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Molecular Characterization of *Vigna unguiculata* (L.) Walp Collection based on ISSR Markers

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ABSTRACT

Background: In Azerbaijan, cow pea is one of the fundamental legumes. This study was carried out at the Institute of Genetic Resources, Azerbaijan National Academy of Sciences to evaluate genetic diversity in 28 cowpea accessions from Azerbaijan national gene bank. The aim of this study was to evaluate the genotypic diversity, relatedness and population structure among local cow pea resources available in Azerbaijan and the cultivated VIR accessions based on ISSR markers.

Methods: The studies were conducted in 2017-2019 at the Institute of Genetic Resources (IGR) of the National Academy of Sciences (NAS) of Azerbaijan. 28 samples were used as research material: 10 of them were local forms and 18 were samples obtained from VIR. Sowing of collection samples was carried out in duplicate with an area of food of one plant 10 × 45 cm at the optimum time, in the at the end of April.

Result: In the study, 28 alleles were synthesized for the collection with five ISSR markers, with an average polymorphism index of 75%. The rich genetic diversity (GMO = 0.80; PIC = 0.34) was found in the collection. Statistical analyzes have shown that the efficiency of the UBC 835 and the UBC 818 premieres was high. The average genetic distance index in the cow pea collection was 0.33. 71% of the samples were genetically identifiable and 29% revealed that the genotype was identical across the studied loci. PCoA and cluster analysis identified 3 genetically distinct groups within the collection, with the first two coordinates explaining 57% of this genetic variation.

Key words: Biodiversity, Genotype, ISSR markers, Polymorphism, *Vigna unguiculata*.

INTRODUCTION

Vigna Savi, one of the richest breeds of the Legumes chapter, combines a number of important cultural and wild species from economic point of view. *Vigna unguiculata* (2n = 2× = 22) is believed to have originated in Africa, where a large genetic diversity of wild types occurs throughout the continent, particularly southern Africa, however the greatest genetic diversity of cultivated cowpea is found in west Africa (Prota, 2006). Pasquet, (1999) reported that cowpea was domesticated in Northeast Africa and a secondary center of domestication was in West Africa and the Indian sub-continent. At present, cowpea is an essential crop in developing coun-tries of the tropics and subtropics, especially in sub-Saharan Africa, Asia, Central and South America (Singh *et al*., 1997).

In Azerbaijan, cow pea is one of the fundamental legumes and it is crucial to evolve new accessions that are able to tolerate the biotic and abiotic stresses accountable for low productivity. The display of modern genetic tools with improved agronomic traits and increased genetic variation leads to the expansion of high-yielding accessions (Hwang *et al*., 2008). In this circumstance, a convenient description of the genetic divergence at the morphology and molecular levels in the accessible local cow pea accessions is indispensable for additional improvement of yield and stress tolerance. The agro-morphological traits have limitations for the development of gene pools because these traits are affected by environmental factors and stage of plant development, or they demonstrate merely limited diversity (Terzopoulos and Bebeli, 2008). The molecular techniques profitably employed to appraise the genetic relationship, are restriction fragment length polymorphism (RFLP), inter-simple sequence repeat (ISSR) amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) methods, target region amplification polymorphism (TRAP), single nucleotide polymorphism (SNP) and simple sequence repeats (SSRs). SSR markers are often applied in plant diversity and population structure because they are easy, PCR-based, highly polymorphic and can be used to differentiate closely related accessions. To develop new cow pea accessions, an introductory description, estimation and comparison of the genetic variation among regional and VİR accessions could be helpful for the imposition of this genetic variability in breeding programs.

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The aim of this study was to evaluate the genotypic diversity, relatedness and population structure among local cow pea resources available in Azerbayjan and the cultivated VIR accessions based on ISSR markers.

MATERIALS AND METHODS

Genotype collectýon

The studies were conducted in 2017-2019 at the Institute of Genetic Resources (IGR) of the National Academy of Sciences (NAS) of Azerbaijan. 28 samples were used as research material: 10 of them were local forms and 18 were samples obtained from VIR (Table 1). Sowing of collection samples was carried out in duplicate with an area of food of one plant 10 x 60 cm at the optimum time, in the a write the end of April.

