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从普通野生稻硅胶干燥的小量叶片中制备 DNA 用于 RAPD 分析和总 DNA 库的建立*

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摘要 普通野生稻(*Oryza rufipogon* Griff.)的基因资源对水稻的育种起着至关重要的作用。报道了从其硅胶干燥的小量叶片中制备 DNA 的方法。用此方法制备的 DNA 分子量大(40 ~ 45 kb),产率也较高(50 ~ 200 μg/g),且成功地进行了 RAPD 扩增。用制备的 44 个居群,1 168 个个体的总 DNA 建立了中国普通野生稻的总 DNA 库作长期冷冻保存,可用于基于 PCR 的 DNA 水平上的各种目的的研究。根据实验结果,从在室温下贮存 1 周、3 个月、6 个月、1 年的硅胶干燥的叶片中提取的 DNA 用于 RAPD 扩增所得的扩增产物没有差异;模板 DNA 浓度在 3.1 ~ 50 ng 的范围内均得到很好的 RAPD 扩增结果。这说明了从硅胶干燥的叶片中提取的普通野生稻的 DNA 用于 RAPD 扩增的产物很稳定,将其用于群体遗传分析具有很好的可比性和可靠性。同时也讨论了模板 DNA 的纯度和浓度对 RAPD 扩增的影响。

关键词 制备 DNA,总 DNA 库,RAPD,普通野生稻

Preparation of DNA from Silica Gel Dried Mini-amount of Leaves of *Oryza rufipogon* for RAPD Study and Total DNA Bank Construction*

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Abstract Gene resources of *Oryza rufipogon* Griff. play a crucial role in rice breeding, and hence to study their conservation is of utter importance. The authors describe a method for preparation of DNA from mini-amount of the silica-gel-dried leaves of *Oryza rufipogon*. The high molecular weight DNAs of 1 168 individuals representing 44 populations have been obtained with high yields, which could be used for RAPD PCR and construction of total DNA bank of this species. The template DNA from silica-gel-dried leaves stored for one year at room temperature gave the same RAPD results as that from the newly prepared silica-gel-dried leaves. The optional template DNA concentrations for amplification ranged from 3.1 ng to 50 ng. In addition, the quality and quantity of the template DNAs that affect RAPD results are also discussed.

Key words DNA preparation, Total DNA bank, RAPD, *Oryza rufipogon*

DNA analysis by PCR has been widely adopted in plant science. Most studies used fresh or frozen materials as a source of DNA. It is often necessary to preserve the plant materials on ice and transport quickly to the laboratory, or to freeze and store it in liquid nitrogen before carrying back to the laboratory. Both methods can be used successfully when the plants studied are nearby or in easily accessible regions. This, however, is not convenient for the large-scale experiments involving materials from wild populations collected in remote regions, especially for genetic studies. Furthermore, liquid nitrogen may neither be available nor be convenient for shipping. Thus, seeking an alternative to these methods seems most desirable. Rogers and Bendich[1] reported a method for extracting DNA in milligram amounts of herbarium; Doyle and Dickson[2] successfully obtained high quality of DNA from dry leaves. Chase and Hills[3] used an ideal material, the silica gel, for field preservation of leaf samples for DNA studies. Silica gel can be regenerated when heated by various ways in the

