# Research on Genetic Diversity of Pepper Germplasm Resources by Inter-simple Sequence Repeat Molecular Markers

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**Abstract** Eight Capsicum annuum were studied for genetic diversity using ISSR markers. After screen of 30 ISSR primers, 21 pairs could amplify clear bands. And 382 bands were amplified by using above 21 pair of primers, of which 270 bands (70.6%) presented polymorphism. According to Nei and Li, the genetic similarity coefficient (GS) and genetic distances (GD) of eight kinds of materials were analyzed. Based on the analysis of clustering SPSS16.0 software system, the corresponding clustering map was constructed.

## Introduction

Capsicum annuum, alias pepper, has been introduced into China from the Ming and Qing Dynasties, it has been 300 years of cultural history [1]. Chinese pepper cultivation area is around six hundred acres, it spreads mainly in Fujian millet pepper, chili peppers in Sichuan, the horn pepper of Southern Yangtze, cherry pepper Henan, large red peppers of Shandong, etc.. it is indispensable seasoning in the food cooking process possessing its spicy fragrance, warm, irritation, simultaneously, its annual export amount and economic benefits ranked first in the field of vegetable crops since as it is a favorite condiment with high nutritional value[2,3].

A lot of simple tandem repeats (simple sequence repeat, SSR) distribution is decentralized [4] in eukaryotic genomes. SSR is usually constituted of more than 4 tandem repeat units forming by 1 to 4 bases. Inter-simple sequence repeat (ISSR) [5] is a new kind of marker technology developed on the basis of SSR. The basic principle is added a anchor bases formed by 1 to 4 bases at the 5 'or 3' terminal of SSR sequence, and using it as a primer amplified genomic DNA at both ends of the reverse arrangement SSR. ISSR technology is a fast and reliable marker technology with better stability and repeatability comparing SSR. It has been widely used in the item of identification, genetic mapping, gene mapping and genetic analysis diversity on major crops, such as rice [6], wheat [7] and cotton [8]. Certainly, ISSR marker technology also has some drawbacks, it mainly targeted plant, and rarely reported in the animal terms. Pepper attracted more and more scientist's attention with its unique position as a vegetable and condiment. In present work, genetic diversity of eight pepper seeds from different sources were analyzed systematically by ISSR technology.

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## Material and Methods

### Instruments and equipments

Eppendorf AG22331 PCR amplification was purchased from Hamburg. Both DYY-12 multifunctional electrophoresis with Sanheng PC system and Gel imaging system TANON-200 were supplied by Tanon Science & Technology Co., Ltd.,Shanghai, China. Both deoxynucleotide triphosphate and Taq DNA polymerase were synthesized by Tiangen Biotech (Beijing) CO.,LTD.

### Treatment of Materials

Materials

Eight pepper seeds and its corresponding information in this experiment were shown in Table 1-1, ISSR-PCR amplification system is shown as Table 1-2. The sequence of PCR primer was displayed in Table 1-3.

**Table 1-1 Eight pepper materials**

| Number | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| Name | 2011-4 | New 9A | Guo 228 | Fengsan A | Xixian | Wangyi | 261A | Color 2011-2 |

**Table 1-2 ISSR-PCR amplification system**

| | |
|---|---|
| DNA template | 2.0 μL |
| 10×Taq PCR buffer（$Mg^{2+}$ ） | 2.5 μL |
| dNTP(2.5 mM) | 2.0 μL |
| ISSR primer(50 ng/μL) | 2.0 μL |
| Taq enzyme（5 U/μL) | 0.5 μL |
| $ddH_2O$ | 16.0 μL |
| Total volume | 25 μL |

Treatment of materials

After these eight pepper seeds grown into seedlings in the laboratory condition, harvest seedlings and put it into the refrigerator (-80 ℃ :, 24 hours), then quickly take it out and grind into powder, immediately load in the tubes (1.5mL).

### Methods

Extraction and detection of DNA

Pepper DNA was extracted by modified CTAB method. The integrity of pepper DNA was detected by agarose gel electrophoresis.

ISSR-PCR amplification system

PCR gene amplification reaction system was 25 uL, the detail information of reaction system was listed in table 1-3. PCR amplification conditions were as following: 95 ℃ : denaturation, 5min **à** 94 ℃ : denaturation, 30s **à** annealing (temperature determined by the character of primer), 30s **à** 72 ℃ : extension 60s, 40 cycles **à** 72 ℃ : constant temperature, 10min.