DNA extraction and electrophoresis

All plants were grown in field at IGR of ANAS. Genomic DNA was extracted from leaf tissue (2 g) according to modified CTAB method by Rogers. The quality and quantity of extracted DNA was determined by spectrophotometer. The DNA concentration was adjusted to 20 ng µl-1 to use for PCR. Five ISSR primers were used for diversity analysis in cowpea. PCR was carried out in 25 µl reaction volume containing 2.5 µl 10 × PCR buffer, 2 µl dNTP (5 mM), 2 µl primer (10 µM), 1.5 µl $MgCl_2$ (50 mM), 0.2 µl Tag polymerase and 20 ng extracted DNA. PCR condition was as following: initial denaturation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 1 min, annealing at 50-55°C for 45 seconds, extension at 72°C for 1 min and final extension at the same temperature for 7 min. Amplification was performed in T100 thermal cycler (BioRad) in following conditions: initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 35-37°C for 45 seconds, extension at 72°C for 1 min and final extension at 72°C for 5 min. PCR products were analyzed using 1.8% agarose gel, stained with ethidium bromide and documented using BIO-RAD gel documentation system. A 100 bp ladder was used as a molecular size standard. Fragments amplified by ISSR primers were scored for presence or absence and coded as (1) or (0) respectively. WEIR's genetic diversity index (GDI) was calculated based on binary matrix (1990). Cluster analysis was performed using the unweighted pair-group method with arithmetical average (UPGMA) and dendrograms were constructed on the basis of Jaccard's genetic similarity index (1908) using SPSS (2003) software package.

Molecular marker (ISSR) assay

Five ISSR primers were used for PCR reaction (Table 2).

Scoring and statistical data analysis

The scramble bands were coded manually as either present (1) or absent (0). Scored data were applied to the calculation of Jaccard's similarity coefficient using XLSTAT 11 software. The Jaccard's coefficient was converted to a dissimilarity matrix to create dendrogram using the Unweighted Pair-Group Method with Arithmetic averages (UPGMA) by using XLSTAT 11 software. Polymorphism Information Content (PIC), gene diversity and major of allele frequency were computed by using Power Marker version 3.25 software. To determine the relationship between different accessions, the Principal Component Analysis (PCA) was conducted using XLSTAT 11 software. Gen AlEx version 6.5 software was also used to estimate the molecular variance among and within populations (Peakall *et al*., 2012). Marker Index (MI) and Resolving Power (RP) were measured according to Powell *et al*. (1996) and Prevost and Wilkinson (1999). To population structure, a model analysis was fulfilled to infer

Table 1: Cow pea accessions examined in the study and their origin.

Accessions	Origin
K- 262	VIR
K- 252	VIR
K- 259	VIR
K- 771	VIR
K- 273	VIR
K- 268	VIR
K- 263	VIR
K- 264	VIR
K- 272	VIR
K- 261	VIR
K- 267	VIR
AG- 342	VİR
K- 1832	VİR
K- 1292	VİR
AG- 340	AZE
AzeVİG- 17	AZE
AzeVİG- 36	Ganja
K- 1190	VIR
K- 1138	VIR
AzeVIG- 1	Jalilabad
AzeVIG- 2	Karabakh
KARA	AZE
K- 424	VIR
K- 271	VIR
sps. *sesguipedalis*	Lankaran
sps. *sesguipedalis*	Lankaran
AzeVİG- 3 (local)	Ganja
Ayla	IGR

Table 2: Primer names, sequences, motif and annealing temperature of SSR markers used in this work.

Primer name	Sequence: 5′-3′	Annealing temperature (°C)
UBC 810	GAGAGAGAGAGAGAGAT	45
UBC 812	GAGAGAGAGAGAGAGAA	45
UBC 818	CACACACACACACACAG	48
UBC 835	AGAGAGAGAGAGAGAGYC	50.2
UBC 857	ACACACACACACACACYG	54.3

Note: Y = (C, T).

genetic structure and to clarify the number of sub-populations using the STRUCTURE software version 2.3.4 (Pritchard *et al*., 2000; Evanno *et al*., 2005).

