Classification and phylogenetic relationship of 10 fig varieties by RAPD and SSR molecular markers

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Abstract: In this experiment, 24 fig varieties were analyzed by RAPD and SSR. Results showed that 80 loci were amplified by 9 RAPD primers, including 70 polymorphic loci. The polymorphic percentage was 87.50%. A total of 34 allelic variations were detected in 10 pairs of SSR primers, 29 of which were polymorphic bands, and the percentage of polymorphic sites was 85.29%. Correlation analysis of the two markers of genetic identity showed significant correlation, indicating that the two markers were highly consistent in the analysis of fig genetic diversity. Cluster analysis revealed that the RAPD markers showed that the 24 fig varieties had a genetic similarity coefficient of 0.7 and could be divided into four groups. SSR markers showed that the 24 fig varieties can be divided into four groups at the genetic similarity coefficient of 0.66. The results show that the two molecular markers are suitable for the genetic diversity analysis of fig, and they are more reliable when combined than when used alone.

1. Introduction

Fig (Ficus carica L.) is called “fig” because its small flowers are hidden in the torus; only the fake fruit is formed by the receptacle, no flowers can be seen, and its edible part is the aggregate formed by the expansion of the container. It is edible and medicinal, and it is also a cultivated plant in the ornamental garden. Eating figs is one of the earliest cultivated fruit trees [1-2]. Fig has a long history of cultivation in China and a wide variety. With the economic growth and the improvement of people's living standards, the nutritional, medicinal, and health care values of figs have attracted considerable attention; it has also been listed as the national economic crop by the agricultural talent introduction and selection project [3].
However, although China has long introduced a large number of new fig varieties abroad, the phenomenon of “same name” and “same name foreign body” is common because of the different planting environments and naming conventions in different countries. Moreover, the study of figs started late in China. It was only around 1980 that the system of collecting, preserving, and utilizing figs was systematically collected. At the beginning of the 20th century, the figs of figs were edited internationally to improve the description of figs. The phenotypic characteristics of fig were described. However, the application of the description still has some limitations because of the small number of features listed. Therefore, figs are still mainly classified through pulp color, peel color, leaf shape, origin, and agronomic traits. However, this standard is too simple to distinguish hundreds of varieties. In recent years, with the development and advancement of genetics and molecular biology, genetic diversity detection methods have continuously improved. Molecular markers are used to directly detect the polymorphism of gene sequences, avoiding the influence of the external environment on phenotypic traits and improving the accuracy of genetic analysis and breeding work. In addition, molecular markers are distributed throughout the genome and are highly polymorphic. Given their wide distribution in non-coding regions, molecular markers do not affect the expression of target traits. Thus, molecular markers are new tools for fruit tree genetic map construction, gene mapping, phylogenetic analysis, and variety classification and identification [4]. In recent years, several molecular technologies have been developed, including RFLP, RAPD, AFLP, CISH, SRAP, TRAP, ESTs, SNP, SSR, and ISSR.

At present, RAPD is a simple molecular marker. SSR molecular markers are effective for genetic diversity analysis. Many studies also focused on the same fruit tree, such as grapes [5-6], Li Apricot hybrids [7], and apples [8]. The above studies show that most of the fruit trees have the phenomenon of “same name” and “same name foreign body.” However, Wang Liang et al. [9] only used RAPD and SSR molecular markers to analyze the DNA fingerprints of 11 fig varieties, and the two analysis techniques can only effectively distinguish three varieties. The other eight varieties were divided into three groups, and the amplification maps of the varieties in each group were identical and could not be effectively distinguished, indicating that the classification and phylogenetic analyses of fig varieties still need further study. In this experiment, two molecular markers, RAPD and SSR, were selected. The phylogenetic classification of 24 fig varieties in Weiyuan fig germplasm was studied and analyzed. The genetic diversity and relationship of figs were compared. The advantages and disadvantages of the classification of varieties were also compared. This study aimed to provide a molecular basis for the identification and classification of these varieties, to promote the preservation and utilization of these fig varieties, to provide a more complete experimental basis for the identification of the 24 varieties and the establishment of the fig classification system, and to assist the figs. Selection and breeding provide an effective basis for laying a good foundation for the scale development of China’s fig industry in the future [10].

