

## Genetic Diversity and Evolutionary Analysis of Okra (*Abelmoschus esculentus* (L.) Moench) Germplasm Resources based on ISSR Markers

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### Abstract

**Objective:** Okra (*Abelmoschus esculentus* (L.)) is a flowering plant in the mallow family, valued for its edible green fruits. The genetic diversity of Okra was analyzed to provide the theoretical basis for the collection, identification, protection and utilization of the germplasm resources of Okra plants.

**Method:** The genetic diversity among 34 accessions of okra from different regions of China was investigated using Inter-simple Sequence Repeat (ISSR).

**Results:** Out of the 128 repeatable bands detected, 90 bands were polymorphic. The percentage of polymorphic bands was 70.3%, with a mean of 7.6% of polymorphic bands per primer. With the 128 bands, the phylogenetic trees were constructed using the Unweighted Pair-Group Method with Arithmetic Average (UPGMA) cluster analysis. The 34 accessions were divided into two major groups in the evolutionary tree, and the two-dimensional matrix also confirmed this conclusion. The polymorphism information content (PIC) values varied from 0.4507 to 0.6801, indicating that the primers used were authentic.

**Conclusion:** The genetic distance among the sample accessions ranged from 0.6 to 0.94. The data showed that there was a high degree of genetic diversity among the okra accessions revealed by the ISSR markers. Our results confirm the potential value of genetic diversity preservation for future breeding of Okra.

**Keywords:** Okra; Genetic diversity; Intersimple sequence repeat (ISSR) markers

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### Introducción

Okra, a kind of annual herb, is a member of Malvaceae family, as well as is called as *Abelmoschus esculentus*, pepper and so on. It originated from East Asia and West Africa, spread later to tropical, subtropical, and warm temperate regions [1]. With the development of the technology, it was cultivated and distributed across the world. It is not considered to be a vital crop until more attention was paid to the improvement of genes, especially its rapid growth cycle, easy cultivation, protection against pests, high yield, and abundant nutritional materials [2]. As more studies on okra reported, it gets more attention owing to its features of high nutritional value, significant health and therapeutic effects function in the recent years [3]. The tender fruit pods of okra are rich in protein, unsaturated fatty acids, minerals, alkaloids, free amino acid, sticky slippery juice and other bioactive components

[4], which can reduce blood sugar, protect the liver, cardiovascular and intestinal effects bringing excellent medicinal health effects in the condition of taking for a long periods of time. The leaf and

seed of okra can be used as traditional medicines [5]. In addition, the beautiful flower of okra is also considered as ornamental plants [6]. Over the past decades, a series of achievements in cytology, introduction and cultivation techniques of okra is reported. However, there are few studies on the genetic diversity of germplasm resources, which is of significant importance for the identification and breeding [7]. Therefore, it is urgent to study the genetic diversity and genetic relationship of okra accessions in the identification, breeding and utilization of okra, which will provide references for the classification of okra [8].

The ISSR marker technique was proposed by Zietkiewicz [9]. This technique is widely used in studies on the genetic diversity of plants, for instance cotton [10], ryegrass [11], and ipomoea [12]. What's more, it possesses advantages, such as good stability, high polymorphism, simple operation and time-consuming. The principle is to use Microsatellite DNA as a primer, and 2 to 4 random nucleotides were extended at the SSR sequence 3' or 5' termini. In the polymerase chain reaction (PCR), the primer caused specific sites annealing after amplified, which resulted in the repetitive sequence DNA fragments [13]. Multiple bands of the amplified ISSR region were resolved by agarose gel electrophoresis or PAGE electrophoresis. ISSR, a dominant marker, reveals some characteristics of the whole genome.

In this study, ISSR molecular marker technique was used to analyze the genetic diversity of 34 okra accessions and make some preliminary explorations for identification of okra germplasm.

## Material and Methods

### Plant materials

Plant materials were collected from various areas of China. JQK and YGF come from Hangzhou, Zhejiang Province. HQK come from Jiaxing, Zhejiang Province. DJWJ come from Weifang, Shandong Province. KLB and TWWF come from Wuxi Jiangsu Province. MRZ come from Cangzhou Hebei Province.

### Genomic DNA extraction

The leaves were collected from the 34 okra accessions. Genomic DNA was extracted from 0.1 g fresh leaves following the improved CTAB protocol [14] and the DNA was detected with 2% agarose gel electrophoresis.

