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Study on the Genetic Relationship of Pepper Based on Fruit Traits and Molecular Markers

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Abstract To explore the genetic diversity of pepper germplasm resources in China and to improve the efficiency of pepper breeding. In this study, 45 pepper germplasm resources that came from USA and Guizhou province, China, were used to explore the quantitative traits of pepper fruits, and the ISSR analysis of germplasm resources genetic diversity. The results showed that the variance among eight quantitative traits of 45 peppers have a significant variation. The average genetic variation coefficient was 70.7%, and there were intricate correlations among the eight quantitative traits. Based on the fruit number traits, 45 pepper germplasms were clearly classified into six taxa by cluster analysis. The genetic diversity of pepper germplasm resources can be evaluated more accurately by molecular markers and phenotypic traits. This study provides a scientific basis for identification and evaluation of pepper germplasm resources, the selection of superior quality traits, and the selection of parents for crossbreeding.

Keywords Pepper; Morphological characteristics; Molecular markers; Genetic relationship

Background

Capsicum spp. belongs with the genus *Capsicum* in the family of Solanaceae, which is native to the tropical regions of Central Latin America. In China, it is mainly distributed in the provinces of Guizhou, Sichuan and Hunan (Wang et al., 2018). More than 30 species have been identified, of which five, *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum* and *C. pubescens*, are commonly cultivated species (Pickersgill, 1997). Nowadays, pepper is an irreplaceable vegetable with high nutritional value that rich in capsaicin, vitamin C and other mineral elements and also is antibacterial and anti-inflammatory, antioxidant and dissolves blood clots that is why they are widely eaten as well as growing around the world (Adams et al., 2009; Jorge et al., 2016; Mirmanto et al., 2017; Shin et al., 2016). Because the special geographical location and typical subtropical humid monsoon climate in Guizhou, the area has four distinctive seasons, sufficient heat and water, which provides a good environment for the pepper growth (Zhan et al., 2014). Guizhou also has abundant germplasm resources and high quality products, the most successful one is the Lao Gan Ma series of pepper products (Zhan et al., 2020). However, commercial cultivation makes pepper varieties tend to be homogenized and reduces the diversity of germplasm resources that are not conducive to excavation and utilization of excellent traits and cannot effectively meet the new market demand for pepper varieties, so the hybridization of introduced excellent pepper germplasm resources and local varieties is an effective way to solve the current problems. To clarify the genetic kinship of resources for different parental, to determine the genetic distance of hybrid parents, and to realize the construction of superior combinations is the prerequisite for selecting and breeding superior varieties (Lu, 2013).

Genetic relation of plants can be usually determined by analyzing various morphological indicators, which achieves the realization of constructing superior hybrid combinations between intraspecific and interspecific species. However, with the deepening of research, the determination of genetic relationships based on phenotypic characters has large errors and cannot accurately define the genetic relationships within plant species (Igwe et al., 2019; Votava et al., 2002). On this basis, combined with molecular biology, the use of conserved fragments in gene sequences to distinguish intraspecific and interspecific genetic relationships is more accurate and reliable than traditional morphological markers. At present, they have been widely used in the breeding of pepper (Huang et al., 2001), wheat (Najaphy et al., 2011), rice (Moonsap et al., 2019), cotton (Abdi et al., 2012) and other crops.

In this study, 45 pepper varieties with good performance were selected as research subjects after preliminary adaptive screening. Subsequently, each variety of fruit shape and quality characteristics was statistically analyzed, and each variety of genetic distance was comprehensively judged by combining ISSR molecular marker technology to lay the foundation for further breeding of excellent new varieties.

1 Results

1.1 Fruit quantitative traits

Each trait was significantly different from the various germplasm materials (Table 1), among which six quantitative traits of FLD, FWD, FT, FFM, FMS, NSF was significantly different and the average genetic variation rate of 70.7%. The Vc content and FFM variation coefficient were 42.8% and 139.2%, respectively. The coefficient of variation in five traits was greater than 60.0%, including FLD, FWD, FT, FFM, FMS and NSF, indicating that the genetic variation of 45 pepper germplasms was large.

Table 1 The basic statistic data and diversity index based on the fruit characters descriptors

QT	AVG	Min.	Max.	S.D.	CV (%)
FLD (cm)	6.598	1.465	19.326	4.325	65.6
FWD (cm)	2.268	0.546	7.237	1.648	72.7
FT (mm)	2.339	0.86	6.44	1.333	57
FFM (g)	18.79	0.51	121.99	26.156	139.2
FMS (g)	1.143	0.1	3.343	0.874	76.5
NSF (grain)	118.267	28	346	74.347	62.9
Vc (mg/g)	0.772	0.311	1.531	0.33	42.8
SS (mg/g)	31.123	9.92	72.23	15.275	49.1
AVG	-	-	-	15.536	70.7

Note: QT: Quantitative Trait; AVG: Average; Min.: Minimum value; Max.: Maximum value; S.D.: Standard Deviation; CV: Coefficient of Variation

1.2 The correlation of among fruit quantitative traits

To analyze the correlation coefficient among quantitative traits in fruits, we can understand the degree of correlation among traits, further improving the breeding efficiency. The results showed that there were intricate relationships among eight quantitative traits (Table 2). The maximum correlation coefficient between FFM and FTD was 0.906 with a significant positive correlation, which indicating the largest factor affects the fresh mass of pepper was FWD. The minimum correlation coefficient between SS content and NSF was 0.001, and there was almost no correlation between them, but there were positive and negative small correlations compared with other traits. The correlation coefficient of Vc content with FLD and FMS were 0.407 and 0.404, respectively, which were significantly correlated, indicating peppers with high Vc content can be selected as parents with a larger NSF and FTD.

