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Full Length Research Paper

Inter simple sequence repeat analysis of genetic diversity of five cultivated pepper species

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This paper studied the genetic diversity of five cultivated pepper species using inter simple sequence repeat (ISSR) analysis. The amplicons of 13 out of 15 designed primers were stable polymorphic and therefore were used as genetic biomarkers. 135 total clear bands were obtained, of which 102 were polymorphic bands with an average polymorphism rate of 75%. The cultivated species *Capsicum annuum* L. had less specific genotype bands compared with two wildtype species; its Nei's gene diversity index, Shannon's diversity index and genetic differentiation index were 0.21, 0.33 and 0.8306, respectively. The genetic variations of these five species were mainly interspecifically rather than intraspecifically. Cluster analysis showed that they belonged to different branches: *C. annuum* L., *Capsicum chinense* Jacquin. and *Capsicum frutescens* L. were in one cluster, whereas *Capsicum baccatum* L. and *Capsicum pubescens* Ruiz and Pavon were in another cluster. *C. annuum* L. had the least genetic polymorphism. Our results suggested that ISSR markers are valid tags in the studies on pepper genetic diversity and that cultivated species identification and gene exchange between cultivated and wild species should be strengthened in pepper breeding to improve heterosis.

Key words: Pepper, inter simple sequence repeat (ISSR), cultivated species, genetic diversity.

INTRODUCTION

Pepper is an important vegetable and condiment. It is widely planted in Southeast Asia, South Asia, Korean Peninsula and Latin America. International Plant Genetic Resources Board (IBPGR) has classified pepper cultivars into five species: *Capsicum annuum* L., *Capsicum baccatum* L., *Capsicum chinense* Jacquin, *Capsicum frutescens* L. and *Capsicum pubescens* Ruiz & Pavon. The near continuous overlap in morphological characteristics among the C. annuum L., C. *chinense* Jacquin and C. *frutescens* L. led various authors to recognize them as a species complex (Pickersgill, 1971; McLeod et al., 1982). Among them, *C. annuum* L. is the most differentiated, widely cultivated one and the focus in pepper breeding worldwide (Pickersgill, 1997). However, its genetic basis is relatively narrow (Prince et al., 1995) and barely contains outstanding characteristics of other cultived speices, which significantly constrained the progress of pepper breeding. Since the 80s of the 20th century, China has bred 100 new pepper cultivars and updated cultivars in the major pepper-producing areas four to five times which improved pepper yield and quality. The heterosis in pepper is very obvious that the yield of elite hybrids normally is 50% higher than that of the traditional cultivars. Therefore, study on the genetic relationship of different cultivated pepper species to find new parental cultivars and extend gene pool is of importance in effective utilization of pepper germplasm and an effective way to resolve the bottleneck of pepper breeding.

Molecular marker-assisted breeding is an important component of modern breeding technology, by which Wang et al. (2004) have clearly demonstrated that, pepper CMS restoration is controlled by one major gene and four minor genes and further located them on pepper molecular genetic map. Many genetic markers have been used in pepper breeding. For example, Tang et al. (2004) developed a molecular marker OPL09-763 that is closely linked to CMS restorer gene (major) by using RAPD-BSA

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Table 1. Source and category of peppers.