RESULTS AND DISCUSSION

In the present investigation, 28 genotypes of cow pea collected from various regions of Azerbaijan and VIR collection, were evaluated using five ISSR markers.

Initially polymerase chain reaction was performed with 5 ISSR primers, amplification product was not synthesized with more than half of the samples with 2 of the primers (UBC 810 and UBC 812) and the amplicons were obtained with most of the samples for other 3 primers. UBC 810 and UBC 812 primers were synthesized in the length of 250-900 nc 8 (5 polymorphs) and 6 (3 polymorphs) paragraphs, respectively. A different profile was obtained for the AG-342 sample with the UBC 810 marker with sequence $(GA)_8T$. UBC 818 primer that realized amphlification of the $(CA)_8G$ microsatellite loci were synthesized in 8 paragraphs with the 28 cow peas samples, 6 of which were polymorphic and the percentage of polymorphism was 75% (Table 3). The genetic diversity index (GDI) and polymorphism information content (PIC-Polymorphism Information Content) for the UBC 818 premieres were 0.827 and 0.36, respectively, which is sufficiently high indicator. Igve *et al*. (2017) used 10 ISSR primers to assess the gentic diversity in the 18 cow pea genotype, only four visible primers were amplified and used for further analysis.

The size of the synthesized items varied in the range of 250-1500 nc. As a result of the different combinations of points obtained, 8 different profiles for 28 genotypes were identified (Fig 1). A unique profile for the genotypes AG-342, K-1138 and the Ayla species was recorded.

For all primers surveyed, the effective multiplex ratio (EMR) based on the polymorphous locus fraction, the potential for discrimination between selected primer genotypes and the medium resolving power (MRP) were calculated. For 818 primers marker index of 1.62, effective multiplex ratio 4.5 were obtained. Resolving power and medium resolving power were 3.36 and 0.56 m respectively. Obtained data shows that mentioned primers were efficient for cow pea.

The next dinucleotide motif synthesized by the UBC 835 primer was 13 bits and the fragment size was 150-3000 nc, a very high percentage of polymorphism (92.3%) was recorded for the locus (Fig 2). Of the 16 profiles, only 4 were observed in 2-8 genotypes, while the remaining 12 profiles were unique and found only in one genotype. The main variability parameters were characterized by high GMO and PIC values and were 0.89 and 0.33 units, respectively. Effective multiplex ratios of other indicators were also high (11.1).

Seven amplicons with length 20-800 nc were synthesized with the UBC 857 primer, of which 3 were monomorphic and 4 were polymorphic. Indicator of polymorphism of locus $(AC)_8YG$ was 57%. The marker's ability to distinguish between genotypes was slightly lower

Table 3: Indicators of ISSR markers in the cow pea collection.

Primer name	Sequence: 5'-3'	Number of synthesized bands	Number of polymorphic bands	Polymorphism %	Genetic diversity ratio	PIC	EMR	MRP	P	MRP
UBC 818	$(CA)_8G$	8	6	75	0.83	0.36	4.5	1.62	3.36	0.56
UBC 835	$(AG)_8YC$	13	12	92.3	0.89	0.33	11.07	3.66	5.96	0.50
UBC 857	$(AC)_8YG$	7	4	57	0.69	0.33	2.3	0.76	1.7	0.43
UBC 810	$(GA)_8T$	8	5	62.5	0.54					
UBC 812	$(GA)_8A$	6	3	50	0.50					
Medium		8.4	6	7.4	0.70	0.34	5.94	2.01	3.67	0.50
General		2	30							

(1.7) than other primers and MRP was 0.43. Other parameters of the premiere awareness, MI (Marker Index (MI), EMR and PIC, were 0.76, 2.3 and 0.33 respectively. Among the genotypes, 3 profiles were recorded and the genetic variance coefficient calculated based on the frequency of profiles was found to be 0.69.