Table 2 Correlation coefficient of fruit quantitative traits

QT	FLD (cm)	FTD (cm)	FT (mm)	FFM (g)	FMS (g)	NSF (grain)	Vc (mg/g)	SS (mg/g)
FLD (cm)	1	-	-	-	-	-	-	-
FTD (cm)	0.339	1	-	-	-	-	-	-
FT (mm)	0.338	0.871**	1	-	-	-	-	-
FFM (g)	0.492**	0.906**	0.766**	1	-	-	-	-
FMS (g)	0.607**	0.763**	0.630**	0.779**	1	-	-	-
NSF (grain)	0.440*	0.901**	0.740**	0.835**	0.843**	1	-	-
Vc (mg/g)	0.407*	0.202	0.147	0.191	0.404*	0.26	1	-
SS (mg/g)	-0.048	0.1	0.183	-0.022	-0.064	0.001	0.051	1

Note: *,**,*** means significant differences at $p<0.05$; $p<0.01$ and $p<0.001$, respectively

1.3 Cluster analysis of fruit quantitative traits

Based on eight traits of 45 pepper germplasms, the Euclidean distance was calculated and the dendrogram was obtained by the cluster analysis (Figure 1). The 45 pepper germplasms were clustered into six taxa at Euclidean distance of 7.5. The mean values of the eight quantitative traits for each taxon are shown in Table 3.

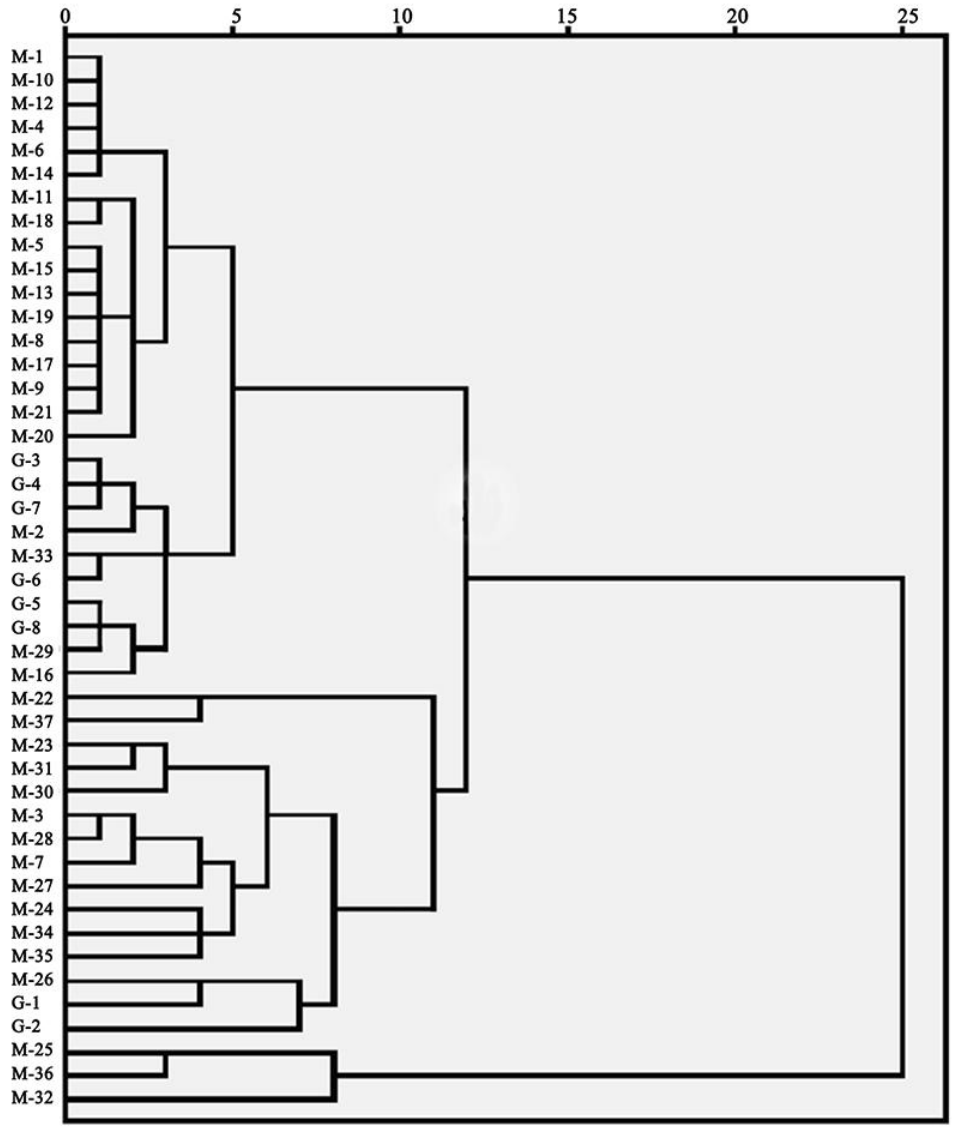

Figure 1 The dendrogram of program using Between-groups Linkage

The first group includes 27 varieties, which can be further divided into 2 subgroups. The first subgroup includes M-1, M-10, M-12, M-4, M-6, M-14, M-11, M-18, M-5, M-15, M-13, M-19, M-8, M-17, M-9, M-21, M-20. The second subgroup includes G-3, G-4, G-7, M-2, M-33, G-6, G-5, G-8, M-29, M-16. The second group includes M-22 and M-37. The third group can be divided into 2 subgroups. The first subgroup consisted of M-23, M-31 and M-30. The second subgroup consisted of M-3, M-28, M-7, M-27, M-24, M-34 and M-35. The fourth group includes M-26, G-1 and G-2. The fifth group includes M-25 and M-36. The sixth group has only one variety, M-32.