Accession or name	Country	Category	Accession or name	Country	Category
PI171565	Turkey	*Capsicum annuum* L.	PI152225	Peru	*Capsicum chinense* Jacquin
PI193472	Ethiopia		PI645555	Niger	
PI407450	South Africa		PI281305	Boliva	
PI555649	Sudan		PI406987	Panama	
PI262172	Germany		PI594139	Belize	
PI645487	Panama		PI209028	Puerto Rico	
PI451762	Israel		PI244667	India	
PI439378	Jamaica		PI281435	United States	
PI640503	Australia		PI215736	Peru	
PI640578	Taiwan		PI438532	Belize	
Grif 9284	Costa Rica	*Capsicum annuum* var.glabriusculum	PI195301	Guatemala	*Capsicum frutescens* L.
PI273425	United State		PI257062	Colombia	
PI311126	Nicaragua		PI263109	Former Soviet Union	
PI406948	Nicaragua		PI631151	Nepal	
PI439325	Nicaragua		PI355395	Ecuador	
PI566812	Mexico		PI640907	Nigeria	
PI631135	Guatemala		PI406847	Honduras	
PI631139	Guatemala	*Capsicum baccatum* L.	Grif9286	Costa Rica	*Capsicum pubescens* Ruiz & Pavon
PI267729	Guatemala		PI159261	United States	
PI215699	Peru		PI593620	Guatemala	
PI215700	Peru		PI593639	Guatemala	
PI281310	Brazil		Co0709	Guatemala	
PI260595	Brazil		Co4895	Guatemala	
PI 293349	Peru		Co2415	Guatemala	
PI281408	Peru	*Capsicum baccatum* var.baccatum	PI439382	Paraguay	*Capsicum baccatum* var.baccatum
PI439369	Bulgaria		PI439411	Uraguay	
PI263258	India		PI593932	Ecuador	
PI439371	Chile		PI594136	Hungary	

method. Wang et al. (2005) developed two RAPD markers possibly linked with sterile genes. These markers have accelerated pepper hybrid breeding to certain extent. Inter simple sequence repeat (ISSR) labeling is a PCR-based molecular biology technique. It requires very small amount of template and is convenient in result recording and highly reproducible (Ziet et al., 1994), therefore, is widely used in species identification, genetic mapping, gene locating, phylogeny and evolution (Laura et al., 2008; Zamani et al., 2011; Dong et al., 2011). In this study, the genetic polymorphisms and relationships of 40 varieties from five cultivated pepper species were evaluated by using ISSR-PCR technology and compared with those of two wild pepper species in order to understand their intra- and inter-species variations, clarify the phylogenetic among the species and provide theoretical support for the accurate identification of pepper germplasm resources and their further appication.

MATERIALS AND METHODS

56 plants of the two wild and five cultivated pepper species were from the United States Department of Agriculture and the Vegetable Institution of Hunan Academy of Agricultural Science (Table 1). The varieties were selected from major continents that pepper species were originated from and cultivated.

ISSR primer screening

A total of 50 primers with length of 17 to 18 bp synthesized by the Sangon Biotec (Shanghai) Co., Ltd. were used in the ISSR-PCR reaction. After a preliminary test, we selected 13 primers; those had clear, repeatable, amplified polymorphic bands under optimal conditions and were used to analyze the polymorphrism of pepper cultivars. Each experiment was performed at least twice. The

Table 2. ISSR Primers and optimal annealing temperature.

Primer	Sequence	Optimal annealing temperature (℃)	Primer	Sequence	Optimal annealing temperature (℃)
ISSR-807	$(AG)_8T$	50.6	ISSR-849	$(GT)_8YA$	50.7
ISSR-811	$(GA)_8C$	52.2	ISSR-851	$(GT)_8YG$	52.1
ISSR-815	$(CT)_8G$	51.9	ISSR-853	$(TC)_8RT$	50.3
ISSR-823	$(TC)_8C$	52.5	ISSR-857	$(AC)_8YG$	51.8
ISSR-825	$(AC)_8T$	50.5	ISSR-861	$(ACC)_6$	60.2
ISSR-827	$(AC)_8G$	51.5	ISSR-892	$HVH(TG)_7$	59.5
ISSR-843	$(CT)_8RA$	50.3			

optimal annealing temperature of each primer pairs was explored separately (Table 2).

PCR amplification and product detection

Genomic DNAs of all the plants were extracted using CTAB method (Rogers and Bendich, 1988) and were used as PCR templates. ISSR-PCR were performed in 25 µl reaction system containing 2.5 µl of 1 × PCR buffer, 1.25 mmol/l Mg^{2+}, 0.32 mmol/l dNTPs, 1.2 µmol/l primer, 1.5 U Taq enzyme and 40 ng template under the following conditions: 95℃ denaturation for 5 min, followed by 40 cycles of 94℃ denaturation for 1 min, 50℃ annealing for 70 s and 72℃ extension for 1.5 min and a final extension at 72℃ for 10 min. Amplicons were separated by 2.0% agarose gel electrophoresis at 5 $V{\cdot}cm^{-1}$ for 1.5 h and photographed by Tanon 4100 gel imaging system.