### ISSR PCR amplification

DNA primers used in PCR amplification were presented in **Table 1**. 10 ng template DNA, 10 mL 2x PCR Master Mix, 2x PCR Buffer, 2x dNTP, and 1 mL of each primer were mixed using ultrapure water. All the oligonucleotides were dissolved in Amplification. PCR was performed using ABI 2720 Thermal Cycler (Applied Biosystems, USA) with the following process: 5 minutes pre-denaturation at 94°C, 50 cycles of denaturation at 94°C for 30s, 1 minute for annealing and 72°C extended for 90s. The PCR products were electrophoresed in a 100V with 2% agarose gel for 1 hour and then photographed in a gel imager.

### Data statistics

ISSR products were established with 0 and 1. The UPGMA method

was used to construct the molecular phylogenetic tree among the accessions via the R program software. PowerMarker software was used to calculate the polymorphism information content (PIC), allele frequency, genetic diversity and genetic distance of each primer. The principal co-ordination analysis (PCoA) of CENTER and EIGEN were obtained in NTSYS-pc [15]. The cluster silhouette plot and optimal number of clusters were obtained by the R program. The two-dimensional array with coordinates was used to identify the 34-point accession differences of okra.

## Results

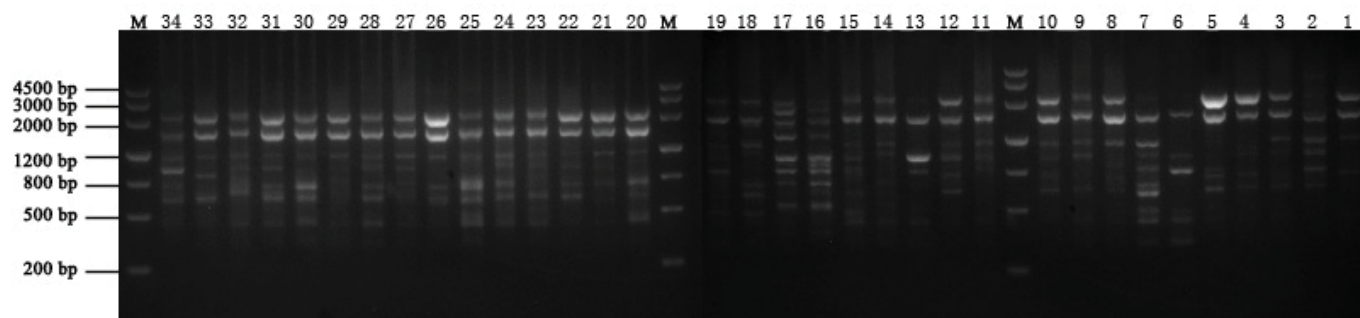
### ISSR polymorphism

The 15 ISSR primers were used to amplify 34 okra accessions. A total of 128 DNA fragments was obtained from the 15 primers, and the average fragments amplified from each primer is 8.5, in which polymorphic fragments were 90, accounting for 70.3%. The size of amplified fragments was between 200 and 3000 bp. Different primer has the different amplified results, the primer 840 produced most amplified DNA fragments, as shown in **Figure 1**. As compared with 840, 830 had the least number of 3 bands with no polymorphism bands. In these 15 primers, there were 7 (GA)<sub>n</sub>, indicating that there are a lot of (GA) dinucleotide repeats in okra genome.