2. Materials and Methods

2.1 Materials

2.1.1 Plant material
Twenty-four young leaves of fig material were collected from the Weiyuan fig germplasm resources in Sichuan. The specific varieties and land acquisition are shown in Table 1.

2.1.2 Reagents
Plant genomic DNA extraction kit, agarose, acrylamide (Acr), Bis, Tris, boric acid, Na2EDTA, APS, TEMED, NaOH, formaldehyde, sodium tetraborate, and silver nitrate were used.

Table 1 Test materials
2.2 Test design

2.2.1 Extracting DNA
The 24 samples of fig DNA were collected by the CTAB method [11] plant genome extraction kit (see Table 1), and DNA purity and concentration were detected by gel electrophoresis. After standardization, the dilution was 25 ng·L⁻¹ and then stored at −20 °C for subsequent test procedures.

2.2.2 RAPD-PCR amplification and product detection
Amplification was carried out using 30 random primers synthesized by Optimus, and RAPD-PCR amplification was carried out on a PTC-200 gene amplification

| Number | Cultivar              | Flesh Color     | Flesh Color | Source    |
|--------|-----------------------|-----------------|-------------|-----------|
| 1      | Purple fruit          | Dark purple     | Red         | Japan     |
| 2      | Chinese fruit         | purple/Amaranth | Yellow      | China     |
| 3      | Orphan                | Golden          | Light Yellow| America   |
| 4      | Jinzao                | Purple brown    | Pink        | Italy     |
| 5      | Fenschn               | Chartreuse      | Light strawberry or amber | France |
| 6      | Brown’s Wick          | Chartreuse      | Reddish brown| France   |
| 7      | Mano’s Dauphine       | Purple to brown | Pink        | America   |
| 8      | Orma                  | Yellow          | Yellow      | Japan     |
| 9      | Kodota                | Chartreuse      | Amber       | Italy     |
| 10     | Yadang                | Reddish brown   | Red         | Japan     |
| 11     | Meiya                 | Yellow          | Light Yellow| America   |
| 12     | B110                  | Light green     | Reddish brown| Japan   |
| 13     | Crispy green skin     | Chartreuse      | Red         | China     |
| 14     | Patanchoe             | Chartreuse      | Red         | America   |
| 15     | M118                  | Yellow          | Amber       | Israel    |
| 16     | M111                  | Amaranth        | Yellow      | Israel    |
| 17     | M121                  | Amaranth        | Red         | Israel    |
| 18     | X29                   | purple          | Red         | Italy     |
| 19     | ALMA                  | Yellow          | Light red or amber| France |
| 20     | Viennetae Bordeaux    | Amaranth        | Red         | Spain     |
| 21     | A1213                 | Yellow green    | Pink        | America   |
| 22     | Granulated sugar      | Purple brown    | Light red   | Italy     |
| 23     | Holy Fruit 1          | Yellow green    | Reddish brown| America |
| 24     | Wenzian               | Reddish brown   | Light red   | Japan     |

Table 2. SSR primer sequence and annealing temperature
method described by Tian Yizhen et al. [12]. After electrophoresis with 1.5% agarose, the buffer solution was subjected to 1×TAE electrophoresis and then stained with EB, and the data were recorded.

2.2.3 SSR-PCR amplification and product detection
The SSR primers from the fig genome (Table 2), which were screened and patented by Guo Zhengbing et al. [13] in 2017, were amplified, and the reaction system was optimized for PCR of genomic DNA of different varieties of figs. The PCR reaction system consisted of 12.5 µL of 2×Taq Master Mix, 0.3 µL of upstream and downstream primers, 2 µL of DNA template strand, and finally fixed to 25 µL with ddH2O. The reaction conditions were pre-denaturation at 94 ℃ for 5 min; 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 56 ℃ for 35 s (see Table 2 for specific primer annealing temperatures), and extension at 72 ℃ for 40 s; and final extension at 72 ℃ for 3 min. [14]. The amplified product was detected by 8.0% denaturing polyacrylamide gel electrophoresis. The electrophoresis buffer was 1×TBE. After electrophoresis was completed by 0.1% silver nitrate solution, the excess AgNO3 was washed away with distilled water and then pre-cooled. Developed in the developer until the strip is clearly visible, and the photo was recorded [15].