We know that in the mean value of FLD, group 5 > group 4 > group 3 > group 1 > group 6 > group 2. In the mean value of FTD, group 6 > group 4 >group 2 > group 3 > group 5 > group 1. In the mean value of FT, group 6 > group 2 > group 4 > group 5 > group 3 > group 1. In the mean value of FFM, group 4 > group 6 > group 5 > group 2 > group 3 > group 1. In the mean value of FMS, group 4 > group 5 > group 3 > group 6 > group 2 > group 1. In the mean value of NSF, group 4 > group 6 > group 2> group 3 > group 5 > group 1. In the mean value of Vc content, group 5 > group 2 > group 4 > group 3 > group 1 > group 6. In the mean value of soluble sugars, group 2 > group 5 > group 3 > group 1 > group 6 > group 4 (Table 3).

Table 3 The most value and average value of eight quantitative characters in all kinds of groups

Group	FLD (cm)	FTD (cm)	FT (mm)	FFM (g)	FMS (g)	NSF (grain)	Vc (mg/g)	SS (mg/g)
1	0.67-13.84	0.54-2.23	0.86-2.34	0.51-11.27	0.10-1.31	35-135	0.31-1.46	9.92-60.39
	4.8	1.19	1.45	3.44	0.58	69.9	0.71	29.05
2	3.89-4.53	4.68-4.69	4.56-5.55	23.21-35.13	0.62-1.72	153-220	0.74-1.19	66.77-72.23
	4.21	4.68	5.06	29.17	1.17	186.5	0.97	69.5
3	3.81-12.48	1.94-4.78	2.49-4.62	14.05-58.74	1.25-3.34	112-269	0.37-1.21	14.64-49.14
	8.12	3.29	3.09	28.36	1.98	184.4	0.8	30.66
4	9.60-10.68	5.54-7.72	4.35-4.39	91.12-121.99	2.84-3.31	255-346	0.75-0.87	16.62-21.65
	10.14	6.39	4.37	106.56	3.07	300.5	0.81	19.14
5	16.61-19.33	2.12-3.19	3.19-3.35	31.06-57.99	1.34-2.57	91-185	0.78-1.53	16.42-57.32
	17.54	2.75	3.29	41.66	2.02	130	1.12	36.46
6	4.73	6.67	6.44	72.58	1.51	226	0.6	23.02

1.4 Genetic diversity analysis of ISSR markers

A total of 149 bands were amplified using the screened 15 ISSR primers on 45 pepper germplasms, with each primer amplifying 6-13 bands, of which primer UBC887 amplified the least number of bands with only 6, while primer UBC899 amplified the most bands with 13, and the length of bands ranged from 200-2000 bp. Among which 89 bands were polymorphic, and the percentage of average polymorphic bands was 59.7%. The minimum percentage of polymorphism amplified by primer UBC808 was 25.0% and the maximum percentage of polymorphism amplified by primer UBC887 was 100.0%. Based on the binary data array of “1” and “0” obtained from the amplification results, the results of genetic diversity parameters were analyzed by POPGENE statistical software (Table 4). For the different primers, the average Shannon index (SI) was 0.3410 and the average Nei's gene diversity index (NGDI) was 0.2316. The primer UBC808 had the lowest SIand NGDI of 0.1673 and 0.1191, respectively. The primer UBC887 had the highest SI and NGDI of 0.5802 and 0.3933, respectively. SI reflects higher genetic diversity than NGDI. The trends in the magnitude of SI and NGDI are consistent with the trends in the percentage of polymorphic sites.

Table 4 Diversity index of ISSR marker

Primer	NAB	NPB	PPB (%)	SI(I)	NDGI(I)
UBC807	10	8	80	0.4337	0.2895
UBC808	8	2	25	0.1673	0.1191
UBC816	8	4	50	0.2749	0.184
UBC825	9	8	88.9	0.489	0.3274
UBC826	10	4	40	0.2214	0.152
UBC835	10	6	60	0.3049	0.2018
UBC856	11	4	36.4	0.1815	0.1202
UBC857	9	4	44.4	0.259	0.1774
UBC864	10	6	60	0.3697	0.2559
UBC881	11	6	54.5	0.3491	0.2446
UBC886	12	8	66.7	0.4066	0.282
UBC887	6	6	100	0.5802	0.3933
UBC889	11	8	72.7	0.4036	0.273
UBC895	11	6	54.5	0.2738	0.1783
UBC899	13	9	69.2	0.4004	0.276
Total	149	89		5.1151	3.4745
AVG	9.9	5.9	59.7	0.341	0.2316

Note: NAB: the number of amplified bands; NPB: the number of polymorphic bands; PPB: the percentage of polymorphic bands; SI: Shannon index; NGDI: Nei gene diversity index; AVG: average