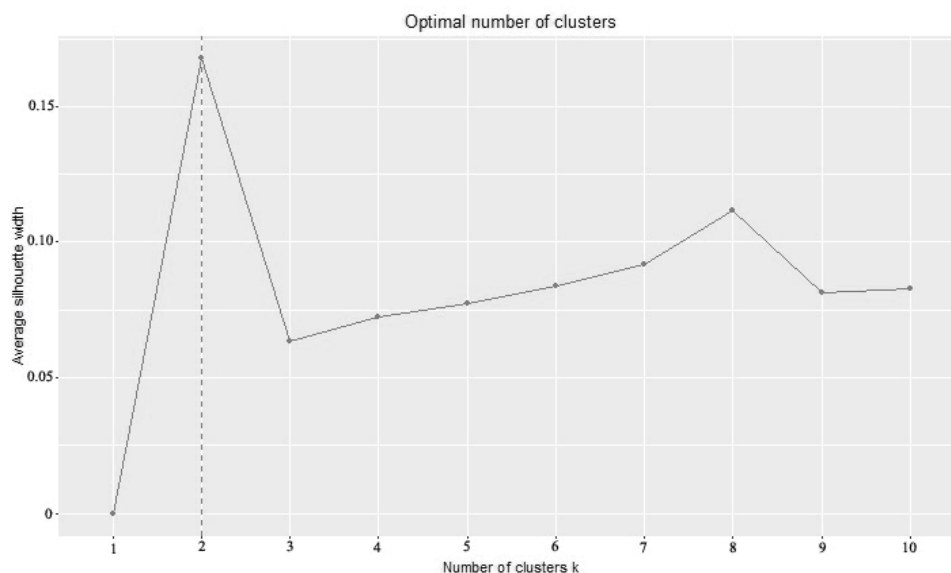
### Cluster analysis

We classify 34 samples against the R program, and the results showed that the two categories were most appropriate (**Figure 2**). It can be seen from the dendrogram that all materials can be divided into two groups at the threshold of 5.87 (**Figure 3**). The populations MRZ1-5 and DJWJ1-4 constitute the first group. The second group consists of TWWF1-5, KLB1-5, HQK1-5, YGF1-5 and JQK1-5. The above clustering results are consistent with its geographical distribution. The results from PCoA revealed the diversity among these accessions (**Figure 4**). The ordination plot indicated clearly distinct groupings and confirmed consistency with those obtained from UPGMA cluster analysis. At the same time, we used the R program to further verify the results and got the cluster silhouette plot (**Figure 5**). When the width was close to -1, the results of cluster analysis was wrong. When the width was close to +1, the results of cluster was more real. The results obtained were 0.13 indicating that it was credible to classify these 34 accessions into two categories.

The Power Marker software was used to calculate PIC values (**Table 2**). The results showed that the allele frequency was about 0.5, the genetic diversity was from 0.6857 to 1, the minimum and maximum values of polymorphism were 0.4507 and 0.6801, respectively, and the value of primer 834 was the highest. Based on the 128 DNA fragments generated by ISSR amplification, the genetic similarity matrix of 34 okra accessions was obtained (**Table 3**). The genetic similarity coefficient (0.94) between HQK-3 and HQK-5 was the highest, which indicated that the genetic similarity between HQK-3 and HQK-5 was the closest. The similarity coefficient between MRZ-3 and KLB-4 (0.6) was the smallest. The results indicated the genetic similarity between MRZ-3 and KLB-4 was the closest, which was consistent with the result of cluster analysis.



**Figure 1** Agarose gel electrophoresis profiles were obtained by using 840 ISSR primers for the 34 okra accessions. Lanes 1-34 are MRZ-1, MRZ-2, MRZ-3, MRZ-4, MRZ-5, TWWF-1, TWWF-2, TWWF-3, TWWF-4, TWWF-5, KLB-1, KLB-2, KLB-3, KLB-4, KLB-5, DJWJ-1, DJWJ-2, DJWJ-3, DJWJ-4, HQK-1, HQK-2, HQK-3, HQK-4, HQK-5, YGF-1, YGF-2, YGF-3, YGF-4, YGF-5, JQK-1, JQK-2, JQK-3, JQK-4, JQK-5, respectively.



**Figure 2** The optimal number of clusters is 2 by R program.

**Table 1** The annealing temperature (Tm) and the total number of bands (TB), number of polymorphic bands (PB) and % of polymorphic bands (PPB) used in the ISSR primers.

Primer	Sequence 5'-3'	Tm (°C)	TB	PB	PPB(%)
811	GAGAGAGAGAGAGAC	47	9	6	0.666666667
823	TCTCTCTCTCTCC	49	6	4	0.666666667
825	ACACACACACACACT	45	9	7	0.777777778
830	TGTGTGTGTGTGTGG	48	8	7	0.875
834	AGAGAGAGAGAGAGCT	50	8	6	0.75
835	AGAGAGAGAGAGAGCC	54	8	6	0.75
840	GAGAGAGAGAGAGACT	49	13	12	0.923076923
841	GAGAGAGAGAGAGACC	50	12	10	0.833333333
842	GAGAGAGAGAGAGATG	49	7	5	0.714285714
855	ACACACACACACACCT	52	7	5	0.714285714
857	ACACACACACACACCG	49	6	5	0.833333333
884	ACTAGAGAGAGAGAGAG	48	10	3	0.3
887	GCATCTCTCTCTCTC	49	11	6	0.545454545
889	ACTACACACACACACAC	49	7	4	0.571428571
891	ACTTGTGTGTGTGTGTG	47	7	4	0.571428571

**Table 2** The allele frequencies, genetic diversity, heterozygosity and polymorphism information content (PIC) of 15 primers.