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field. We had followed the procedure of Doyle and Dickson[2] for preparation of DNA from silica-gel-dried leaves of *O. rufipogon*, but only gained a small amount of DNA which was seriously degraded. Since this is not an efficient procedure for DNA preparation, and that quality of DNA may seriously affect PCR results, it is apparently not a suitable method for analysis of population genetics based on RAPD data. Thus we present a method for DNA preparation from mini-amount of silica-gel-dried leaves of *O. rufipogon* which gave high yields and little degradation. Random amplified polymorphic DNA (RAPD)[4,5] is a molecular marker produced through polymerase chain reaction (PCR) with a single stringed arbitrary DNA nucleotide sequence (primer) to randomly amplify genome regions, being sensitive to the quality and quantity of the template DNA. In order to acquire a high reliability and good reproducibility of amplification, it is important to evaluate the factors, which might affect the results of RAPD. *O. rufipogon*, the putative progenitor of Asian cultivated rice *O. sativa*, is widely distributed in the tropics and subtropics of monsoon Asia[6]. In China, it has been found in eight provinces and autonomous regions: Guangdong, Guangxi, Hainan, Yunnan, Hunan, Jiangxi, Fujian and Taiwan (extincted since 1978[7]). It possesses agronomically useful traits, such as resistance to insects and pathogens, and tolerance of salinity. Therefore, it has been proven to be a precious gene pool for genetic improvement of rice, and contributed much to rice yields of the world. However, human activities have led to rapid extinction of a great number of populations of the species. If proper protective strategies are not taken, it would be fully extincted in China in the next ten to fifteen years[8]. Although *in situ* conservation has been considered as an effective way to maintain genetic diversity of the endangered species, it has obvious disadvantages, e. g. occupying cultivable area, consuming labor and requiring a large amount of investment. Consequently, the construction of a total DNA bank is an economical and useful way to maintain gene resources of the endangered species, for total DNA bank can be stored at −72 ℃ for a long period, and can also be used for various researches at DNA molecular level.

1 Materials and Methods

1.1 Sampling strategy

Young and clean leaves of *O. rufipogon* Griff. were collected from 44 natural populations throughout its distribution range in China. Leaves were collected individually from more than 10 individuals per population varied with population sizes. Because *O. rufipogon* is a perennial herb with a certain degree of colonizing ability, samples were randomly collected at an interval of at least five meters to prevent collecting multiple samples from a single genet.

1.2 Preservation of leaf materials

Leaf preservation mainly followed the method of Chase and Hills[3]. Since *O. rufipogon* grows in rivers, ponds and marshlands, and its leaves contain more water than that of land plants, and are thicker than that of most dicots, additional steps must be taken to ensure thorough leaf drying. In the field, about 5 g of fresh leaves were collected from each individual, and was placed in a small (10 cm × 12 cm) ziplock plastic bag, into which about 50 g of silica gel was added. The bag was shaken to allow the silica gel touch the leaves tightly and evenly, until most of the silica gel in the bag turned to pale pinkish purple in color. Then the gel was removed from the bag. A second 50 g of dried silica gel was put into the bag as the first time and the samples were checked frequently within the next ten hours. When the color of a part of the silica gel in the bag turned to pale pinkish purple, then all the gel was removed immediately. Another 50 g of dried silica gel was poured into the bag, and was retained for 12 h. Thereafter, leaves were checked and a snap with a clear-cut break indicated that the sample was well dried, and most of the silica gel was removed, leaving a small amount of the silica gel as an indicator for sample rehydration. Finally, the ziplock plastic bag containing samples was put in a larger ziplock plastic bag together with a little silica gel, and was stored at room temperature ready for further use.

1.3 Extraction of DNA

The hot CTAB procedure[2, 3] was used with some modifications as following. Step 1. About 0.1 g of dried leaf was frozen in liquid nitrogen before it was ground to fine powder in a precooled mortar; Step 2. The powder was transferred to a sterile 2 mL Eppendorf tube