1.5 Clustering analysis of ISSR markers

The binary data array of "1" and "0" entered into NTSYS-PC2.10 software to calculate the genetic similarity coefficient among the varieties (Table 5). Genetic similarity coefficient is an important index to measure the level of germplasm resources variation, where the similarity coefficient was greater, and the kinship was closer between two varieties, otherwise the kinship was more distant. From the genetic similarity coefficient (Table 5), it can be seen that the similarity coefficient between one of the 45 pepper germplasm resources and another ranged from 0.6577 to 0.9262, with M-8 and G-2, M-13 and G-8 having the lowest similarity coefficient of 0.6577, indicating that M-8 and G-2, M-13 and G-8 are more distantly related to each other. The interspecies genetic similarity coefficient between M-1 and M-12, M-26 and M-27, M-26 and M-28, M-27 and M-29, M-22 and M-37, and M-29 and M-34 were above 0.9000. The genetic similarity coefficient among most of the varieties was around 0.8000, indicating these 45 peppers were closely related to each other. Cluster analysis of genetic similarity coefficient by UPGMA method, followed by TreeDisplay function to draw a cluster analysis dendrogram (Figure 2). The results showed (Figure 2) that M-5 and M-17 clustered into one group first with the maximum genetic similarity coefficient between them of GS=0.9262, as well as all peppers clustered into one group with GS=0.6577. The 45 pepper germplasms were classified into five categories at GS = 0.8200. The first type contains M-1, which has cherry-shaped, skyward, purple fruits. The second type contains G-5, which has finger-shaped, skyward, green fruits. Similarly, in the third type there is only one variety, M-25, which has cone-shaped, prone, yellow fruits. The fourth type contains G-2 and G-8, and they are fine wire peppers, prone and green. The fifth type contains 40 varieties except the above four types, the fruit is mostly small conical, pointed at the apex, clustered, more yellow and purple, and individual varieties are green, while there are also individual wire peppers such as M-2, M-26, M-33 and M-34 and lantern peppers such as M-24, M-32 and M-35 in this group.

Table 5 Genetic similarity of molecular markers among forty-five genotypes of pepper according the Jaccard coefficient of similarity