Primer	Major Allele.		Genotype No	Allele No	Gene Diversity	Heterozygosity	PIC
	Frquency						
811	0.5000		4.0000	5.0000	0.6600	1.0000	0.6115
823	0.5143		6.0000	6.0000	0.6776	0.9714	0.6447
825	0.5000		5.0000	6.0000	0.6763	1.0000	0.6370
830	0.5000		6.0000	7.0000	0.6861	1.0000	0.6519
834	0.5000		6.0000	7.0000	0.7045	1.0000	0.6801
835	0.5000		6.0000	7.0000	0.6669	1.0000	0.6224
840	0.5000		8.0000	9.0000	0.6886	1.0000	0.6556
841	0.5000		8.0000	9.0000	0.6996	1.0000	0.6728
842	0.5000		5.0000	6.0000	0.6653	1.0000	0.6191
855	0.5143		5.0000	5.0000	0.6633	0.9714	0.6219
857	0.5429		6.0000	6.0000	0.6445	0.9143	0.6064
884	0.6571		4.0000	4.0000	0.5057	0.6857	0.4507
887	0.5000		6.0000	7.0000	0.6706	1.0000	0.6283
889	0.5000		3.0000	4.0000	0.6057	1.0000	0.5293
891	0.5143		6.0000	6.0000	0.6718	0.9714	0.6357
<b>Mean</b>	0.5464		5.3125	5.9375	0.6179	0.9071	0.5792

**Table 3a** Genetic similarity coefficient matrix of 34 okra accessions.

	MRZ-1	MRZ-2	MRZ-3	MRZ-4	MRZ-5	TWWF-1	TWWF-2	TWWF-3	TWWF-4	TWWF-5	KLB-1	KLB-2
<b>MRZ-1</b>	1											
<b>MRZ-2</b>	0.85	1										
<b>MRZ-3</b>	0.85	0.78	1									
<b>MRZ-4</b>	0.72	0.76	0.78	1								
<b>MRZ-5</b>	0.75	0.74	0.76	0.81	1							
<b>TWWF-1</b>	0.66	0.67	0.67	0.71	0.69	1						
<b>TWWF-2</b>	0.65	0.66	0.67	0.7	0.67	0.87	1					
<b>TWWF-3</b>	0.67	0.69	0.69	0.73	0.73	0.78	0.8	1				
<b>TWWF-4</b>	0.65	0.64	0.71	0.7	0.65	0.76	0.77	0.86	1			
<b>TWWF-5</b>	0.71	0.7	0.74	0.71	0.72	0.84	0.82	0.87	0.87	1		
<b>KLB-1</b>	0.74	0.71	0.71	0.7	0.74	0.76	0.77	0.81	0.81	0.84	1	
<b>KLB-2</b>	0.71	0.71	0.67	0.7	0.71	0.79	0.78	0.8	0.78	0.82	0.89	1
<b>KLB-3</b>	0.63	0.73	0.65	0.74	0.71	0.81	0.78	0.84	0.82	0.8	0.82	0.85
<b>KLB-4</b>	0.64	0.65	0.6	0.66	0.66	0.78	0.78	0.79	0.74	0.8	0.82	0.87
<b>KLB-5</b>	0.65	0.67	0.6	0.68	0.64	0.71	0.72	0.69	0.72	0.7	0.78	0.72
<b>DJWJ-1</b>	0.67	0.75	0.71	0.74	0.78	0.67	0.71	0.74	0.71	0.71	0.74	0.71
<b>DJWJ-2</b>	0.74	0.74	0.78	0.73	0.81	0.7	0.72	0.74	0.75	0.78	0.75	0.77
<b>DJWJ-3</b>	0.71	0.66	0.78	0.74	0.74	0.65	0.67	0.61	0.69	0.68	0.69	0.69
<b>DJWJ-4</b>	0.73	0.72	0.71	0.67	0.65	0.67	0.64	0.64	0.74	0.7	0.71	0.71
<b>DJWJ-5</b>	0.76	0.69	0.75	0.73	0.67	0.7	0.69	0.72	0.72	0.74	0.75	0.75
<b>HQK-1</b>	0.78	0.74	0.71	0.69	0.75	0.74	0.78	0.74	0.73	0.74	0.81	0.74
<b>HQK-2</b>	0.76	0.77	0.75	0.76	0.78	0.78	0.78	0.77	0.74	0.79	0.78	0.77

Table 3b Genetic similarity coefficient matrix of 34 okra accessions.