containing preheated 900 μL CTAB extraction buffer [2% (W/V) hexadecyltrimethylammonium bromide (CTAB, Sigma), 100 mmol/L Tris-HCL (pH 8.0), 1.4 mol/L NaCl, 20 mmol/L EDTA, 1.5 % polyvinyl-pyrrolidone (PVP, Sigma), 0.5 % 2-mercaptoethanol (Sigma)], which was well mixed by inverting the tube several times; Step 3. The tube was then incubated in 60 ℃ water bath for 30 ~ 45 min; Step 4. 900 μL 24 : 1 chloroform : isoamyl alcohol was added to the tube and was mixed by shaking gently about 10 min. Then it was centrifuged at 8 000 r/min for 10 min at room temperature; Step 5. The aqueous phase (upper) was transferred to another 2 mL Eppendorf tube; the organic phase (lower) was piped out, and then the remaining phase (middle) was combined with 400 μL CTAB extraction buffer and mixed well; The step 3 was repeated; Step 6. 500 μL 24 : 1 chloroform : isoamyl alcohol was added to the tube and was mixed by shaking gently about 10 min. Then it was centrifuged at 8 000 r/min for 10 min at room temperature; Step 7. The aqueous phase was transferred to the 2 mL Eppendorf tube of Step 5, an equal volume of 24 : 1 chloroform : isoamyl alcohol was added and mixed by shaking gently about 6 min. It was centrifuged at 8 000 r/min for 10 min at room temperature; Step 8. The aqueous phase was transferred to a new 1.5 mL Eppendorf tube, and 2/3 volumes of cold (-20 ℃) isopropanol was added and mixed by inverting the tube several times. The tube was stored at -20 ℃ for at least 30 min. Overnight precipitation increases DNA yields. In fact, most of the large DNA fragments were precipitated in the first 30 min; Step 9. The tube was centrifuged at 12 000 r/min for 5 min at 4 ℃; then the supernatant was discarded; Step 10. The precipitated DNA was washed twice with 75% ethanol, and the ethanol was carefully decanted after centrifugation; Step 11. The DNA pellet was air dried for 30 min (being careful to avoid over-drying), and was resuspended in 200 μL TE buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA (pH 8.0)], and then was stored at 4 ℃; Step 12. The suspension was RNased by adding 1 ~ 2 μL 10 g/L RNase to the solution which was incubated in water bath for 1 h at 37 ℃; Step 13. 2 ~ 4 μL 10 g/L Proteinase K was added to the solution which was incubated for 1 h at 55 ℃; Step 14. 150 μL sterile ultrapurified water and 360 μL 24 : 1 chloroform : isoamyl alcohol was added to the solution which was mixed well by shaking gently about 6 min. Then it was centrifuged at 8 000 r/min for 10 min at room temperature; Step 15. The aqueous phase (upper) was transferred to a new 1.5 mL Eppendorf tube, 1/10 volume of 3 mol/L cold (-20 ℃) sodium acetate (pH 5.2) was added to it. DNA was then precipitated by the addition of 2 volumes of cold absolute ethanol. The tube was stored at -20 ℃ for at least 30 min, and then was centrifuged at 12 000 r/min for 6 min at 4 ℃; the supernatant was discarded; Step 16. The step 10 was repeated; Step 17. The DNA pellet was air dried at least 30 min or overnight, and was resuspended in 100 ~ 200 μL TE buffer [1 mmol/L Tris-HCl (pH 8.0), 0.1 mmol/L EDTA (pH 8.0)], and was stored at 4 ℃.

Concentrations and sizes of extracted DNA were estimated by comparison with ethidium bromide stained, unrestricted lambda (48.5 kb) DNA standard concentration in 0.8 % agarose gel.

1.4 PCR

The suitability of DNA preparation for PCR was tested using random primers (OPB-14, 19, 20, Sengong Biotech Company). RAPD PCR was run on the Air Rapidcycler (ARC) in a reaction volume of 10 μL containing 1.2 μmol/L primer, 10 ng DNA template, 50 mmol/L Tris-HCl (pH 8.3), 0.5 g/L BSA, 2 mmol/L $MgCl_2$, 0.5 Units of *Tag* DNA polymerase, 200 μmol/L dNTPs, 1% Ficoll and 1 mol/L Tartrazine. The initial two amplification cycles were carried out with 1 min at 94 ℃, 10 sec at 35 ℃ and 20 sec at 72 ℃. Subsequent 45 cycles were 2 sec at 94 ℃, 10 sec at 35 ℃ and 1 min at 72 ℃. A final 4 min extention at 72 ℃ followed the last cycle.

The amplification products were electrophoresed through 1.4% agarose gel (Promega) containing 0.5 μg/mL ethidium bromide, and were visualized and photographed on an UV transilluminator. Molecular weight of RAPD fragments was estimated using a 100 bp ladder DNA (Pharmacia Biotech) as a standard.