	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8	M-9	M-10	M-11	M-12	M-13	M-14	M-15	M-16	M-17	M-18	M-19	M-20	M-21	M-22	M-23	M-24	M-25	M-26	M-27	M-28	M-29	M-30	M-31	M-32	M-33	M-34	M-35	M-36	M-37	G-1	G-2	G-3	G-4	G-5	G-6	G-7	G-8
M-1	1.0000																																												
M-2	0.7718	1.0000																																											
M-3	0.7517	0.7919	1.0000																																										
M-4	0.7718	0.8523	0.7651	1.0000																																								
M-5	0.8188	0.8322	0.8389	0.8188	1.0000																																						
M-6	0.7919	0.8591	0.8389	0.8322	0.8523	1.0000																																					
M-7	0.7852	0.8523	0.7919	0.8255	0.8591	0.8322	1.0000																																			
M-8	0.7450	0.8121	0.7919	0.8121	0.8725	0.8591	0.8389	1.0000																																	
M-9	0.7852	0.8792	0.8188	0.8523	0.8859	0.8591	0.8523	0.8389	1.0000																																
M-10	0.7450	0.8121	0.8322	0.7987	0.8859	0.8725	0.8389	0.8523	0.8792	1.0000																															
M-11	0.8054	0.8322	0.8255	0.8054	0.8523	0.8121	0.8054	0.8054	0.8591	0.8591	1.0000																														
M-12	0.7718	0.7987	0.8188	0.8255	0.8725	0.8591	0.8389	0.8389	0.8658	0.9195	0.8725	1.0000																													
M-13	0.7584	0.7987	0.8054	0.7987	0.8591	0.8322	0.8121	0.8389	0.8389	0.8523	0.8859	0.8658	1.0000																												
M-14	0.7584	0.8523	0.8054	0.8389	0.8456	0.8725	0.8658	0.8926	0.8792	0.8658	0.8456	0.8523	0.8389	1.0000																											
M-15	0.7919	0.8591	0.8389	0.7919	0.8523	0.8658	0.8456	0.8591	0.8725	0.8591	0.8523	0.8725	0.8054	0.8859	1.0000																										
M-16	0.7919	0.8725	0.7852	0.8322	0.8926	0.8523	0.8322	0.8591	0.8725	0.8725	0.8658	0.8456	0.8591	0.8591	0.8255	1.0000																									
M-17	0.8255	0.8255	0.8322	0.8389	0.9262	0.8456	0.8523	0.8523	0.8792	0.8926	0.8591	0.8792	0.8792	0.8658	0.8054	0.8993	1.0000																								
M-18	0.8121	0.8389	0.8859	0.8255	0.8859	0.8591	0.8523	0.8255	0.8389	0.8523	0.8456	0.8792	0.8389	0.8255	0.8591	0.8591	0.8792	1.0000																							
M-19	0.8054	0.8322	0.8658	0.8054	0.8658	0.8523	0.8456	0.8322	0.8054	0.8322	0.8255	0.8322	0.8456	0.8322	0.8792	0.8255	0.8456	0.8725	1.0000																						
M-20	0.8322	0.8591	0.8523	0.8591	0.8926	0.8523	0.8456	0.7919	0.8456	0.8322	0.8658	0.8725	0.8322	0.8322	0.8255	0.8658	0.9128	0.8993	0.8658	1.0000																					
M-21	0.8054	0.8456	0.7987	0.9128	0.8523	0.8523	0.8456	0.8456	0.8591	0.8456	0.8792	0.8591	0.8322	0.8859	0.8255	0.8658	0.8725	0.8188	0.8255	0.8926	1.0000																				
M-22	0.7718	0.7987	0.8322	0.8389	0.8322	0.8188	0.8255	0.8523	0.8658	0.8523	0.8725	0.8792	0.8255	0.8389	0.8054	0.8188	0.8658	0.8255	0.8188	0.8456	0.8859	1.0000																				
M-23	0.7181	0.7987	0.8591	0.7852	0.7919	0.8188	0.7852	0.7718	0.8121	0.8121	0.7919	0.8121	0.7584	0.8121	0.7919	0.7919	0.8255	0.8121	0.7919	0.8456	0.8054	0.8523	1.0000																			
M-24	0.7315	0.8121	0.8859	0.7718	0.8322	0.8188	0.7987	0.8121	0.8389	0.8389	0.8054	0.8121	0.7852	0.7987	0.8188	0.8188	0.8121	0.8658	0.8322	0.7919	0.7785	0.8255	0.8523	1.0000																		
M-25	0.7651	0.7919	0.8523	0.7651	0.8389	0.8121	0.8188	0.7785	0.8456	0.8456	0.8255	0.8322	0.8188	0.8054	0.8389	0.7852	0.8322	0.8322	0.8389	0.7987	0.7852	0.8456	0.7785	0.8456	1.0000																	
M-26	0.7584	0.8523	0.8188	0.8389	0.8456	0.8188	0.8255	0.8255	0.8792	0.8523	0.8322	0.8523	0.8389	0.8389	0.7919	0.8456	0.8792	0.8523	0.7919	0.8456	0.8322	0.8658	0.8389	0.8523	0.7919	1.0000															
M-27	0.7785	0.8456	0.8255	0.8322	0.8523	0.8389	0.8456	0.8322	0.8859	0.8725	0.8523	0.8591	0.8591	0.8591	0.8121	0.8792	0.8859	0.8591	0.8121	0.8658	0.8792	0.8859	0.8456	0.8591	0.7987	0.9128	1.0000													
M-28	0.7584	0.8255	0.8188	0.8255	0.8456	0.8456	0.7852	0.8389	0.8792	0.8523	0.8322	0.8523	0.8389	0.8389	0.8054	0.8456	0.8523	0.8121	0.7919	0.8188	0.8456	0.8658	0.8389	0.8523	0.7919	0.9195	0.8859	1.0000												
M-29	0.7517	0.8054	0.8389	0.7651	0.8658	0.8523	0.8456	0.8456	0.8725	0.8859	0.8389	0.8456	0.8456	0.8456	0.8121	0.8389	0.8591	0.8322	0.8121	0.8255	0.8255	0.8591	0.8188	0.8456	0.8389	0.8456	0.9060	0.8591	1.0000											