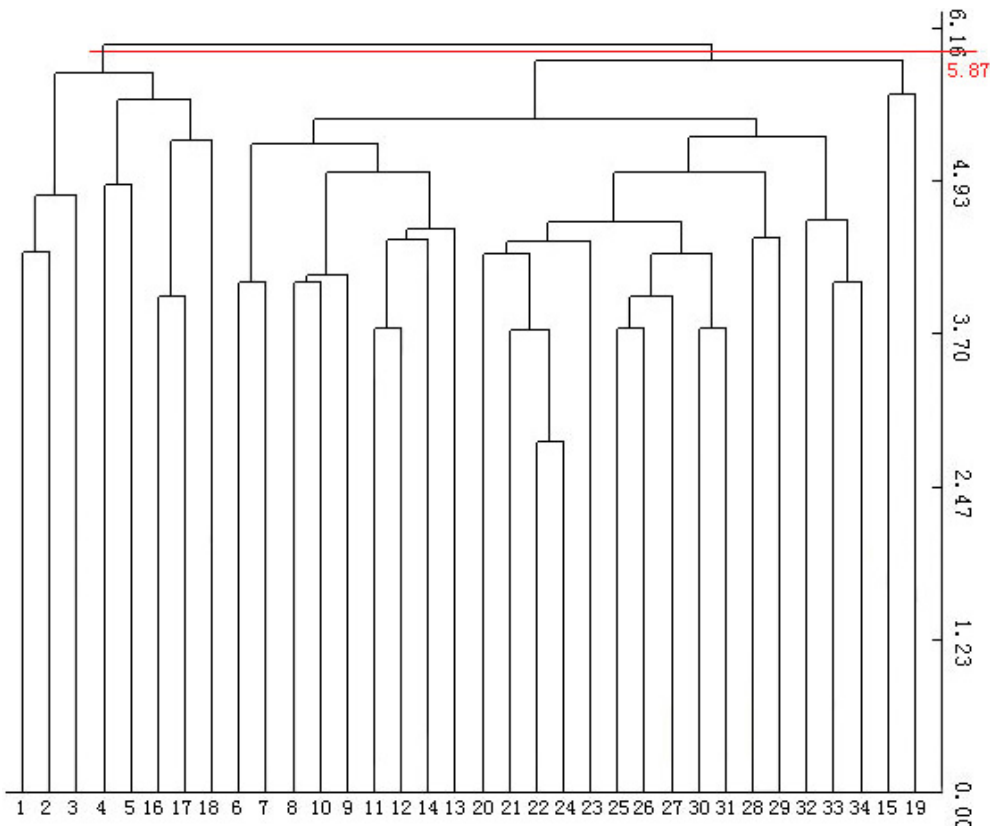
	MRZ-1	MRZ-2	MRZ-3	MRZ-4	MRZ-5	TWWF-1	TWWF-2	TWWF-3	TWWF-4	TWWF-5	KLB-1	KLB-2
HQK-3	0.69	0.71	0.7	0.74	0.75	0.81	0.79	0.79	0.76	0.81	0.84	0.85
HQK-4	0.77	0.76	0.71	0.71	0.74	0.72	0.73	0.76	0.78	0.8	0.84	0.82
HQK-5	0.71	0.71	0.71	0.74	0.75	0.78	0.79	0.79	0.78	0.81	0.84	0.82
YGF-1	0.74	0.74	0.76	0.77	0.75	0.77	0.76	0.76	0.79	0.81	0.79	0.76
YGF-2	0.72	0.73	0.73	0.74	0.71	0.78	0.78	0.79	0.78	0.8	0.76	0.76
YGF-3	0.76	0.78	0.77	0.73	0.74	0.76	0.77	0.81	0.77	0.78	0.8	0.77
YGF-4	0.65	0.67	0.66	0.71	0.7	0.76	0.74	0.8	0.77	0.76	0.77	0.77
YGF-5	0.7	0.71	0.69	0.71	0.7	0.73	0.71	0.78	0.75	0.79	0.77	0.74
JQK-1	0.78	0.77	0.75	0.73	0.71	0.76	0.78	0.78	0.77	0.79	0.8	0.77
JQK-2	0.74	0.78	0.74	0.76	0.71	0.78	0.8	0.81	0.8	0.81	0.81	0.8
JQK-3	0.73	0.66	0.72	0.7	0.71	0.71	0.74	0.74	0.74	0.74	0.74	0.75
JQK-4	0.71	0.72	0.69	0.7	0.71	0.73	0.72	0.75	0.77	0.76	0.74	0.74
JQK-5	0.67	0.68	0.68	0.69	0.71	0.74	0.74	0.79	0.79	0.81	0.74	0.74