Statistical analysis and data processing

Each experiment was performed in duplicates and those with stable and clear bands were analyzed. Every DNA fragment, regardless strong or weak was considered as one molecular marker that represented one primer binding site. Existence of binding site (dominant) was quantified as 1 and no band (hidden) was considered as 0. The populations of polymorphic loci (PPL), Nei's genetic diversity index (H), Shannon's information index (I), genetic differentiation coefficient (Gst), total gene diversity (Ht) of cultivated species and genetic diversity within cultivated species (Hs) were calculated by POPGEN32 software. Nei's genetic distances were used to perform UPGMA cluster analysis and construct phylogenetic trees.

RESULTS AND ANALYSIS

Genetic diversity of the five cultivated pepper species

A total of 135 amplicons were obtained in 28 pepper plants by ISSR-PCR using 13 primers. These amplicons had molecular weights between 100 and 2000 bp and 102 polymorphic loci, accounting for 75% of the total loci. Each primer amplified the average 10.38 bands, among which, 8.50 were polymorphic bands with Nei's gene diversity index (H) of 0.21 and Shannon's information index (I) of 0.33.

The percentages of polymorphic loci within the cultivated species were between 12.21 and 25.08%. In detail, that of *C. chinense Jacquin* was the highest (25.08%), followed by 24.12% of *C. frutescens L.*, that of *C. pubescens Ruiz* & *Pavon* was the lowest (10.57%), followed by *C. baccatum* L. (12.21%) and *C. annuum* L. (12.58%). The Nei's genetic diversity indices (H) were between 0.03 and 0.08 and Shannon's information indices (I) were between 0.06 and 0.13. The percentages of polymorphic loci of the two wild species were 34.8% for *C. annuum var. glabriusculum* and 38.9% for *C. baccatum var.baccatum* and were much higher than those of the five cultivated species, indicating that the two wild species had higher genetic diversity.

Specific band analysis of the five cultivate pepper species

Comparative analysis of the PCR specific bands of the five cultivated pepper species showed that each species had 1 to 3 specific bands, which can be used as molecular markers to label and identify different cultivated species (Table 3). Wildtype *C. annuum var. glabriusculum* and *C. baccatum* var. baccatum had 4 and 6 specific bands, respectively.

Genetic differentiation of the five cultivated pepper species

Genetic differentiation coefficient (Gst) is the ratio of genetic differences between cultivated species to total genetic variation and was calculated according to the genetic diversity of the cultivated species (Hs) and the total genetic diversity (Ht). The total gene diversity (Ht) of the five cultivated pepper species was 0.35 and the genetic diversity within the cultivated species (Hs) was 0.11. The genetic differentiation coefficient between the cultivated species (Gst) was 83.06 and 16.94%, respectively, indicating that, genetic differentiation within the cultivated species was relatively low and that between the cultivated species was relatively high.

UPGMA dendrogram showed that, the five cultivated pepper species could be divided into two large branches.

Table 3. Specific bands of different population.

Category	Numbers of specific band	Primer	Fragment size (bp)
Capsicum annuum L.	2	807	300,350
		823	420
Capsicum annuum var.*glabriusculum*	4	857	1250
		892	240
		861	300
		853	400
Capsicum baccatum L.	3	811	240
		827	410
		853	130
Capsicum baccatum var.*baccatum*	6	861	250
		827	200
		851	160
		851	500
		815	200
		843	400
Capsicum chinense Jacquin	1	853	200
Capsicum frutescens L.	2	849	420
		857	340
Capsicum pubescens Ruiz & Pavon	3	815	300
		823	430

Cluster *C. annuum L.* and *C. annuum var. glabriusculum* and cluster *C. chinense Jacquin* and *C. frutescens L.* merged into one branch; Cluster *C. baccatum L.* and *C. baccatum var.baccatum* and cluster *C. pubescens Ruiz & Pavon* constituted the other branch (Figure 1). These results indicated a clear difference in the genetic relationship between the cultivated species: species with closer genetic relationships cluster first and that with distant genetic relationship cluster later.