M-30	0.7651	0.8054	0.8121	0.8054	0.8121	0.7987	0.8188	0.8054	0.8456	0.8322	0.8121	0.8456	0.8054	0.8456	0.7852	0.8255	0.8456	0.8322	0.7584	0.8523	0.8389	0.8322	0.8322	0.8054	0.7987	0.8725	0.8658	0.8456	0.8658	1.0000										
M-31	0.7114	0.7919	0.8523	0.8322	0.8389	0.8121	0.8322	0.8054	0.8322	0.8456	0.7987	0.8456	0.8054	0.8322	0.8389	0.8255	0.8188	0.8591	0.7987	0.8523	0.8523	0.8188	0.8322	0.8054	0.7852	0.8054	0.8523	0.8188	0.7987	0.8389	1.0000									
M-32	0.7651	0.8188	0.8389	0.7785	0.8658	0.7852	0.8322	0.8188	0.8456	0.8054	0.8121	0.7919	0.8188	0.8054	0.7987	0.8389	0.8456	0.8322	0.8255	0.8255	0.8121	0.8188	0.8054	0.8725	0.8255	0.8591	0.8523	0.8591	0.8389	0.7987	0.7987	1.0000								
M-33	0.7919	0.8456	0.8255	0.8188	0.8658	0.8255	0.8322	0.8188	0.8322	0.8456	0.8523	0.8456	0.8054	0.8591	0.8523	0.8389	0.8725	0.8859	0.8121	0.8792	0.8523	0.8322	0.8188	0.8188	0.7852	0.8725	0.8658	0.8322	0.8255	0.8658	0.8523	0.8389	1.0000							
M-34	0.7651	0.7785	0.8255	0.7651	0.8389	0.8523	0.8054	0.8456	0.8456	0.8859	0.8255	0.8456	0.8322	0.8725	0.8389	0.8255	0.8322	0.8188	0.8121	0.7987	0.8121	0.8188	0.8054	0.8322	0.7987	0.8188	0.8523	0.8322	0.9060	0.8389	0.7852	0.8255	0.8255	1.0000						
M-35	0.7718	0.7852	0.8322	0.7987	0.8456	0.8188	0.7852	0.8523	0.8389	0.8523	0.8188	0.8255	0.8389	0.8389	0.7919	0.8591	0.8792	0.8389	0.8188	0.8322	0.8456	0.8389	0.8255	0.8523	0.7919	0.8792	0.8859	0.9060	0.8859	0.8591	0.8054	0.8859	0.8456	0.8993	1.0000					
M-36	0.7651	0.7919	0.9195	0.7785	0.8658	0.8121	0.8054	0.8188	0.8591	0.8591	0.8658	0.8456	0.8188	0.8322	0.8255	0.8389	0.8591	0.8725	0.8389	0.8389	0.8121	0.8322	0.8322	0.8859	0.8255	0.8725	0.8926	0.8591	0.8792	0.8255	0.8255	0.8658	0.8389	0.8658	0.8859	1.0000				
M-37	0.7315	0.8121	0.8591	0.8255	0.8188	0.7919	0.8121	0.8121	0.8658	0.8255	0.8456	0.8255	0.7852	0.8389	0.8054	0.8054	0.8389	0.8255	0.7919	0.8188	0.8456	0.9060	0.8658	0.8389	0.8054	0.8658	0.8725	0.8523	0.8456	0.8188	0.8188	0.8188	0.8322	0.8188	0.8523	0.8859	1.0000			
G-1	0.7919	0.8658	0.8389	0.7987	0.8591	0.8456	0.8658	0.8523	0.8389	0.8658	0.8658	0.8255	0.8121	0.8658	0.8591	0.8456	0.8658	0.8591	0.8725	0.8725	0.8591	0.8658	0.8389	0.8255	0.8054	0.8523	0.8993	0.8389	0.8859	0.8188	0.7919	0.8322	0.8591	0.8322	0.8389	0.8389	0.8591	1.0000		
G-2	0.7248	0.7248	0.7315	0.7383	0.6779	0.7047	0.6980	0.6577	0.7248	0.6846	0.6913	0.7114	0.6980	0.6711	0.7181	0.6913	0.6980	0.7785	0.7315	0.7584	0.6644	0.7248	0.7517	0.7248	0.7584	0.7517	0.7181	0.7114	0.6644	0.7450	0.7315	0.7047	0.7315	0.6644	0.6846	0.7047	0.7383	0.6913	1.0000	
G-3	0.7919	0.7919	0.8792	0.7919	0.8792	0.8389	0.8188	0.8054	0.8322	0.8591	0.8255	0.8456	0.8054	0.8322	0.8658	0.8255	0.8725	0.8859	0.8255	0.8658	0.8255	0.7919	0.8188	0.8188	0.8389	0.8054	0.8121	0.8054	0.8255	0.8389	0.8792	0.8389	0.8658	0.7987	0.8456	0.8389	0.8188	0.8255	0.7450	1.0000
G-4	0.7450	0.7852	0.8322	0.7718	0.8322	0.8054	0.7584	0.8121	0.8121	0.8389	0.8054	0.8255	0.7718	0.8121	0.7919	0.8054	0.8121	0.8121	0.7785	0.8054	0.8188	0.8523	0.8255	0.8389	0.7785	0.8658	0.8456	0.8792	0.8456	0.8725	0.8054	0.8322	0.8859	0.8456	0.8926	0.8725	0.8658	0.8121	0.7114	0.8456	1.0000
G-5	0.7987	0.7584	0.7785	0.7450	0.7785	0.7785	0.7181	0.7315	0.7450	0.7718	0.7651	0.7718	0.7315	0.7584	0.7919	0.8054	0.7852	0.8255	0.7919	0.7785	0.7383	0.7450	0.7584	0.7584	0.7651	0.7315	0.7517	0.7047	0.7248	0.7517	0.7785	0.7248	0.8054	0.7651	0.7584	0.7919	0.7718	0.7383	0.7919	0.8188	0.7852	1.0000
G-6	0.8255	0.7987	0.8188	0.8121	0.8456	0.8188	0.7987	0.8523	0.8255	0.8658	0.8993	0.8523	0.8255	0.8658	0.8456	0.8725	0.8792	0.8389	0.8322	0.8591	0.8725	0.8389	0.7852	0.7852	0.7785	0.8255	0.8322	0.8389	0.8188	0.8188	0.7919	0.8054	0.8591	0.8591	0.8792	0.8591	0.8389	0.8591	0.7114	0.8591	0.8389	0.7987	1.0000
G-7	0.8054	0.7919	0.8523	0.7785	0.8658	0.7987	0.7919	0.8054	0.8591	0.8456	0.8658	0.8456	0.8456	0.8054	0.8121	0.8255	0.8859	0.8456	0.8255	0.8389	0.8389	0.8456	0.8054	0.8188	0.8523	0.8591	0.8792	0.8591	0.8523	0.8389	0.8121	0.8523	0.8389	0.8121	0.8859	0.8926	0.8725	0.8389	0.7315	0.8658	0.8591	0.7785	0.8591	1.0000
G-8	0.7383	0.6980	0.7450	0.7248	0.6779	0.7047	0.6846	0.6846	0.7248	0.6711	0.6779	0.6711	0.6577	0.6980	0.7047	0.6644	0.6711	0.7517	0.7181	0.7047	0.6913	0.7248	0.7383	0.7517	0.7315	0.7383	0.7181	0.7114	0.7047	0.7450	0.7047	0.7181	0.7450	0.7315	0.7383	0.7450	0.7517	0.6913	0.8255	0.7315	0.7785	0.7785	0.7248	0.7450	1.0000

