Genetic diversity of 24 ancient litchi germplasm resources using ISSR molecular marker

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Abstract：【Objective】The present study explored the genetic diversity and relationships of Guangxi ancient litchi germplasm resources in order to provide references for utilization of germplasm resources and variety breeding. 【Method】 The genetic relationships of 24 ancient litchi varieties were analyzed using ISSR molecular marker. 【Result】Thirteen primers with clear bands and favorable repeatability were selected from 100 ISSR primers；96 bands were amplified altogether and 43 of them were polymorphic bands with a polymorphism proportion of 44.79%, and 7.4 bands were amplified by each primer on average. The genetic similarity coefficients among the tested ancient litchi germplasm resources ranged from 0.43 to 1.00 with average relative genetic similarity coefficient of 0.61. UPGMA cluster analysis results indicated that, the 24 tested germplasm resources of ancient litchi could be classified into three groups when genetic similarity coefficient was 0.66. Group Ⅰ came from Lingshan county, Guangxi；Group Ⅱ derived from Guiping city, Beiliu cuty, Yulin city, Lingshan county and Daxin county in Guangxi；Group Ⅲ came from Beiliu city and Lingshan county in Guangxi. 【Conclusion】 The hereditary basis among the 24 varieties of ancient litchi in Guangxi is extensive, and they can serve as excellent materials for cross breeding of litchi in the future.

Key words：ancient litchi；ISSR molecular marker；genetic diversity；cluster analysis

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24份古荔枝种质资源ISSR遗传多样性分析

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摘要：【目的】了解广西古荔枝种质资源的遗传多样性及其亲缘关系，为其种质资源利用和品种选育等提供参考。【方法】采用ISSR分子标记技术对24份古荔枝种质资源的遗传多性及亲缘关系进行分析。【结果】从100条ISSR引物中筛选出13条带形清晰、重复性好的引物，共扩增出96条条带，其中有43条为多态性条带，多态性比例为44.79%，平均每条引物扩增的条带数为7.4条。供试古荔枝种质资源间的遗传相似系数范围为0.43~1.00，平均相对遗传相似系数为0.61。UPGMA法聚类分析结果表明，在遗传相似系为0.66处供试的24份古荔枝种质资源被分为三大类群。类群Ⅰ均来自广西灵山县，类群Ⅱ来自广西桂平市、北流市、玉林市、灵山县和大新县，类群Ⅲ来自广西北流市和灵山县。【结论】24个广西古荔枝品种资源间的遗传基础宽，可作为今后荔枝杂交选育的优良种质资源。