DISCUSSION

The genetic diversity and relationship of the five cultivated pepper species was extensively studied previously based on morphology and cytology. Egawa (1985, 1986) studied the chromosome pairing of interspecific pepper hybrids during meiosis. He observed a very small number of univalents during meiosis of the interspecific hybrids of *C. chinense Jacquin* and *C. frutescens* L., indicating that, their chromosomes were highly homologous. *C. frutescens* L. was closest to *C. chinense Jacquin*, followed next by *C. annuum* L. and then by *C. baccatum* L. Bahadur (2003) found that pollen fertile rate of hybrid of *C. frutescens* L. and *C. chinense Jacquin* was only 28%, whereas that of the intraspecific hybrid was 75%. Thus, they concluded that *C. frutescens* L. and *C. chinense Jacquin* were distant relatives. Levebvre et al. (1993) studied genomic DNA polymorphism of 13 cultivars of *C. baccatum* L and one cultivar of *C. annuum* L by RFLP technology and found that interspecific genetic variation of *C. annuum* L. and *C. baccatum* L. was great. Prince et al. (1995) analyzed Southern hybridization bands of a total of 21 cultivars of the five cultivated pepper species; *C. annuum* L., *C. baccatum* L., *C. chacoense*, *C. chinense* Jacquin and *C. frutescens* L. by RFLP technology and found that these 21 cultivars had 4 distinct clusters, three of which were closely connected clusters presented in *C. annuum* L., *C. frutescens* L. and *C. baccatum* L. and the other one was only presented in *C. chinense* Jacquin and *C. chacoens*, indicating a closer relationship among the former three species and between the latter two species. This study showed that *C. annuum* L., *C. frutescens* L. and *C. chinense* Jacquin were closely related and had distant relationship to the other two species, in agreement with Egawa (1985) conclusions. ISSR markers have been widely used to analyze plant genetic diversity because of their good stability and high genetic polymorphisms. Our results showed that *Capsicum pubescens* Ruiz & Pavon