Table 3c Genetic similarity coefficient matrix of 34 okra accessions.

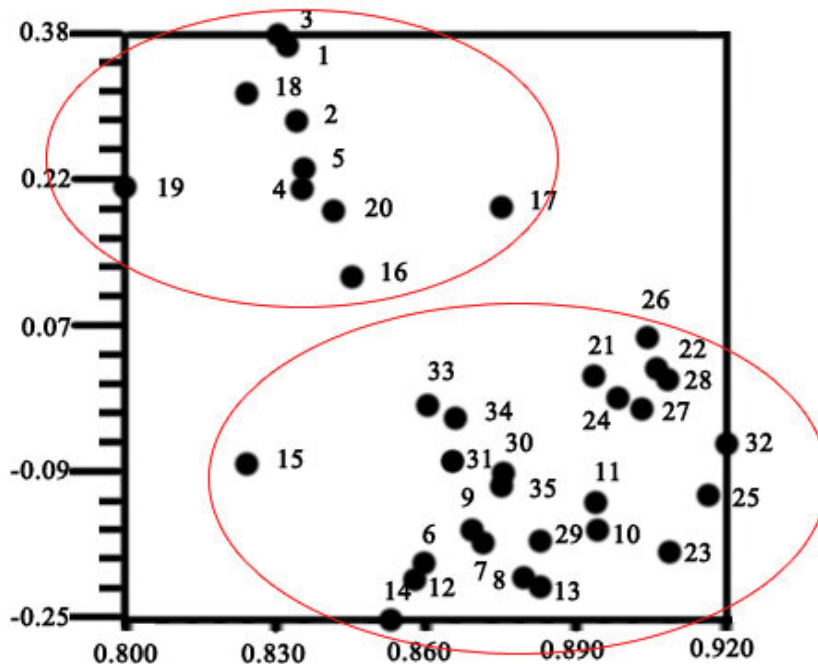
	KLB-3	KLB-4	KLB-5	DJWJ-1	DJWJ-2	DJWJ-3	DJWJ-4	DJWJ-5	HQK-1	HQK-2	HQK-3	HQK-4
KLB-3	1											
KLB-4	0.84	1										
KLB-5	0.73	0.73	1									
DJWJ-1	0.73	0.7	0.67	1								
DJWJ-2	0.73	0.73	0.67	0.88	1							
DJWJ-3	0.67	0.65	0.67	0.74	0.83	1						
DJWJ-4	0.73	0.68	0.71	0.63	0.72	0.75	1					
DJWJ-5	0.71	0.7	0.71	0.66	0.74	0.8	0.83	1				
HQK-1	0.74	0.77	0.79	0.76	0.79	0.71	0.67	0.71	1			
HQK-2	0.78	0.74	0.69	0.78	0.78	0.74	0.69	0.72	0.87	1		
HQK-3	0.83	0.81	0.73	0.74	0.76	0.7	0.67	0.68	0.83	0.88	1	
HQK-4	0.8	0.77	0.76	0.74	0.79	0.71	0.71	0.74	0.84	0.84	0.84	1
HQK-5	0.81	0.8	0.73	0.78	0.79	0.73	0.67	0.73	0.86	0.9	0.94	0.86
YGF-1	0.78	0.74	0.73	0.76	0.79	0.79	0.73	0.78	0.81	0.84	0.8	0.81
YGF-2	0.78	0.75	0.73	0.73	0.78	0.74	0.73	0.78	0.84	0.85	0.83	0.8
YGF-3	0.81	0.73	0.71	0.75	0.77	0.72	0.69	0.77	0.85	0.86	0.82	0.78
YGF-4	0.81	0.76	0.75	0.74	0.72	0.67	0.64	0.71	0.76	0.77	0.82	0.74
YGF-5	0.78	0.73	0.71	0.74	0.72	0.69	0.61	0.71	0.78	0.81	0.82	0.78
JQK-1	0.76	0.74	0.75	0.75	0.78	0.74	0.67	0.77	0.87	0.81	0.82	0.85
JQK-2	0.82	0.74	0.75	0.78	0.78	0.71	0.69	0.74	0.84	0.86	0.85	0.84
JQK-3	0.71	0.71	0.71	0.67	0.74	0.72	0.63	0.67	0.78	0.77	0.79	0.79
JQK-4	0.73	0.7	0.74	0.74	0.74	0.69	0.67	0.67	0.78	0.8	0.79	0.76
JQK-5	0.74	0.72	0.73	0.74	0.78	0.7	0.65	0.71	0.77	0.79	0.83	0.78