3. Results and Analysis

3.1 PCR amplification product results and genetic diversity analysis
The amplification results obtained by RAPD primer 22 and SSR primer FCUP066-7 for the 24 fig varieties collected from the Weiyuan fig germplasm resources in Sichuan are shown in Figures 1 and 2, respectively. The number of RAPD-PCR amplified bands is large and clear. A similarity table for each genetic diversity parameter was obtained from the two molecular markers (see Table 3). From the analysis, 9 RAPD primers amplified 80 repetitive bands, and 8.8 bands were detected on average. The polymorphic bands were up to 70, the percentage of polymorphic sites was 87.50%, and an average of 8.8 bands were detected per primer. A total of 34 highly repetitive bands were amplified by SSR primers, 29 of which were polymorphic bands. The percentage of polymorphic sites was 85.29%, and 3.4 bands were detected on each pair of primers. Both RAPD and SSR are suitable for fig polymorphism analysis, but their results are different. The band polymorphism amplified by RAPD was slightly higher than that amplified by SSR, and the number of alleles (Na) and effective alleles (Ne) was slightly higher than that of SSR markers. However, the average of Nei's gene diversity index (H) and Shannon's information index (I) of SSR markers was higher than that of RAPD markers.

| SSR primer | Base sequence | Annealing temperature (℃) |
|------------|--------------|---------------------------|
| FCUP066-4(F) | CAATTCCTTCTTCATCTAAGA | 58 |
| FCUP066-4(R) | GTCCTCACGTTGAATCTCTGA | 59 |
| FCUP084-4(F) | CTTCTCCATTCATCTCAGGA | 55 |
| FCUP084-4(R) | ATCTTCTCAGGATCTCGAA | 55 |
| FCUP086-5(F) | CTTCTCAGAAGAGAAACCA | 58 |
| FCUP086-5(R) | CTACAGAAAATGAGCTCTCA | 58 |
| FCUP089-4(F) | CCGGAACACGCAATCTCCAA | 55 |
| FCUP089-4(R) | CAAGGGTGCCAGCTGCTGAA | 55 |
| LMC270(F) | ATCTTCCACTTGGTGXGAA | 56 |
| LMC270(R) | CTCTTGCTGCTAAGAAGCTTT | 58 |
| LMC270(F) | AATGTCTTCTACGTXGART | 56 |
| LMC270(R) | AGACATCAGAAGACACAG | 56 |
| LMC270(F) | TGTCTGTCTACGTXGART | 58 |
| LMC270(R) | CTCTTGCTGCTAAGAAGCTTT | 58 |
3.2 Analysis of kinship

A genetic distance diversity analysis was performed using NTSys 2.10e cluster analysis software to calculate the genetic similarity coefficient between the materials (Figures 3 and 4). The maximum number of genetic similarities obtained by RAPD markers was 0.85, and the minimum was 0.60. The genetic similarity coefficient obtained by the SSR marker had a maximum value of 0.91 and a minimum value of 0.47. Both molecular markers indicated differences in the genetic relationship among the fig varieties, but the genetic similarity coefficient of the SSR markers was higher than that of the RAPD markers.
Table 4. RAPD analysis of genetic similarity coefficients among 24 fig varieties

| 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  | 23  | 24  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1   | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 2   | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 3   | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 4   | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

Table 5. SSR analysis of genetic similarity coefficients among 24 fig varieties

| 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  | 23  | 24  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1   | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 2   | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 3   | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 4   | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