**Table 1-3 Sequence of ISSR primers**

| Name | Sequence（5'--3'） | Name | Sequence（5'--3'） |
|---|---|---|---|
| ISSR-01 | ATATATATATATATATT | ISSR-16 | GTGTGTGTGTGTGTGTC |
| ISSR-02 | ATATATATATATATATG | ISSR-17 | GTGTGTGTGTGTGTGTT |
| ISSR-03 | ATATATATATATATATC | ISSR-18 | TCTCTCTCTCTCTCTCC |
| ISSR-04 | TATATATATATATATAC | ISSR-19 | TCTCTCTCTCTCTCTCG |
| ISSR-05 | TATATATATATATATAG | ISSR-20 | ACACACACACACACACT |
| ISSR-06 | AGAGAGAGAGA AGAGT | ISSR-21 | ACACACACACACACACC |
| ISSR-07 | AGAGAGAGAGAGAGAGC | ISSR-22 | ACACACACACACACACG |
| ISSR-08 | AGAGAGAGAGAGAGAGG | ISSR-23 | TGTGTGTGTGTGTGTGC |
| ISSR-09 | GAGAGAGAGAGAGAGAT | ISSR-24 | TGTGTGTGTGTGTGTGG |
| ISSR-10 | GAGAGAGAGAGAGAGAC | ISSR-25 | ACCACCACCACCACCACC |
| ISSR-11 | CTCTCTCTCTCTCTCTT | ISSR-26 | AGCAGCAGCAGCAGCAGC |
| ISSR-12 | CTCTCTCTCTCTCTCTA | ISSR-27 | AGTAGTAGTAGTAGTAGT |
| ISSR-13 | CTCTCTCTCTCTCTCTG | ISSR-28 | ATGATGATGATGATGATG |
| ISSR-14 | CACACACACACACACAT | ISSR-29 | CCGCCGCCGCCGCCGCCG |
| ISSR-15 | CACACACACACACACAG | ISSR-30 | CTCCTCCTCCTCCTCCTC |

Detection of ISSR products

Amplification products were detected using 3% agarose gel electrophoresis, and saved the pictures using gel imaging system.

Data Analysis

Statistics of bands: the clear and the recurring weak bands counted yes, otherwise no. Using NTSYS-pc statistical analysis software (Rohlf, 2000) calculated genetic similarity (GS) and distance (GD). $GS = 2N_{ij} / (N_i + N_j)$ and $GD = -\ln GS$ , where $N_{ij}$ is the total number bands of material i and j; Ni and Nj is the number bands of material i and j, respectively. Spss16.0 analysis software system was utilized to carry out clustering, and constructed the corresponding genetic clusters map.

## Results and Discussion

### Quality and integrity of pepper DNA

Eight DNA of pepper seedlings were extracted by reformed CTAB method. The result of agarose gel electrophoresis was shown as Fig.2-1: extracting DNA bands bright and intact without tailing phenomenon, corresponding name please refers Table 1-1. It showed that extracted DNA received little contamination by protein or polysaccharide. The extracted genomic DNA quality meets the requirements of ISSR technology.

### Amplification of effect of primers

Genetic polymorphic analysis of 8 Pepper based on amplification using 30 ISSR primers showed that 21 ISSR primers exception of ISSR-01, 02, 03, 23, 25, 26, 27 and 29 obtained stable polymorphic bands and finally amplified 382 clear bands as shown in Table 2-1. Fragment size rang from 250 ~ 2000bp, and amplified bands numbers of every primer is 1 to 8. There are 270 polymorphic bands and the average polymorphism ratio is 70.7%, see Fig. 2-2.

**Table 2-1 Amplification bands collection of ISSR primers**

| Primers | Statistics of bands numbers amplification by ISSR | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | Total |
| ISSR-01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ISSR-02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ISSR-03 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ISSR-04 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 3 |
| ISSR-05 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 2 | 12 |
| ISSR-06 | 0 | 1 | 2 | 1 | 2 | 2 | 2 | 0 | 10 |
| ISSR-07 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 6 |
| ISSR-08 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 15 |
| ISSR-09 | 3 | 3 | 3 | 2 | 2 | 2 | 4 | 4 | 23 |
| ISSR-10 | 3 | 3 | 3 | 3 | 2 | 3 | 2 | 3 | 22 |
| ISSR-11 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 4 |
| ISSR-12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ISSR-13 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 12 |
| ISSR-14 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 32 |
| ISSR-15 | 5 | 6 | 5 | 6 | 5 | 5 | 4 | 4 | 40 |
| ISSR-16 | 2 | 2 | 2 | 2 | 0 | 1 | 0 | 0 | 9 |
| ISSR-17 | 3 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 15 |
| ISSR-18 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 5 |
| ISSR-19 | 5 | 5 | 5 | 5 | 0 | 3 | 0 | 5 | 28 |
| ISSR-20 | 3 | 4 | 4 | 3 | 2 | 4 | 2 | 3 | 25 |
| ISSR-21 | 5 | 6 | 5 | 5 | 6 | 5 | 0 | 6 | 38 |
| ISSR-22 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 14 |
| ISSR-23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ISSR-24 | 0 | 2 | 2 | 0 | 2 | 0 | 0 | 0 | 6 |
| ISSR-25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ISSR-26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ISSR-27 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ISSR-28 | 3 | 3 | 3 | 3 | 4 | 3 | 3 | 4 | 26 |
| ISSR-29 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ISSR-30 | 8 | 7 | 6 | 6 | 2 | 8 | 0 | 0 | 37 |
| Total | 56 | 60 | 58 | 52 | 41 | 47 | 29 | 39 | 382 |