关键词：古荔枝；ISSR分子标记；遗传多样性；聚类分析

0 Introduction

【Research significance】Litchi (*Litchi chinesis* Sonn), belonging to Sapindaceae family, *Litchi* genus, is a typical subtropical evergreen fruit tree native to and widely distributed in southern China. With the reputation of "Fruit King in South of the Five Ridges", litchi is renowned throughout the world for its bright color, juicy and tender flesh. Litchis carry abundant germplasm resources from the diversified environments they live in, and such resources include wild, semi-wild and cultivated ancient litchi trees distribute in different areas. ISSR(Inter-simple sequence repeat) is a new type of DNA molecular marker based on SSR sequence information in plant genomes created by Zietkiewicz et al.(1994). Its main advantages are as follows: no DNA sequence information is required, easy to utilize with favorable repeatability and rich polymorphism. The analysis of genetic diversity and relationship of ancient litchi germplasm resources with ISSR can help with understanding of diversity and genetic relationship of litchi germplasm resources, germplasm innovation and variety breeding of litchi. 【Research progresses】Currently, ISSR marker has been widely applied to variety identification, genetic diversity analysis and drawing of plant genomes in plant genetics and breeding. Yang et al.(2013) analyzed the genetic relationships of 13 varieties of *Camellia semiserrata* Chi with ISSR, and the results indicated that the 13 varieties(series) of *C. semiserrata* Chi could be classified into three groups and hereditary basis among the variety resources of *C. semiserrata* Chi in Guangxi was extensive. Lu et al. (2013) analyzed genetic diversity of *Dendrobium* germplasm resources in different areas with ISSR, and 24 *Dendrobium* samples from different areas were classified into six groups with rich genetic diversity. Feng et al.(2014) analyzed genetic diversity and relationship of germplasm resources of Chinese chestnut with ISSR. The results indicated that the 49 varieties of Chinese chestnut could be classified into two main groups, and the first of which can be divided into two sub-groups; varieties coming from the same region were almost in the same group. Vu et al.(2011) analyzed genetic diversity of corn germplasm resources with ISSR, and the 21 corn varieties could be classified into three main groups with the similarity coefficients ranging from 0.52 to 0.90. 【Research breakthrough point】Currently, the genetic diversity analysis of litchi mainly focused on cultivated varieties, and few was analyzed on germplasm resources of hundred-year-old ancient litchi. Furthermore, the related researches of ancient litchi mostly focused on the flowering characteristics and fruit quality, and no studies on germplasm resources and genetic diversity of ancient litchi from molecular level have been reported. 【Solving problems】With 24 varieties of ancient litchi in Guangxi as the samples, ISSR molecular marker was adopted to analyze their genetic diversity and relationship in order to provide theoretical guidance for protection of germplasm resources of ancient litchi and lay foundation for their effective utilization and breeding of new litchi varieties.

1 Materials and methods

1.1 Experimental materials

The 24 tested ancient litchi germplasm resources were taken from wild, semi-wild and cultivated ancient litchi trees over 100-year-old in various litchi-producing areas in Guangxi. They are all locally representative or of excellent features such as large fruit, early blossoming, aborted seeds and good quality (Table 1).

The used reagents SDS, Tris, EDTA, CTAB and β-mercaptoethanol were all imported analytical reagents purchased from Beijing Solarbio Science & Technology Co.,Ltd.; the conventional chemical reagents were domestic analytical reagents; and dNTP, DL2000 DNA Marker and *Taq* enzyme were purchased from Sangon Biotech(Shanghai) Co., Ltd.

1.2 Experimental methods

1.2.1 Sample collection Young and mature leaves with no diseases or insect pests were collected from strong ancient litchi trees growing in different areas of Guangxi , and they were quick-frozen by liquid nitrogen and stored under -20 ℃ for total DNA extraction.

1.2.2 Extraction of litchi genome DNA The modified CTAB method(Lichtenstein and Draper,1985) was adopted to extract genome DNA from healthy litchi leaves. Its mass was tested by 0.8% agarose gel electrophoresis(AGE) and the concentration and purity were tested by ultraviolet spectrophotometer; in the end, the DNA was diluted to 100 ng/μL and put into a refrigerator under -20 ℃.

1.2.3 Primer selection and ISSR amplification The DNA templates of four typical varieties(series) were selected from the 24 ancient litchi resources for PCR amplification of 100 universal ISSR primers to select those with favorable polymorphism. The 20.00 μL ISSR-PCR reaction system included 1.00 μL of 40