2 Results and Discussion

2.1 Construction of total DNA bank

We used the leaves from 1 168 individuals for constructing total DNA bank. These individuals belong to

44 populations of *O. rufipogon* (Table 1), covering its entire distribution range in China. 5 ~ 20 μg high molecular weight DNA (about 40 ~ 45 kb) was obtained from 0.1 g dried leaves following our procedure of DNA extraction (Fig. 1). The second extraction (step 5 to step 6) increased one-third of the amount of DNA. So it is an essential part of our procedure. The amount of DNA of each individual is enough for various research based on PCR. The quality of DNA is also good enough for conducting PCR.

These DNA samples of *O. rufipogon* are preserved at −72 ℃ or −40 ℃ in our laboratory.

Table 1 Sampled populations for construction of total DNA bank

Population No	Locality	No of individuals	Population No	Locality	No of individuals
02	Dongxiang, Jiangxi	18	03	Dongxiang, Jiangxi	19
04	Zencheng, Guangdong	18	05	Zencheng, Guangdong	27
06	Zencheng, Guangdong	18	07	Boluo, Guangdong	38
10	Huiyang, Guangdong	42	11	Huidong, Guangdong	12
12	Huidong, Guangdong	45	13	Haifeng, Guangdong	22
14	Haifeng, Guangdong	24	18	Fugang, Guangdong	22
19	Yingde, Guangdong	31	24	Kaiping, Guangdong	45
25	Taishan, Guangdong	28	28	Enping, Guangdong	17
29	Gaozhou, Guangdong	45	34	Zhanjiang, Guangdong	25
36	Suixi, Guangdong	20	37	Qiongshan, Hainan	24
38	Qiongshan, Hainan	23	39	Wenchang, Hainan	34
40	Qionghai, Hainan	25	41	Qionghai, Hainan	24
43	Lingshui, Hainan	25	45	Sanya, Hainan	25
46	Sanya, Hainan	10	47	Ledong, Hainan	34
49	Dongfang, Hainan	36	51	Lin'gao, Hainan	28
52	Chengmai, Hainan	32	53	Wuxuan, Guangxi	32
54	Wuxuan, Guangxi	28	55	Wuxuan, Guangxi	21
56	Wuxuan, Guangxi	36	57	Wuxuan, Guangxi	29
59	Tengxian, Guangxi	26	60	Hezhou, Guangxi	26
61	Yulin, Guangxi	27	62	Hepu, Guangxi	23
64	Tiandong, Guangxi	28	66	Shanglin, Guangxi	24
68	Yuanjiang, Yunnan	12	69	Chaling, Hunan	20

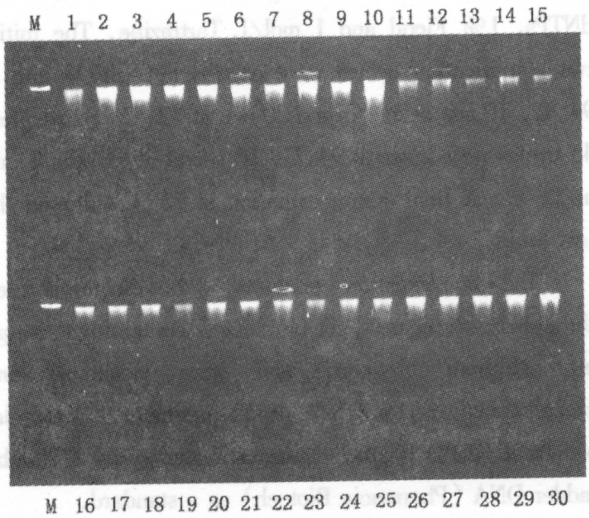

Fig. 1 Preparation of DNA from silica-gel-dried leaves of *Oryza rufipogon*
M. 100 ng standard λDNA; 1 ~ 30. No of individual of population 10.