Table 3d Genetic similarity coefficient matrix of 34 okra accessions.

	HQK-5	JQK-1	JQK-2	JQK-3	JQK-4	JQK-5
HQK-5	1					
YGF-1	0.83					
YGF-2	0.83					
YGF-3	0.84					
YGF-4	0.81					
YGF-5	0.84					
JQK-1	0.87	1				
JQK-2	0.85	0.89	1			
JQK-3	0.78	0.83	0.84	1		
JQK-4	0.76	0.78	0.84	0.86	1	
JQK-5	0.81	0.82	0.84	0.81	0.87	1

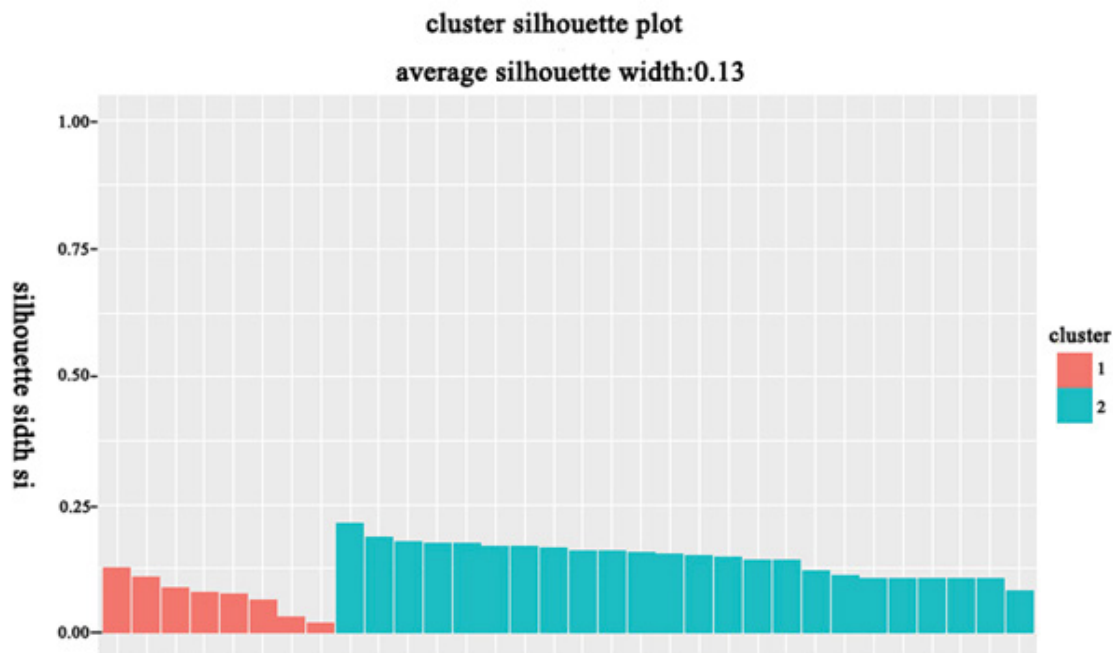


**Figure 3** Molecular phylogenetic tree based on genetic distance of 34 okra accessions. Numbers 1-34 are MRZ-1, MRZ-2, MRZ-3, MRZ-4, MRZ-5, TWWF-1, TWWF-2, TWWF-3, TWWF-4, TWWF-5, KLB-1, KLB-2, KLB-3, KLB-4, KLB-5, DJWJ-1, DJWJ-2, DJWJ-3, DJWJ-4, HQK-1, HQK-2, HQK-3, HQK-4, HQK-5, YGF-1, YGF-2, YGF-3, YGF-4, YGF-5, JQK-1, JQK-2, JQK-3, JQK-4, JQK-5, respectively.