Thus, a total of 42 points were synthesized in five ISSR primers in the studied cow pea collection, with 8.4 amplicon per primer. The total number of polymorphic fragments ranged from 30 to 3-12 (average grade 6.0). Generally, the percentage of polymorphism on microsatellite locus in the cow pea genotypes was 67.4%. Ghalmi *et al.* (2009) obtained 63% polymorphism for a collection of 20 Algerian chickpea genotypes with 12 ISSR premiers (Ghalmi *et al*., 2009). Obtained results were in line with the experience of Igve *et al*. (2017); also coincides with experiments where four ISSRs synthesized, 32 alleles for the 18 *V. unguiculata* genotype of the Nigerian origin, with amplicons ranging from 4-14.

In the present study, the average indicators of the MI, EMR, RP (Resolving Power -RP) and MRP for 3 loci were 2.01, 5.94, 3.67 and 0.50 units, respectively. High genetic diversity was identified in the collection of 28 samples. The average polymorphism data capacity and genetic diversity coefficient for the collection were 0.34 and 0.70, respectively. On the one hand, this indicator showed that the collection is genetically rich and on the other hand, the used primers are effective. Among the premieres, the UBC 835 is characterized by the highest values on all indicators, except PIC. All three primers used in the analyzes can be successfully applied in future molecular-genetic studies on cowpea.

Defining genetic relationship in the cowpea collection by ISSR markers

Genotypes in dendrograms for cowpea samples based on ISSR profiles are grouped into 4 major clusters (Fig 3). Among the samples, the Jakkard genetic distance index ranged from 0-0.73 to an average of 0.33 units. 100% genetic similarity was observed within a group of genotypes. The maximum genetic distance was recorded between the genotypes of AzeVIG-2 and AG-342.

The first cluster consisted of 11 genotypes and is divided into 2 sub-clusters. The AzeVIG-3 (local) and Ayla species were present in this cluster. The closest genetically similar accessions in the first sub cluster were the genotypes AzeVIG-3 and AzeVIG-2, with the Ney genetic distance index being 0.27 units. Among the 11 genotypes, the K-263 sample was more genetically different from the other samples. The second sub cluster contains 4 specimens, where K-264 and K-424 specimens were similar to each other upon all the loci and were identical. This result may also be due to the small number of primaries involved in the study.

The second cluster consisted of 9 samples, which in turn were subdivided into 2 sub-clusters. The second sub cluster was composed of four genotypes with 100% similarity (K-252, K-771, K-1292 and K-1832), whereas the genetic distance index between the five samples in the first sub cluster ranged between 0-0.32. In the sub-cluster, the AG-342 sample, unlike other genotypes, formed a separate independent group. It should be noted that, the lowest Ney distance index between this genotype and the other 27 samples is 0.24 and the highest is 0.73, which is the maximum value for the collection.

The third cluster combines the K-267, K-273, K-271 and AzeVIG-17 genotypes. The closest genotypes in the cluster were K-267 and K-273 (Ney genetic distance index = 0.1) and the farthest genotypes were K-271 and K-267 (GDI = 0.21).

Finally, the fourth cluster was composed of AzeVIG-36, K-268 and 2 *V. unguiculata sps. sesquipedalis* specimens. Thus, the sameness of several genotypes was found in the

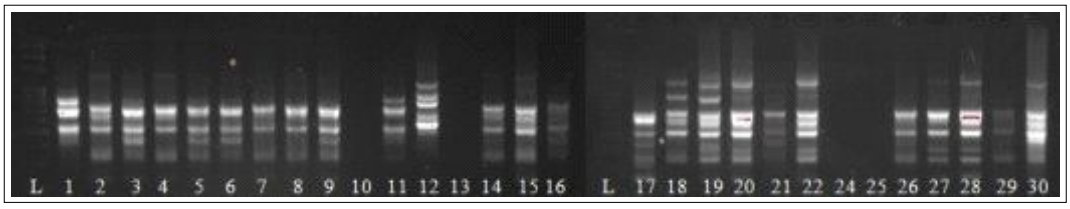
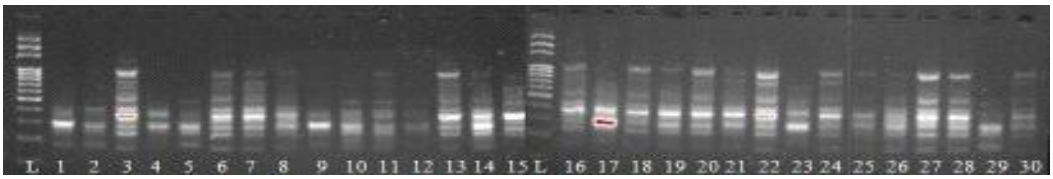


Fig 1: Molecular-genetic profiles obtained for cow bean collection with the UBC 818 primer.