2.2 Quality and quantity of template DNA with RAPD results

The extraction of DNA from dried plant materials has been demonstrated by several researchers. Rogers and Bendich[1] acquired DNA with a maximum length of 20 ~ 30 kb and an average length of 0.1 ~ 1.0 kb from herbarium materials ranging from 20 ~ 95 years in age. The DNAs they obtained degraded with the yield of 10 ~ 90 ng/mg, and were not qualified for RAPD analysis. Doyle and Dickson[2] extracted DNA from the leaves of *Solanum glutinosum* and *Glycine tabacina*, which were dried up to three and ten months, respectively. They successfully gained DNAs with the length of above 23 kb, which was competable with that from fresh samples. But the yields varied depending on the efficiency of grinding. Moreover, the DNAs were successfully used in experiments of restriction endonuclease. Pyle and

Adams[3] studied various methods of DNA preservation for plant materials, and found that the yields of genomic DNA (30 ~ 50 kb) could be obtained from dried leaves, which was as good as those from fresh and frozen leaves. In addition, they found that leaves dried for two months appeared to be some loss of DNA. According to our experiments, the quantity and quality of DNA extracted from silica-gel-dried leaves stored for one year at room temperature are almost equal to those from newly silica-gel-dried leaves. In contrast to dried materials, Rogers and Bendich[10] prepared DNA from fresh leaves of several species, and acquired DNA with the length of 50 ~ 100 kb and yields of 15 ~ 50 ng/mg fresh weight. Compared with their result, the efficiency of our DNA preparation is higher, and is almost equal to those obtained from fresh leaves. The molecular weight of DNA obtained from the silica-gel-dried leaves of *O. rufipogon* is also higher than that from herbarium specimens, but is lower than that from fresh leaves. Therefore, silica gel is an ideal desiccant for field preservation of leaves of both land and aquatic plant for DNA studies. The protocol we recommend here may be most appropriate for preparation of DNA of high quantity and quality from the silica-gel-dried mini-amount of leaves of *O. rufipogon*.

In order to acquire the best efficiency in reproducibility and amplification pattern, we investigated several factors related to template DNA that might affect the PCR results. Different template DNAs extracted from the silica-gel-dried leaves stored at room temperature for one week, three months, six months and one year, respectively, were used for RAPD PCR. No difference was observed from those amplification profiles with three random primers (OPB-14, 19, 20) (Fig. 2). In addition, results from RAPD PCR using the unpurified DNAs (step 12 to step 17) as the templates showed that all the fragments except those with high molecular weight (1 100 ~ 2 700 bp) were amplified in some individuals (Fig. 3). This may produce false polymorphic fragments in genetic analysis. Skroch and Nienhuis[11] suggested that the template DNA should be purified and RNased in order to eliminate most macromolecular substances, and to ensure the stability of reaction system of PCR. On the other hand, Wang *et al*[12] indicated that the template DNA which contained RNA and certain amount of protein did not affect amplification of PCR when they conducted RAPD PCR using the samples of *Cathaya argyrophylla*. Furthermore, when the template DNAs used in our experiment were less than 15 kb in length due to certain degree of degradation, consequently, the amplified fragments were unclear, and all the high molecular fragments (1 200 bp ~ 1 700 bp) were not amplified. In addition, low molecular fragments (400 ~ 600 bp), which were not shown using the template DNA of high molecular weight, were produced (Fig. 4). Wang *et al*[12] also found that the high molecular fragment was not amplified

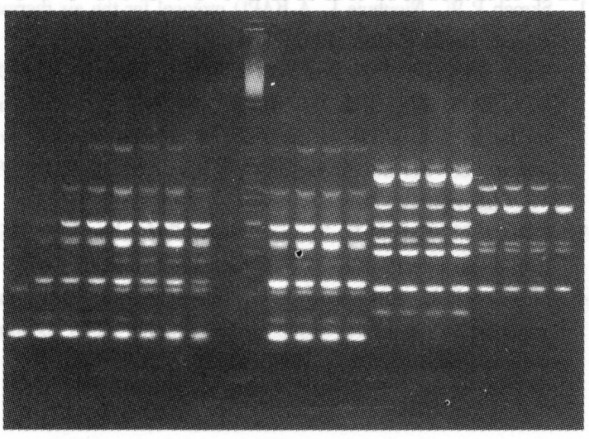

Fig.2 RAPD profile using different template DNA
M. 100 bp molecular ladder DNA; Lanes 1 ~ 9. Amplification results using primer of OPB-20 and template DNA at 200, 100, 50, 25, 12.5, 6.2, 3.1, 1.6, 0.8 ng, respectively. Lanes 10 ~ 21. Amplification results using template DNA from silica-gel-dried leaves stored at room temperature for 1 week (10, 14, 18), 3 months (11, 15, 19), 6 months (12, 16, 20) and for 1 year (13, 17, 21) with the primers OPB-20, OPB-19 and OPB-14, respectively.