**Figure 4** Two - dimensional matrix of 34 okra accessions.





**Figure 5** The cluster silhouette plot among 34 okra accessions.

## Discussion

Microsatellite markers, a significant tools for genetic analyses, can be distinguished with a high degree of variability. However, the common protocols used for separating Microsatellites suffer from cost, time consuming, laborious [16,17] and inefficiency, which limit their applications. To overcome the limitation and further improve yield, ISSR-PCR were introduced. Furthermore, in genome only the regions that are abundant in Microsatellite motifs are targets for ISSR. The structure of Microsatellite was efficient for the strategy of ISSR-PCR with anchored 3' and 5' terminal in the application of generating polymorphic loci among different species, which are certified succeed due to more than 86% consisted of internal microsatellite sequence among all the products except these at terminals. Agarose gel electrophoresis is the most common approach for isolating PCR products, which can achieve polymorphic bands with lower percentage, but have the advantages of cheap and time saving. Therefore, in this study we choose agarose gel for separating different bands generating in ISSR-PCR.

Molecular basis of polymorphism and their distribution across the genome is quite important for study on genetic diversity and population structure. In this study, the genetic diversity among 34 accessions of okra was analyzed using ISSR markers. A total of 128 DNA fragments and 90 polymorphic fragments (70.3%) were obtained by PCR amplification from 34 accessions, indicating that the genetic diversity of okra was relatively abundant. These fragments were processed to construct the molecular phylogenetic tree. The accessions of okras were divided into two major groups at the threshold of 5.87, which was consistent with the results obtained by the R program. Meanwhile, from the two-dimensional matrix obtained by NTSYS-PC software, the 34 okras were more appropriately divided into two groups. The above results showed that the two taxa were reliable.

From the constructed molecular dendrogram and the genetic similarity coefficient matrix, it can be seen that the HQK-3 and HQK-5 accessions had the closest genetic relationship, while the MRZ-3 and KLB-4 had the furthest genetic relationship. The phylogenetic relationships of different accessions are sometimes not obvious, but the genetic relationship of the same accessions is very different, which indicated that the genetic relationship between okra germplasms was complex.

Information on genetic diversity and population structure is essential for a better understanding of the breeding and genetic relationships of crop germplasm. The values of allele frequency, genetic diversity, and heterozygosity were high, which showed that the ISSR primers can produce some measurable fragments. PIC provided an estimate of the discriminant ability of markers based on marker NA and allele relative frequencies to distinguish genotypes [18] PIC was used to determine the genetic diversity of okra. High, moderate and low polymorphisms were expressed as  $PIC > 0.5$ ,  $0.5 > PIC > 0.25$  and  $PIC < 0.25$ , respectively [19,20]. The PIC value ranged from 0.4507 to 0.6801, with an average value of 0.5745 in this study, indicating that the primers could form high polymorphisms, which were useful for genetic variation in the genotype studied in this study. The above results confirm the potential value of genetic diversity preservation for future breeding of Okra.

## Conclusion

The phenotypic and genotypic variations are important for the development of new improved lines and cultivars. It would be a good tool of selecting okra breeding program to study the characterization of the genetic variability among okra germplasm. In the present study, the considerable genetic diversity among 34 okra accessions was observed at both the morphological

and molecular levels, which is of importance for germplasm classification, management, and further utilization. The results of this paper showed the ISSR markers would be a useful tool for okra genetic diversity studies and in breeding programs. The phylogenetic trees based on genetic distance indicated the 34 accessions were divided into two major groups. Moreover, the two-dimensional matrix also confirmed clustering results. On the whole, the data showed that there was a high degree of genetic diversity among the okra accessions revealed by the ISSR markers. Our results confirm the potential value of genetic diversity preservation for future breeding of Okra.

## Conflict of Interest

The author confirms that this article content has no conflict of interest.

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