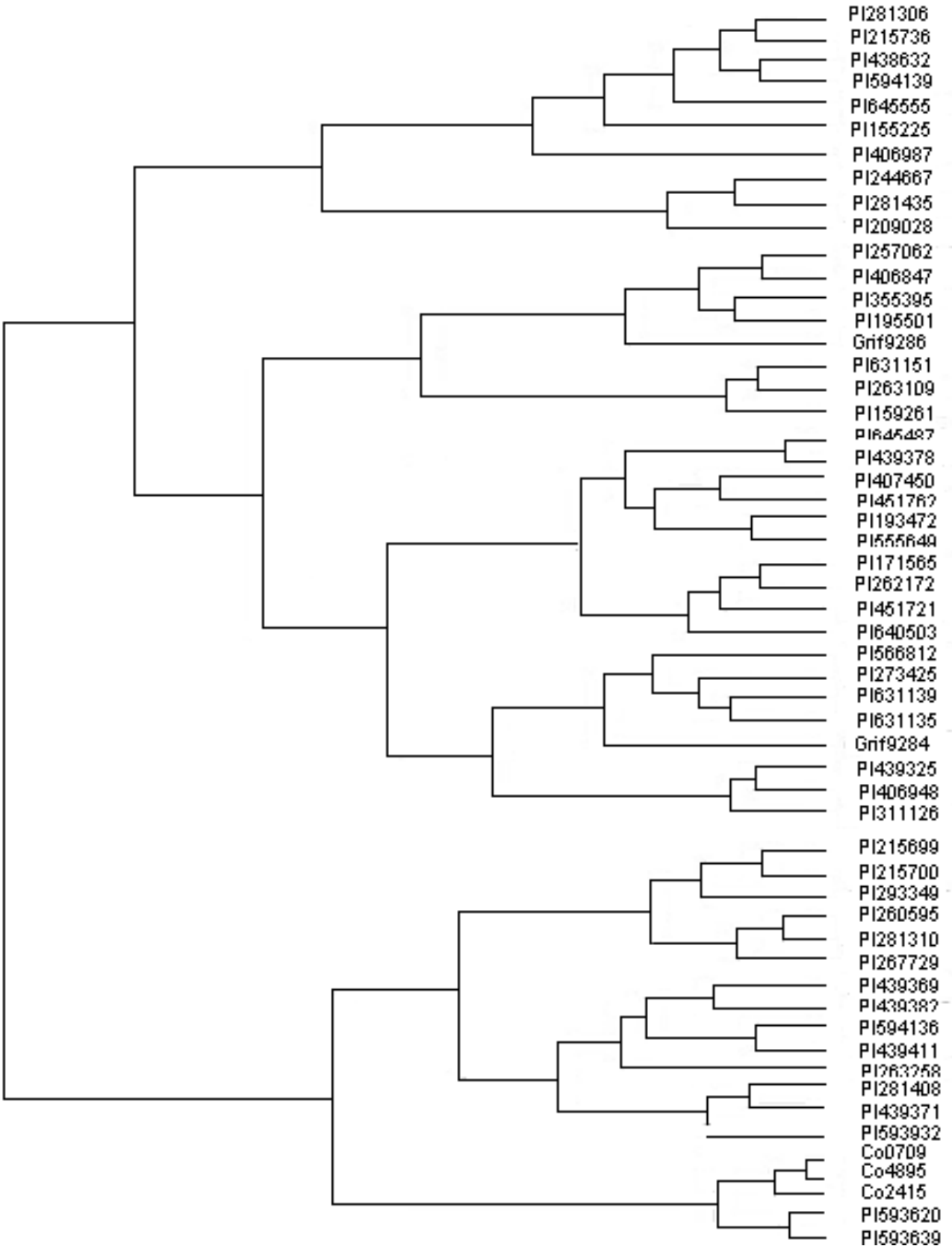

Figure 1. Homologous tree of peppers based ISSR markers. Cluster C. annuum L. and C. annuum var. glabriusculum and cluster C. chinense Jacquin and C. frutescens L. merged into one branch and Cluster C. baccatum L. and C. baccatum var.baccatum and cluster C.pubescens Ruiz & Pavon constituted the other branch.

had the lowest genetic polymorphism. It might be that the mainly distributed plateau and narrow distributation affected its gene flow. ISSR markers reveal the law from the molecular level and it is more reliable than the morphological characteristics, therefore, our results revealed the phylogenetic relationships of the five cultivated species more authentically.

The major cultivated species currently used in pepper production is *C. annuum* L. However, further studies on cross breeding found that this species lacks genetic polymorphism and heterosis is hardly obtained. For examples, Levebvre et al. (1993) concluded based on RFLP results that the sweet pepper cultivars in *C. annuum* L. have homogenous genetic background and lack genetic polymorphism. Heras et al. (1996) analyzed 11 pepper cultivars using RAPD analysis and concluded that *C. annuum* L. has uniformed genetic background and lack genetic diversity. Using RAPD marker analysis, Wang et al. (2003) tested genetic diversity of inbred and hybrid lines of *C. annuum* L including bell pepper, long pepper, cone pepper and tufted pepper and found that less than 50% of the bands are polymorphic, indicating narrow genetic base among the tested peppers. Consistent with those findings, this study also shows low genetic polymorphism within *C. annuum* L. This study also found that the genetic diversities of all the five cultivated species were less than those of the two wild species. it is known that *C. frutescens* L. is resistant to blight and verticillium wilt, *C. chinense* Jacquin is resistance to verticillium wilt and *C. baccatum* L. is resistant to TMV, CMV and blight. Therefore, breakthrough in breeding is more difficult to achieve by cross breed among the cultivated species. Gene exchange between cultivated and wild species should be strengthened in pepper breeding to improve heterosis.

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