3.3 UPGMA clustering results

3.3.1 RAPD genetic similarity and cluster analysis

After cluster analysis, the 24 fig germplasm resources can be divided into four groups (I–IV). Group I contains only one fig variety, A1213. Group II contains two fig varieties, Orma and ALMA. Group III includes 11 fig varieties, such as Ka Duo Tai, Mei Yu, and Gorgeous. Among them, Ka Duo Tai, Adam, Green Morning, Shengguo No. 1, and Wenxian Fruit are closely related, Meiyu. M111, M121, crispy green, and sugar are more closely related. Group IV includes 10 fig varieties, such as purple fruit, Chinese purple fruit, and Jin Aifen, among which Lanrik and Masi Taofen are closely related. Purple fruit, Chinese purple fruit, Jin Aifen, M118, X29, purple Bordeaux, Jinzao, and Fenghuang are closely related. (RAPD clustering analysis is shown in Figure 3)

3.3.2 SSR genetic similarity and cluster analysis

After cluster analysis, the 24 fig germplasm resources can be divided into four groups (I–IV). Group I includes six species, including Jin Aifen, ALMA, and Orma. Among them, Jin Aifen, ALMA, Jinzao, and Fenghuang are closely related, and Olma is closely related to Gorgeous. Group II includes two fig varieties, M121 and purple Bordeaux. Group III includes eight fig varieties, such as Kadaitai, Adam, M118, and M111. Among them, Meiyu and A1213 are closely related, and M118 and M111 are closely related. Adam, Green, and X29, are closely related. Group IV includes eight fig varieties, such as purple fruit, Branrick, and crispy green, among which purple fruit Branrick, Crispy Green, Chinese Purple Fruit, and Masi Taofen are closely related. Purple fruit and Branrick are closely related. Sugar, St. Fruit No. 1, and Wenxian Fruit are closely related. (SSR clustering analysis is shown in Figure 4)
4. Discussion and conclusion

The results of this experiment show that both RAPD and SSR molecular marker techniques are suitable for the analysis of fig genetic diversity, although the two markers have many differences. First, the strips of RAPD markers are clear and numerous, whereas those of SSR markers are less and messy. The reason for this result may be that only 10 SSR primer pairs were obtained from previous studies, which may result in insufficient comprehensive results. Second, genetic diversity data were based on predecessors. All values of RAPD markers were superior to those of SSR markers [5]. In addition, the SSR marker data were better than the RAPD marker data. The results of this experiment show that the genetic diversity data is not a numerical value is a certain marker is better than another marker. The possibility of the difference in the test is still related to the first point; the third and the two types of marker cluster analysis. The classification of fig varieties was not consistent, such as the fig varieties “Jade” and “gorgeous” in the RAPD analysis. The relationship was very close, but it was not clustered in the SSR analysis. This result may be attributed to the number of primers, detection sites, and genetic information. In general, the difference between the two molecular markers may be due to the lack of primer selection, which produced insufficient results. This situation may be improved by strict screening of primers and annealing temperature, increasing the number of primers, and increasing the number of detection points and genetic information [16].

As far as the experimental technology system is concerned, RAPD and SSR have their own advantages and disadvantages. RAPD is simple in operation, time saving, and labor saving, and the reagents used are less toxic. In general, it cannot cause harm to the human body, but its stability, accuracy, and repeatability are poor. SSR markers have the advantages of high polymorphism, co-dominance, and high sensitivity, showing unique advantages in DNA fingerprinting construction, genetic diversity analysis, and variety identification [17]. Compared with RAPD markers, SSR markers have better stability and reproducibility, but their experimental operation is difficult. Primers are not easy to obtain and costly. For some weaker species of genomic basic research, you can learn from their related species and even other species. Primer. The results show that the two molecular markers are suitable for the genetic diversity analysis of fig, and they are more reliable when combined than when used alone. In general, the results of the classification and phylogenetic analysis of the 24 fig varieties using RAPD and SSR molecular markers showed the feasibility of the experiment. Combined analysis enhanced the
accuracy and authenticity of the test. This study may serve as a basis for strengthening the management and protection of fig planting resources.

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