Table 1　Twenty－four ancient litchi varieties（lines）and their sources

Code	Sample name	Collecting location	Code	Sample name	Collecting location
1	*Dendrobenthamia japonica* var. Chinensis	Team 2 of Kantou village，Qianjin Village Committee，Lingcheng town	13	Y-5	Jiangning town，Bobai，Guangxi
2	Dingxiang	Zhonghe village，Xinfeng town，Beiliu	14	Dingxiang	Lianshan village，Madong town
3	Dazao	Farmland in Luozheng village，Beiliu	15	Sour litchi	Shihui Mountain，Lu village，Madong town
4	Dahongpao	Farmland in Luozheng village，Beiliu	16	He litchi	Modaotang，Lu village，Madong town
5	Water litchi	Madongshi village	17	He litchi	Guannan village，Xinfeng town，Beiliu
6	Red litchi	Yunshan Temple in Luo village，Beiliu	18	Millennium litchi	Dengjia village，Xinwei town，Lingshan
7	He litchi	Zhonghe village，Xinfeng town，Beiliu	19	Huazhi litchi	Huangyidui，Shajing Village Committee，Tanxu town，Lingshan
8	Y-6	Xialei town，Daxin，Guangxi	20	Areca-nut litchi	Team 1 of Daguang village，Longkutang Village Committee，Tanxu town，Lingshan
9	Y-2	Jiangning town，Bobai，Guangxi	21	Dashui litchi	Shuijingwu,Longkutangvillage,Tanxutown,Lingshan
10	Y-1	Jiangning town，Bobai，Guangxi	22	Dazao	Pojing Garden，Team 6,Dali village，Xinxu town，Lingshan
11	Y-3	Jiangning town，Bobai，Guangxi	23	Jinfeng	Zhongqitang，Baishui Village Committee，Lingcheng town，Lingshan
12	Y-4	Anning township，Jingxi，Guangxi	24	Nuomici	Changgang Mountain，Lingcheng town，Lingshan

ng/μL template DNA，0.50 μL of 2.5 mmol/L dNTPs，0.16 μL of 5.0 U/μL *Taq* polymerase，1.00 μL of 10 μmol/L primer，2.50 μL of 10×PCR Buffer solution，and ddH$_2$O was increased to 20.00 μL. The PCR reaction procedure was as follows：3 min of initial denaturation under 95 ℃；40 s of that under 95 ℃，40 s of that under 55 ℃ and 2.5 min of that under 72 ℃ for 35 cycles；and then 10 min of extension under 72 ℃. The amplification products were added with 4.00 μL 6× loading Buffer and separated by electrophoresis with 1.8% agarose gel（5.00 μL of Gold View™ DNA dye was added to each 100 mL）；DL2000 DNA Ladder was adopted as the standard molecular weight for comparison；the electrophoretic voltage and current were 120 V and 48 mA，respectively；and observation and photographing were conducted in the gel imaging system.

1. 3　Statistical analysis

The amplification products from the same primer with the same electrophoretic mobility were considered homologous. The obtained images were counted manually；1 was marked if there was a band at the same sized band location，while 0 if not to establish the Excel tabular database；DICE method in NTSYS 2.10e was adopted to calculate the genetic similarity coefficient and cluster analysis was conducted using unweighted pair group method arithmetic averages（UPGMA）.

2　Results and analysis

2. 1　Extraction and test results of litchi genome DNA

High－quality DNA was crucial for the stability and reliability of experiment results. The electro－phoretic detection results indicated that CTAB method in this experiment could steadily extract the genome DNA of litchi leaves，whose OD$_{260}$/OD$_{230}$ measured by ultraviolet spectrophotometer was larger than 2.0 and OD$_{260}$/OD$_{280}$ was about 1.8. The results of 0.8% agarose gel electrophoretic detection indicated that the DNA bands were clear and integral with no trail（Fig.1）. The above results suggested that genome DNA of litchi leaves extracted by the modified CTAB method was of pure and high quality，thus it could meet the require－ments of subsequent experiments.
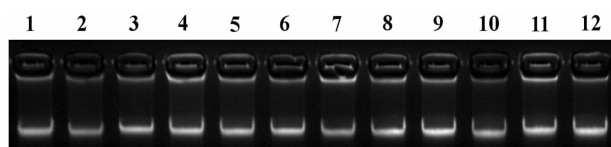

Fig.1　Genomic DNA extracted from leaves of samples 1–12

2. 2　Analysis on polymorphism of primer amplifi－cation

Through the optimized reaction system,13 primers with clear band form and favorable repeatability were selected from 100 ISSR primers for statistical analysis. The results indicated that 96 bands were amplified from the 13 primers，and 43 of them（44.79%）were polymorphic bands. Each primer could amplify 5 to 11 bands，7.4 on average，with the most（11 bands）from UBC844 and the least（4 bands）from UBC815（Table 2）；the amplification products ranged from 300 to 2000 bp. Most of the amplified fragments varied from 350 to 1600 bp. The amplification results of primers UBC817 and UBC818 were shown in Fig.2. The above

results indicated that ancient litchi in China enjoyed high genetic diversity which was possibly related to the complex genetic background due to long－term cross pollination and diversified ecological environments.