1, 1 – K-262; 3- K-252; 4, 5- K-259; 6- K-771; 7- K-273; 8- K-268; 9, 10- K-263; 11- K-264; 12- K-272; 13- K-261; 14- K-267; 15- AG-342; 16- K-1292; 17- AG-340; 18- AzeVIG-17; 19- AzeVIG-36; 20- K-1190; 21- K-1138; 22- AzeVIG-1; 23- AzeVIG-2; 24- Kara; 25- K-424; 26- K-271; 27-28- sps. *sesquipedalis*; 29- AzeVIG-3 (local); 30- Ayla.

Fig 2: Molecular-genetic profiles obtained for cow bean collection with the UBC 835 primer.

cowpea collection, but 19 genotypes were identified with different profiles. The presence of different samples in the same clusters is due to the smaller number of locus analyzed, than their similarity. The samples that appear in different clusters in cluster analysis can be used in future hybridization as genetic divergent forms.

The main coordinates analyzed on the basis of the Ney genetic distance index between the samples and the relative nature of the genotypes over each other on flatness is presented in Fig 4. The results of PCoA were consistent with cluster analysis and it was revealed that some of the studied cowpea genotypes were genetically close or identical to each other.

The first three coordinates accounted for 57.42% and the first five coordinates for 71.5% of the genetic variation.

Genotypes included in the same cluster were also close to each other in PCoA analysis. The K-262 genotype, unlike other genotypes, is located far away from them - in the upper corner of the upper left quadrant. Other genotypes that appear to be genetically different include AzeVIG-3 (local), K-259, AzeVIG-2, K-1832. The Ayla sort is situated closer to the K-267 (Fig 4).

