSR markers is much higher than that reported by Chen et al. [11] who recorded low polymorphism from five ISSR markers across 45 accessions of winged bean and obtained an average of 65.7% polymorphism. The total number of polymorphic bands for all four primers was 127 with an average of 31.75 whereas Mohanty et al. [1] reported a total of 86 polymorphic bands for 24 winged bean accessions using 7 ISSR primers while Chen et al. [11] reported a total of 44 polymorphic bands from 45 winged bean accessions using five ISSR markers. The number of potential ISSR markers depends on the frequency of microsatellites, which changes with species. They are highly polymorphic and have been used in innumerable crop plants to study the genetic diversity, phylogeny, gene tagging and genome mapping [1]. According to the result of this study, the ISSR markers used were highly polymorphic when compared to those used by Chen et al. [11] and Mohanty et al. [1]. ISSR-PCR is usually conducted with an annealing temperature (T_a) of 45°C-60°C depending on the melting temperature of the ISSR primer [25]. However, Ng and Szmidt [26] obtained promising results using touchdown PCR method to obtain good amplifications with ISSR primers that were difficult to optimise using the standard PCR methods. In this study the method of Reddy et al. [25] was applied since the annealing temperatures of the primers had to be optimized to obtain scorable amplicons. In the work of Chen et al. [11], ISSR primers UBC827 and UBC841 amplified a total of 16 and 13 bands with percent polymorphisms of 56.3% and 69.2% respectively. The annealing temperatures used for both markers were set at 51.4°C and 54.5°C. In another study by Mohanty et al. [1], UBC827 and UBC841 gave 93.3% and 100% polymorphism respectively at an annealing temperature of 55°C. However, in the present study, UBC827 amplified 27 bands which were all polymorphic (100%) at an annealing temperature of 53°C but UBC841 failed to amplify any band despite optimisation of the annealing temperature between 45°C to 60°C. Heterozygosity and polymorphic information content are important in the determination of the degree of polymorphism in molecular markers [27]. Markers with polymorphic information content above 0.5 are considered highly informative [28,29]. The average PIC value of the 4 markers used in this study was 0.718, with PIC values greater than 0.5 across all the markers 0.500 (UBC811) - 0.874 (UBC855) indicating high resolving power of the ISSR markers. The PIC values obtained are higher than the range of 0.203-0.354 reported by Mohanty et al [1]. Wong et al. [7] recently reported PIC values ranging from 0.250-0.840 using SSR markers for diversity study in winged bean. The ISSR markers used in this study were able to amplify a minimum of 9 (UBC811) to a maximum of 18 (UBC855) accessions and all recorded 100% polymorphism. This was in line with the reports of Dagneu et al. [23] who also reported 100% polymorphism in

genetic diversity study of 12 accessions of common bean using seven ISSR markers. The markers have also been useful in detecting diversity in other crops such as Tef [30], Coffee [31], Lentils [32], Sesame [13] and Walnut [33]. The FCoA grouped the 20 winged bean accessions into five clusters and the grouping was similar to that of the dendrogram. However, in the dendrogram, accessions TPt9, TPt5 and TPt2 were closely related and TPt10 genetically distant from the other accessions in cluster III but accessions TPt2, TPt9 and TPt10 were closely related and TPt5 genetically isolated from the other accessions in Group III of the FCoA. In both analyses TPt2 and TPt9 were closely related. This implies that accession TPt9 of unknown origin could have originated from Nigeria. Accession TPt5 was genetically isolated from the other three accessions in Group III of the FCoA probably because it had black seed coat colour while accessions TPt2, TPt9 and TPt10 had brown seed colour. On the other hand, accession TPt10 was genetically isolated from TPt2, TPt5 and TPt9 on the dendrogram because these accessions originated from Nigeria while TPt10 originated from Papua New Guinea. Accession TPt18 in cluster 1 and Group 1 in both the dendrogram and FCoA was genetically isolated from other accessions in the cluster/group, its origin remains unknown. In subcluster 2.2 of the dendrogram, accessions TPt33 and TPt26 were closely related. Accession TPt33 could have originated from Nigeria as TPt26 and both accessions have similar seed coat colour. Similarly, in cluster IV of the dendrogram and group IV of the FCoA, accessions TPt154 and TPt11 were closely related and the only accessions in the group/cluster. Since accession TPt11 originated from Costa Rica, there is a possibility that Costa Rica could also be the origin of TPt154. In the research of Wong et al. [34] on improving winged bean productivity, accessions TPt17 (potassium: 3310 mg), TPt33 (phosphorus: 492.5 mg and copper: 2.48 mg), and TPt51 (zinc: 8 mg) were identified as accessions with the highest amount of mineral nutrients. Accessions TPt17 in this study was placed in Cluster I of the dendrogram and Group I of the FCoA. Other accessions in this group could also have higher potassium content. Similarly, accession TPt51 with high zinc content was placed in Group II of the FCoA and subcluster 2.1 of the dendrogram. The other accessions in this group could also be rich in zinc content, while accession TPt33 in Group V of the FCoA and subcluster 2.2 of the dendrogram could mean that accessions TPt26, TPt31 and TPt53 in the same group and cluster may also be rich in phosphorous and copper. Clustering patterns in the dendrogram and FCoA analysis revealed considerable genetic variations among the 20 winged bean accessions and also showed that the grouping is not based on geographical location. This agrees with the report of Chen et al. [11] that there was no significant correlation between the genetic distance and land under cultivation for winged bean investigated. However, seeds with similar seed colour (Brown) were grouped together in cluster I, but that wasn't the case with the other clusters. The grouping of the accessions could have been in respect to specific phytochemical contents, the traits that they exhibit and their responses to various physiological conditions. Further research is needed to confirm this. Winged bean is a promising alternative to protein-rich soybean especially in the tropics where protein deficiency is high [6]. The knowledge of the genetic diversity and molecular characterization of the crop can help in identifying potential elite genotypes. Genetic diversity study will offer significant aid for targeted genetic improvement of nutritional and other quality traits in winged bean which will give rise to a high quality, legume-based protein diet to those areas with high protein deficiency [6].

Conclusion

The present study highlighted significant information on the genetic relationship among the 20 accessions of winged bean and confirmed the potential use of ISSR markers to reveal the extent of genetic variability. This opens up an opportunity for future winged bean improvement programmes. ISSR marker UBC855 was found to be the most polymorphic and informative ISSR marker for genetic diversity studies in winged bean, it revealed 100% polymorphism and identified the highest number of winged bean accessions (18). Assessment of genetic variations and relationships may play a significant role in genetic improvement programmes to improve key quality traits of winged bean such as grain yield, oil and protein content. In general, ISSR markers are useful markers for the assessment of genetic diversity in winged bean germplasm, which could enhance the effectiveness of germplasm management for future winged bean improvement programmes and conservation.

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