Table 2 Amplified results of 24 ancient litchi varieties (lines) and polymorphism analysis

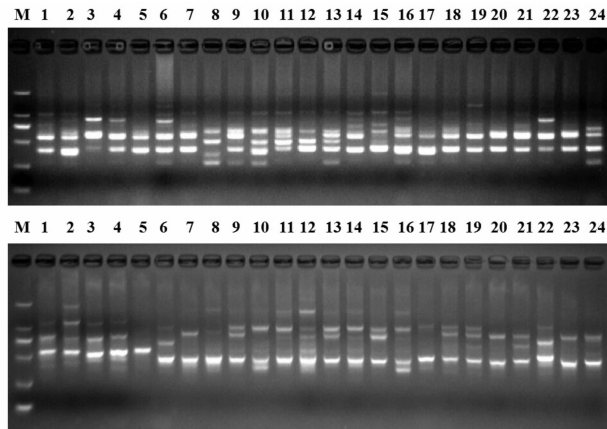| Primer total	Number of bands	Number of polymorphic bands	Percentage of polymorphic bands(%)
UBC807	8	5	62.40
UBC808	8	2	25.00
UBC809	7	3	42.86
UBC811	9	7	77.78
UBC815	4	2	50.00
UBC817	7	5	71.43
UBC818	6	3	50.00
UBC835	5	3	60.00
UBC840	10	5	50.00
UBC841	8	2	25.00
UBC844	11	5	45.45
UBC848	5	2	40.00
UBC857	8	4	50.00
Total	96	43	
Average	7.4	3.3	44.79

Fig.2 PCR amplification electrophoretogram of primers UBC817 (up) and UBC818 (down) for 24 ancient litchi varieties(lines)

M:DL2000 DNA Marker;1-24:24 ancient litchi varieties(lines)

2. 3 Cluster analysis on genetic similarity

NTSYS 2.10e was adopted to calculate ISSR amplification of germplasm resources of 24 ancient litchi resources. The results indicated that the genetic similarity coefficient between each two tested resources of ancient litchi ranged from 0.43 to 1.00 with the average of 0.61. It suggested that hereditary basis between the resources was extensive. The dendrogram of genetic relationship was established by UPGMA. According to the results of Fig.3, the 24 litchi resources could be classified into three groups with similarity of 0.66. The first group included *Dendrobenthamia japonica*

var. chinensis, Millennium litchi, Nuomici, Huazhi litchi and Dashui litchi from Lingshan county, Qinzhou. The second group included Dingxiang, He litchi, Water litchi from Madong town, Guiping, Guigang; He litchi, Dingxiang, Sour litchi and He litchi from Beiliu, Yulin; Y-6 from Daxin; Y-2, Y-1, Y-3 and Y-5 from Jiangning town, Bobai county of Yulin city; Y-4 from Anning township, Jingxi as well as Jinfeng and Areca-nut litchi from Lingshan county. The third group included Dazao, Dahongpao and Red litchi from Beiliu city, and Dazao from Lingshan of Yulin city.
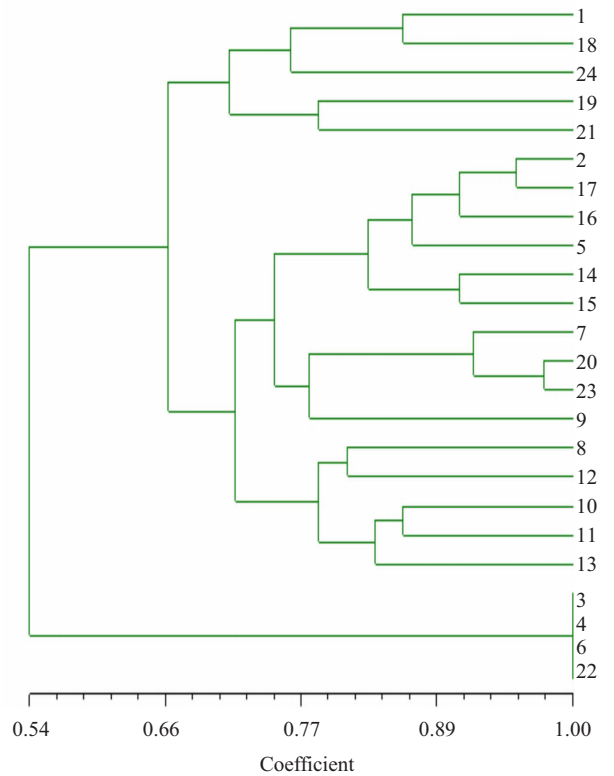