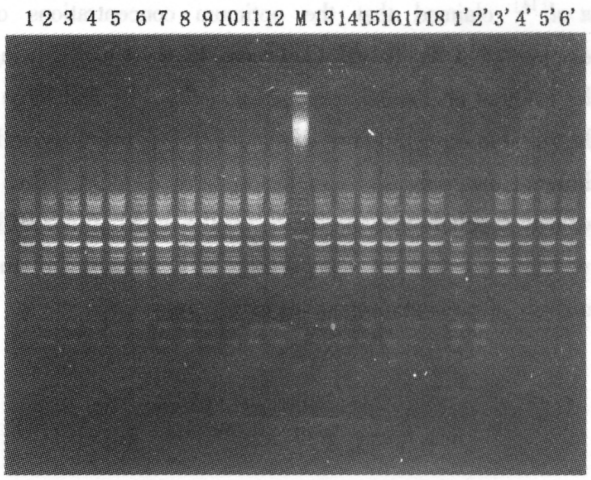

Fig.3 RAPD profile using purified and unpurified template DNA with primer OPB-17
1 ~ 18. Number of purified DNA sample from population 1 as contrast; 1′ ~ 6′. Number of unpurified DNA sample from population 1; M. 100 bp molecular ladder DNA.

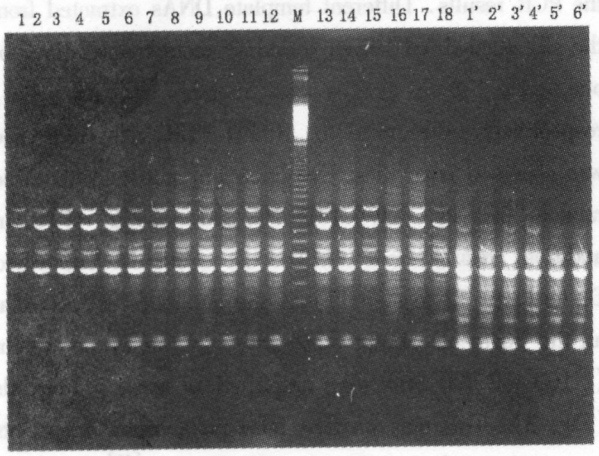

Fig. 4 RAPD profile using high molecular weight DNA and degraded DNA as templates with primer OPB-10
1 ~ 18. Number of DNA sample with high molecular weight from population 1 as contrast; 1′ ~ 6′. Number of degraded DNA sample from population 1; M. 100 bp molecular ladder DNA.

using the degraded template DNA. Therefore, the quality of template DNA is very important for stability of PCR results.

When different concentrations of template DNA (200, 100, 50, 25, 12.5, 6.2, 3.1, 1.6, 0.8 ng, respectively) were tested in our RAPD PCR, it was demonstrated that the template concentrations, ranging from 3.1 to 25 ng, gave rise to amplification profiles with the best reproducibility (Fig. 2). Devos and Gale[13] tried different concentrations of template DNA varying from 2.5 to 2 500 μg/L and found that the optimal concentrations of template DNA which produced identical PCR results were from 200 to 400 μg/L. Ellsworth *et al*[14] claimed that the optimum concentrations of template DNA for RAPD PCR were 12 ~ 40 mg/L using the material of *Tympanuchus phasianellus*. By and large, the suitable concentrations of template DNA may vary with different materials and reaction systems of PCR. Thus, we may conclude that the RAPD results based on our RCP reaction systems are comparable and reliable for the analysis of population genetics of *O. rufipogon*.

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