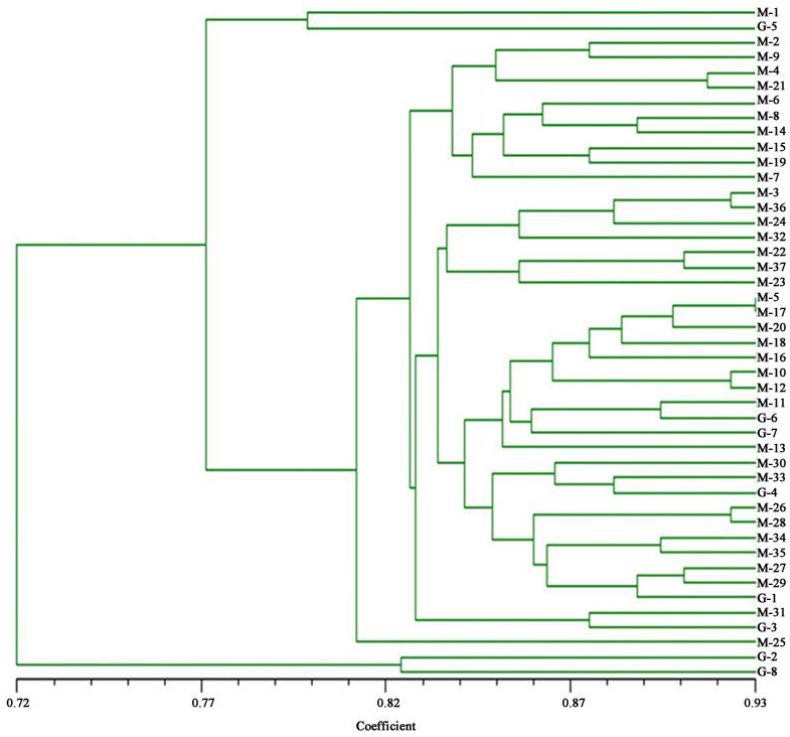

Figure 2 The dendrogram of forty-five genotypes of pepper based on molecular markers

2 Materials and Methods

2.1 Plant materials

In this experiment, 45 pepper materials were used, of which 37 peppers were purchased from Ball, USA and 8 peppers were provided by the Institute of Pepper Research, Guizhou Academy of Agricultural Sciences (GZ Aca. of Agr. Sci.) (Table 6).

Table 6 Description of the test materials

Species number	Name	Sources	Number	Name	Sources
M-1	Hot Ornament Sangria	USA	M-24	Sweet banana	USA
M-2	Cayenne Large Thick	USA	M-25	Whitney	USA
M-3	Big Bertha	USA	M-26	Portugal Hot	USA
M-4	Variegata	USA	M-27	Gypsy	USA
M-5	Thai	USA	M-28	Cherry Pick	USA
M-6	Super Chili	USA	M-29	Hungarian Yellow Wax	USA
M-7	Chevenne	USA	M-30	Jalapeno M	USA
M-8	Peppa Purple Tangerine	USA	M-31	Jalapeno Early Hot	USA
M-9	Cappa Cone White Red	USA	M-32	Alma Paprika	USA
M-10	Masquerade	USA	M-33	Cayenne Long Thin	USA
M-11	Chilly Chill	USA	M-34	Cubanelle	USA
M-12	Red Missile	USA	M-35	Anaheim	USA
M-13	Medusa	USA	M-36	Big Bertha	USA
M-14	Cappa Topfruit White Red	USA	M-37	Big Bomb	USA
M-15	Garda Tricolor	USA	G-1	Guizhou Horn Hepper	GZ Aca. of Agr. Sci.
M-16	Pepper Pepperoni	USA	G-2	Duyun Line Pepper	GZ Aca. of Agr. Sci.
M-17	Garda Hocus Pocus	USA	G-3	Yunnan Wild Pepper	GZ Aca. of Agr. Sci.
M-18	Sangria	USA	G-4	Dangwu Pepper	GZ Aca. of Agr. Sci.
M-19	Garda Fireworks	USA	G-5	Single As Desired	GZ Aca. of Agr. Sci.
M-20	Treasures Red	USA	G-6	Dushan Wrinkled Pepper	GZ Aca. of Agr. Sci.
M-21	Variegata	USA	G-7	Cluster Pepper	GZ Aca. of Agr. Sci.
M-22	Red Cherry Hot	USA	G-8	Dafang Line Pepper	GZ Aca. of Agr. Sci.
M-23	Serrano	USA	-	-	-

Note: GZ Aca. of Agr. Sci.: Guizhou Academy of Agricultural Sciences

2.2 Pepper seedlings growth

Seeds from forty-five pepper samples were soaked in 75% alcohol for 30 seconds and subsequently rinsed them with sterile water, and then seeds were soaked in constant temperature water at 25°C for 20 min, and finally soaked in a raising seeding dish after forcing treatment of bud in thermostat at 25°C for 2 days. Subsequently, seedlings grew in the greenhouse and then controlled the nutrient solution temperature at 25℃~30℃. When the pepper seedlings were reaching at 15~20 cm, they were moved from greenhouse to field. The field trials were arranged with randomized groups, with each variety distributed in the same plot, and each plot was three replicated with a line spacing of 70 cm and a plant spacing of 80 cm, and 10 plants were planted in each plot.

2.3 Field investigation

Five plants were randomly selected from each experimental plot, and two green ripe fruits were randomly selected from each plant to obtain a total of 10 fruits at the fruiting stage of pepper. The fruit longitudinal diameter (FLD), fruit transverse diameter (FTD), flesh thickness (FT), fruit fresh mass (FFM), fresh mass per seed (FMS), number of seeds per fruit (NSF), Vitamin C (Vc) and soluble sugar (SS) content was measured separately.

2.4 DNA extraction

The DNA was extracted by the modified CTAB method. Firstly, add about 0.2 g of young pepper leaves into liquid nitrogen in the first centrifuge tube and ground them into powder. Then added 650 μL of 2% CTAB buffer preheated at 65℃ and 15 μL of β-mercaptoethanol into the first centrifuge tube. To put them in a water bath at 65℃ after carefully inverting the mixture and mixing them well. Now and again, we shook the tube during the heating of water bath. Waited 50-60min before removing them and cooled them for 2 min. Next, added a total of 700 μL of chloroform and isoamyl alcohol in the ratio of 24 to 1 in the tube, then gently shook well. Finally, centrifuged them at 4℃ and 10 000 rpm for 10 min, and the supernatant were carefully put into the second centrifuge tube. The above operation was repeated once more, after which 2 times the volume of cooled anhydrous ethanol and 1/2 volumes of 5 M NaCl added. The mixture was gently shaken and the DNA was precipitated in -20℃ refrigerator for 2 h, and the supernatant was discarded after centrifugation at 10,000 rpm for 10 min at 4℃. The precipitate was washed twice with 70% ethanol, and then solubilized with 100 μL of low salt TE buffer after air-drying. Finally, RNase with a final concentration of 15 μg/mL added, kept in the oven at 37℃ for 30 min, and put in the refrigerator at -20℃.