Fig.3 Dengrogram of 24 ancient litchi varieties(lines) based on ISSR maker analysis

3 Discussion

Based on SSR, ISSR marker is a new molecular marker with the advantages of both RAPD and SSR (Zietkiewicz et al., 1994; Daniel et al., 2002). ISSR marker is widely adopted because of good repeatability and being more stable than RAPD(Ge and Sun, 1999), and it also presented higher polymorphism by detecting the differences among multiple genetic loci (Zietkiewicz et al., 1994; Blair et al., 1999). Studies on the genetic diversity of plant germplasm resources such as corn(Vu et al., 2011), walnut(Chen et al., 2012) and dendrobe (Lu et al., 2013) indicated that ISSR is very sensitive, reliable and easy to operate. Litchi has a long cultivation history and enjoys abun-

dant germplasm resources in China. According to *Litchi Record of Guangxi*（1986）and previous investigations by us, seedling litchi resources are widely distributed in southwestern Guangxi and ancient litchi tree resources are also concentrated in this area. However, there is a lack of ancient trees with advantageous hereditary features despite the abundant germplasm resources. It is of great significance to the preservation and utilization of germplasm resources to conduct analysis on the genetic diversity of ancient litchi resources with molecular marker. In the present study, 13 ISSR primers were adopted to analyze the genetic diversity of 24 ancient litchi resources in Guangxi and 96 bands in total were amplified,43 of which（44.79%）were of polymorphic bands, indicating the polymorphism of genome DNA in ancient litchi groups. The variation amplitude of genetic similarity coefficients among the 24 varieties of ancient litchi resources was higher, suggesting that the hereditary basis among ancient litchi resources in Guangxi was extensive.

      It was also found that different kinds of Dazao collected from different areas had close genetic distance, thus they were placed in the same group; the genetic distances among litchi from different areas were far from each other; the several varieties of wild litchi with close genetic distances in this experiment were placed in the same group; *Dendrobenthamia japonica* var. Chinensi, Millennium litchi, Nuomici, Huazhi litchi and Dashui litchi were placed in the same group for their close genetic distances. Furthermore, ISSR analysis indicated that the complex genotype was found among different ancient litchi resources which collected from different areas of Guangxi, and multiple polymorphism genetic loci were also found in them. These analysis results of the genetic diversity of ancient litchi in Guangxi, the main litchi producing area, could provide research foundation for the collection, identification, evaluation and innovation of germplasm resources, and expand the hereditary basis of breeding materials by introduction, seedling variation selection and mutation breeding with the support of abundant ancient litchi resources in Guangxi, in order to provide germplasm resources for the breeding of new and improved litchi varieties.

4   Conclusion

      There are abundant ancient litchi resources with high genetic diversity in Guangxi. The 24 varieties of ancient litchi resources collected in this experiment

can be classified into three main groups by ISSR molecular marker, suggesting that the hereditary basis is extensive among the germplasm resources of ancient litchi in Guangxi, and they can serve as excellent materials for cross breeding of litchi in the future.

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