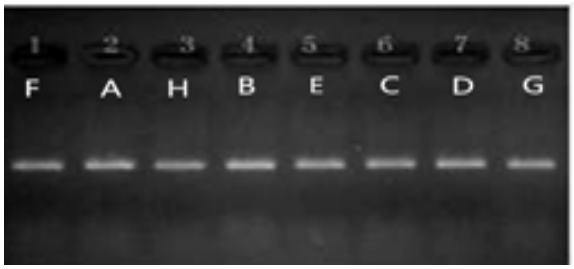



Fig. 2-1 DNA gel electrophoresis of eight pepper varieties

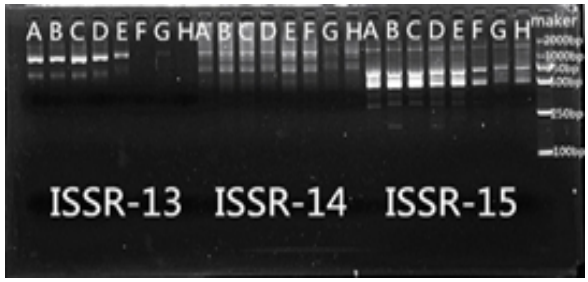



Fig.2-2 Gel electrophoresis of amplification products of selected pepper ISSR primers

**Genetic similarity coefficient of different experimental materials**

To elaborate the genetic similarity coefficient of different materials, the experiment related primer ISSR-15 was selected as a representation. Table 2-2 listed the amplification bands number using ISSR-15 primer. Table 2-3 displayed the genetic diversity bands numbers of pepper molecular marker. Based on Table 2-2 and Table 2-3, genetic similarity coefficient and genetic distance obtained between any two pepper varieties. Table 2-4 showed the comparison between any two pepper varieties. Table 2-5 presented the genetic distance comparison between two pepper varieties. 40 amplified bands of the eight pepper were obtained using ISSR-15 primers amplification. 29 GS values were obtained through the comparison between any two pepper of eight material, and genetic similarity coefficient rang from 0.55 to 0.91 between any two varies. An average genetic similarity coefficient was 0.806, and the average genetic distance is 0.227. Based on the principle that the difference of the genetic similarity coefficients more lager, the genetic relationships more close between any two pepper varieties: Genetic similarity coefficient of eight pepper varieties varied from 0.55 to 0.91.

**Table 2-2 Amplification bands collection of ISSR-15 primers**

| Primer | Statistics of ISSR bands numbers | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2011-4 | New 9A | Guo 228 | Fengsan A | Xixian | Wangyi | 261A | Color 2011-2 | Total |
| ISSR-15 | 5 | 6 | 5 | 6 | 5 | 5 | 4 | 4 | 40 |

**Table 2-3 Genetic diversity collection of pepper molecular marker technology**

| Material | 2011-4 | New 9A | Guo 228 | Fengsan A | Xixian | Wangyi | 261A | Color 2011-2 |
|---|---|---|---|---|---|---|---|---|
| 2011-4 | - | - | - | - | - | - | - | - |
| New 9A | 5 | - | - | - | - | - | - | - |
| Guo 228 | 4 | 5 | - | - | - | - | - | - |
| Fengsan A | 4 | 5 | 5 | - | - | -- | - | - |
| Xixian | 5 | 5 | 4 | 5 | - | - | - | - |
| Wangyi | 3 | 3 | 3 | 3 | 3 | - | - | - |
| 261A | 4 | 4 | 4 | 4 | 4 | 3 | - | - |
| Color 2011-2 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | - |

**Table 2-4 Comparison of genetic similarity coefficient between two pepper varieties**

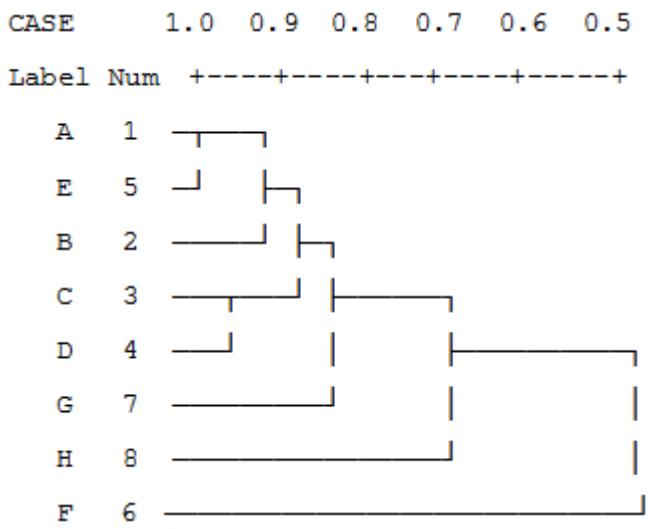
| Material | 2011-4 | New 9A | Guo 228 | Fengsan A | Xixian | Wangyi | 261A | Color 2011-2 |
|---|---|---|---|---|---|---|---|---|
| 2011-4 | - | - | - | - | - | - | - | - |
| New 9A | 0.91 | - | - | - | - | - | - | - |
| Guo 228 | 0.80 | 0.91 | - | - | - | - | - | - |
| Fengsan A | 0.73 | 0.83 | 0.91 | - | - | - | - | - |
| Xixian | 0.91 | 0.91 | 0.80 | 0.91 | - | - | -- | - |
| Wangyi | 0.60 | 0.73 | 0.60 | 0.55 | 0.60 | - | - | - |
| 261A | 0.89 | 0.80 | 0.89 | 0.80 | 0.89 | 0.67 | - | - |
| Color 2011-2 | 0.89 | 0.80 | 0.89 | 0.80 | 0.89 | 0.89 | 0.75 | - |

**Table 2-5 Comparison of genetic distance between two pepper varieties**

| Material | 2011-4 | New 9A | Guo 228 | Fengsan A | Xixian | Wangyi | 261A | Color 2011-2 |
|---|---|---|---|---|---|---|---|---|
| 2011-4 | - | - | - | - | - | - | - | - |
| New 9A | 0.094 | - | - | - | - | - | - | - |
| Guo 228 | 0.223 | 0.094 | - | - | - | - | - | - |
| Fengsan A | 0.315 | 0.186 | 0.094 | - | - | - | - | - |
| Xixian | 0.094 | 0.094 | 0.223 | 0.094 | - | - | - | - |
| Wangyi | 0.511 | 0.315 | 0.511 | 0.598 | 0.511 | - | - | - |
| 261A | 0.117 | 0.223 | 0.117 | 0.223 | 0.117 | 0.400 | - | - |
| Color 2011-2 | 0.117 | 0.223 | 0.117 | 0.223 | 0.117 | 0.117 | 0.288 | - |

**Cluster analysis**

The genetic similarity of eight peppers was carried out clustering by using ISSR-primers amplification, as shown in Fig. 3. Eight pepper varieties were divided into three categories according to Table 2-2. The first category is pepper 2011-4, New 9A and Xixian with the genetic similarity coefficient of 0.91, therefore the kinship among these three pepper varieties is very close. The second category: genetic similarity relationships of Guo 228, 261A and color 2011-2 pepper were moderate. As a result of the genetic similarity coefficient of Fengsan A and Wanyi is 0.55, the kinship of these two pepper varieties is relatively far.




(A-2011-4 B-New 9A C-Guo 228 D-Fengsan A E-Xixian F-Wangyi G-261A H-color 2011-2)

Fig.3 Clustering analysis dendrogram of eight pepper DNA amplification by ISSR-15 primer

## Conclusion

Among 30 screened ISSR primers, 21 primers presented clearly visible bands using ISSR molecular markers analyzing genetic diversity of pepper. Total 382 bands in eight material were obtained employed above 21 primers, which 270 bands (70.6%) presented polymorphism. In addition, selected ISSR-15 primer as a example to analyze the genetic relationship among eight pepper varieties, and the eight pepper varieties are divided into three categories, The first category is pepper 2011-4, New 9A and Xixian with the genetic similarity coefficient of 0.91, therefore the kinship among these three pepper varieties is very close. The second category: genetic similarity relationships of Guo 228, 261A and color 2011-2 pepper were moderate. As a result of the genetic similarity coefficient of Fengsan A and Wanyi is 0.55, the kinship of these two pepper varieties is relatively far. Thus, according to the formulas about genetic similarity coefficient (GS) and genetic distance (GD), using spss16.0 analysis software system to cluster, constructed subsequently genetic correlation clustering patterns, finally it provides an important theoretical reference for pepper disease-resistant and breeding as well as integrated control. Due to its high polymorphism, strong stability, simple operation and other advantages, ISSR marker technology will be bound to be widely applied in more species and fields in the future.

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