2.5 ISSR amplification

A total of 100 primers from UBC801-UBC900 designed by Columbia University (synthesized by Shanghai biotechnology) screened with the optimized ISSR-PCR reaction system, from which 15 primers with good band-shape, high polymorphism, good stability and reproducibility (Table 7). The total volume of PCR reaction was 20 μL, containing Taq DNAase 1.0 U, dNTPs 0.20 mmol/L, primers 0.5 μmol/L, Mg^{2+} 1.5 mmol/L, template DNA 60.0 ng, 10×Buffer and purified water. We used the following PCR cycling conditions: 94°C for 5 min, then 36 cycles of 94°C for 1 min, optimal temperature of primers for 45 s, and 72°C for 2 min, with a final extension at 72°C for 10 min.

2.6 Statistical analysis

Traits of variance analysis, mean value, standard deviation, coefficient of variation, correlation coefficient analysis among traits, Euclidean cluster analysis and kinship dendrogram were done by SPSS 18.0 software. According to the presence or absence of consistent bands of electrophoretic mobility in the amplification products of the same primer to count the ISSR amplification products, and the binary data array of all sites obtained, where the presence of amplified bands was recorded as "1" and the absence of amplified bands was recorded as "0" (Liu et al., 2021). Based on the binary data array, POPGENE 1.32 software was used to evaluate each genetic diversity parameter, and NTSYSpc2.10 software was used to calculate the genetic similarity coefficient (GS) among the materials, and the unweighted pair group method with arithmetic mean (UPGMA) was used for the cluster analysis, and finally, the tree-display function was used to draw a dendrogram for cluster analysis.

Table 7 Screening's primer and sequence

Primer	Sequence
UBC807	AGA GAG AGA GAG AGA GT
UBC808	AGA GAG AGA GAG AGA GC
UBC816	CAC ACA CAC ACA CAC AT
UBC825	ACA CAC ACA CAC ACA CT
UBC826	ACA CAC ACA CAC ACA CC
UBC835	AGA GAG AGA GAG AGA GYC
UBC856	ACA CAC ACA CAC ACA CYA
UBC857	ACA CAC ACA CAC ACA CYA
UBC864	ATG ATG ATG ATG ATG ATG
UBC881	GGG TGG GGT GGG GTG
UBC886	VDV CTC TCT CTC TCT CT
UBC887	DVD TCT CTC TCT CTC TC
UBC889	DBD ACA CAC ACA CAC AC
UBC895	AGA GTT GGT AGC TCT TGA TC
UBC899	CAT GGT GTT GGT CAT TGT TCC A

3 Discussion

3.1 Fruit quantitative traits

The variability among eight traits showed significant or even highly significant variation, which was inconsistent with the non-significant difference in flesh thickness reported by the study of Qiao (2006a). It indicated that we should consider introducing foreign peppers with related traits to breed pepper varieties with abundant differences in FT in China. The variation coefficient of five traits was greater than 60.0%, including FLD, FTD, FFM, FMS and NSF, all of which were greater than the variation coefficient reported in China (Chen et al., 2009; Qiao et al., 2006b). This indicates that Chinese pepper germplasm resources are narrow (Sheng et al., 2011) and it is necessary to introduce foreign pepper resources. Also in this study, a high variation coefficient was found that the weight and the number of seeds of per fruit for the first time. The 45 peppers are both edible and highly ornamental, and their full utilization will have great significance to improve the efficiency of hybridization breeding and breeding new germplasms of excellent pepper in China. The correlation analysis showed that the correlation coefficient among pepper fruit traits was highly varied and intricate that was consistent with those, the correlation coefficient was same among FLD, FTD, and FFM and other traits' significance of difference, reported by Chen et al. (2009), but the correlation coefficient was slightly different.

3.2 Fruit quantitative trait clustering and ISSR molecular clustering

Molecular markers reveal species differences by DNA sequences with high sensitivity, while morphological markers reveal differences in gene and environment interactions when the test plants are morphologically similar. In the present study, the results of clustering based on quantitative traits of fruits and clustering based on ISSR molecular markers were consistent with the results reported by some researchers (Yu, 2012; Zhou, 2010). This may be due to the close genetic distance among 45 peppers and bad clustering effect of fruit numbers or it may be because the ISSR molecular markers represent differences in the entire DNA sequence. However, the genes of eight fruit number traits were different from the whole DNA sequence, which also indicated the differences between the two clusters were inevitable. Interestingly, both clusters did not cluster the eight Guizhou peppers separately, but they were mixed with American pepper materials that may be related to the frequent gene exchange, as a result of the pepper introduction and hybridization carried out by the Institute of Pepper Research, Guizhou Academy of Agricultural Sciences in recent years.

Authors' contributions

GJY is the executor of the experimental design and study; GJY, KX and WJF completed the first draft of the paper; GJY, KX, WJF, WL, CXL, SF, LYK, ZXX and ZP participated in the data analysis and analysis of the experimental results; TM and YY were the designers and leaders of the project, guiding the experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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