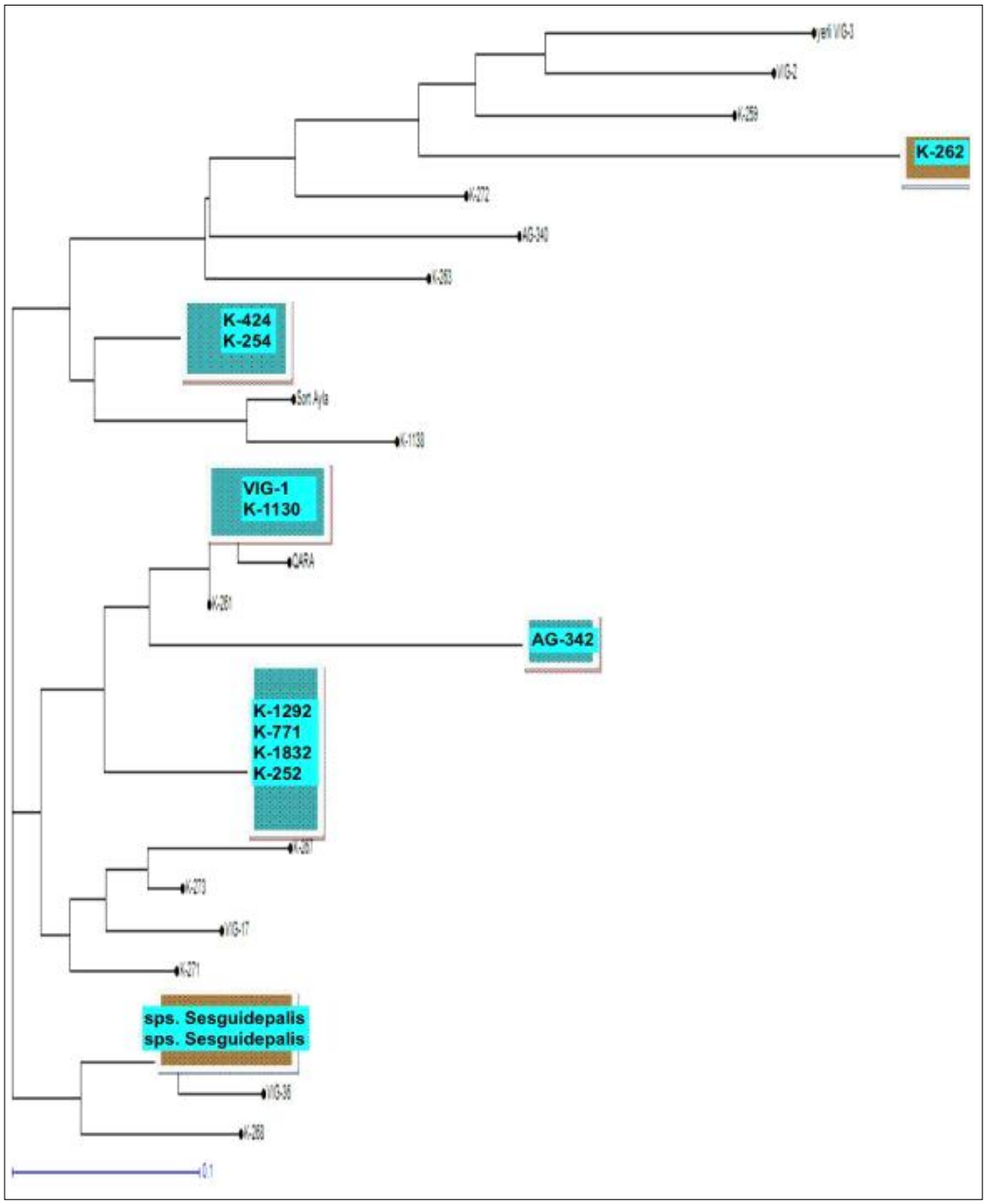


Fig 3: Dendrogram showing genetic affinity in the 28 cow bean sample.

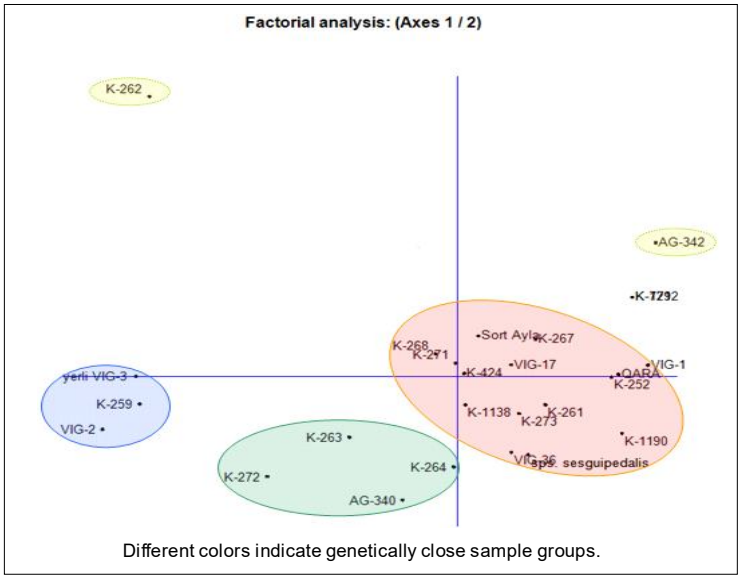


Fig 4: The distribution pattern of the cowpea samples based on the basic coordinate analysis.

The results obtained from the ISSR markers allow preservation of the specimens with highest variation, by rejecting genetically identical or highly similar genotypes.

CONCLUSION

In conclusion, the results demonstrated that the studied *Vigna unguiculata* (L.) Walp collection constituted an important pool of diversity and could be confidently used for further genetic analysis and breeding activities. ISSR markers were efficient to assess genetic diversity within the collection and were able to identify samples with the same botanical varieties. The results of the cluster and PCoA analyzes obtained in the current experiment for the first time, underlined the importance of consideration of botanical variety traits in breeding programs. Information on a high level of genetic diversity and existence of genetically separated groups in the current traits of *Vigna unguiculata* collection can be applied to the develop sentence is incomplete.

Conflict of interest

All authors declare that they have no conflicts of interest.

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