A renewed DNA extraction method for molecular study of *Melampsora larici-populina*

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[**Abstract**] Based on the bi-ball method of DNA extraction, the study renews some steps and component, which makes the PCR more sensible. The changes include: (1) Put the urediniospores into the tube with two steel balls and 1.6 g/L Chelex-100, the uredinia may be picked up with airing host tissues. (2) Add KOH into the extraction buffer instead of NaOH and vortex the tube violently for 30 min. (3) Prick a hole under the bottom instead of picking out of the steel balls, then centrifuge the tube to toss the liquid into the sheathed tube. The DNA achieved by this way can be used as substrates of ITS-nrDNA-PCR, RAPD, SSR. Futher dilution multiples of the extraction liquid is also tested. RAPD and SSR may complete well with over 16× DNA extraction buffer.

[**Key words**] *Melampsora larici-populina*; DNA extraction; bi-ball method; PCR

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Introduction

Melampsora larici-populina is one of the important worldwide diseases occurred in poplar trees. There are many reports about its biological, epidemical features and resistant breeding. Physiological race division is common with 5 races reported in Europe and U. S. A. as well as in China[1,2]. Differences and relations of the races between China and Europe are not clear, as no work has been done on them. Cao Zhimin, et al[2](2005) has studied the pathogenic division based on the poplar host reported by Pinion with one host (*Populus trichocarpa*) failing in discriminating. Whether the two new discriminating hosts (*P. szchuanica* and *P.* × *euramericana* cv. 'Polska 15A') selected in China are suitable for European races is still unknown. No morphological difference has been found between Chinese and the European races[3]. So the molecular study on *Melampsora larici-populina* gets more and more important now. RAPD analysis has been used based on 13 isolations collected from Shaanxi and Qinhai province. 4 RAPD clusters were divided by UPGA method[4]. The RAPD clusters division did not coincide with the pathogenic division, and the reason is still unknown. Many factors may attribute to the reason. Mono-uredinia culture is time consuming and easily polluted by neighbor urediospores, while DNA extraction is difficult and often needs many mono-urediospores, which makes the purity of the extracted DNA uncertain. Usually, Uredionispores are grounded with a mortar and pestle in liquid nitrogen for DNA extraction. The DNA is purified with phenol-chloroform chemical. The CTAB protocol described by Zolan, et al[5] (1986) and the benzyl-chloride protocol described by Zhu Hen, et al[6](1993) are both relatively quick and can yield a high quality and quantity of DNA. However these protocols are time consuming and are easy to be contaminated if there are many samples at disposal. Urediniospores can easily 'boil over' when liquid nitrogen is poured into the mortar, and the DNA can be getting fewer and fewer when the DNA is purified step by step[7]. So a new quick and minim DNA extraction method is developed. Yu Zhongdong, et al[8] has reported a way of extracting DNA from a single uredinia and studied the ITS-

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nrDNA-PCR, but whether the DNA got by this way is fit for random PCR amplifying is not known, and so is the question how many dilution multiples can be acquired if the extraction liquid is stored for future study. This study reported the updating result by the renewed method.

1 Methods and materials

PUT 0.01 g mono-uredinispores of *Melampsora larici-populina* into the tube and grind it by bi-ball method[8]. This study renews some steps and components. The renewed aspects include: (1) Put the urediospores into the tube with two steel balls with 1.6 g/L Chelex-100 (Made in Bio-Rad Biotech. Ltd.), the urediniospores may be picked up with airing host tissues; (2) Add KOH into the extraction buffer instead of NaOH and vortex the tube vigorously for 30 min; (3) Prick a hole under the bottom instead of picking out of the steel balls, then centrifuge the tube to toss the liquid into the sheathed tube. The other steps of dealing the liquid are same as that of YU Zhong-dong, et al[8].

Dilute the upper liquid into 2×, 3×, 4×, 6×, 8×, 16×, 32× and use them as substrates of ITS-nrDNA-PCR, RAPD, SSR. ITS-nrDNA-PCR reaction refering to Gardes & Bruns (1993)[9] and Yu Zhongdong et al (2005)[8], and ITS fragments are digested by restricted enzyme HinfI. The digested ITS is electrophoresis on 8 g/L PAGE with EB dying. RAPD amplification system is 2 μL diluted liquid, 36.5 μL ddH_2O, 2 μL $MgCl_2$ (25 mmol/L), 5 μL 10× Buffer, 0.5 μL dNTPs (10 mmol/L), 4 μL primer (1 μmol/L), 4 U Taqpolymerase (Made in Beijing Dingguo Bio-tech. Ltd.). Reaction condition system is 94 C 1 min, 32 C 1 min, 72 C 1 min 30 s, 5 cycles, then 93 C 3 min, 54.5 C 50 s, 72 C 1 min 30 s, 35 cycles, 72 C 10 min 30 s. SSR amplification system is 2 μL diluted liquid, 36.5 μL ddH_2O, 4 μL $MgCl_2$ (25 mmol/L), 5 μL 10× Buffer, 0.5 μL dNTPs (10 mmol/L), 1 μL M_{13} Primers (10 μmol/L), 5 U Taqpolymerase (Made by Beijing Dingguo Bio-tech. Ltd.). Reaction condition is 94 C 1 min, 49 C 1 min, 72 C 1 min 30 s, 5 cycles, then 93 C 3 min, 48 C 50 s, 72 C 1 min 30 s, 35 cycles, 72 C 10 min 30 s. The thermo-cycler is Hybaid *PX*II.

Products of random primers reaction are checked on 1.5 g/L sugar gel with EB dying.

2 Results and analysis

ALL 1× ~ 6× diluted upper liquid can achieve good ITS amplification products, 1× and 2× have more significant results and over 6× dilution liquid often has no results (Fig. 1). *Hinf* I digested ITS of different tested isolations almost equally (Fig. 2). There is no distinct difference for ITS got by bi-ball method.

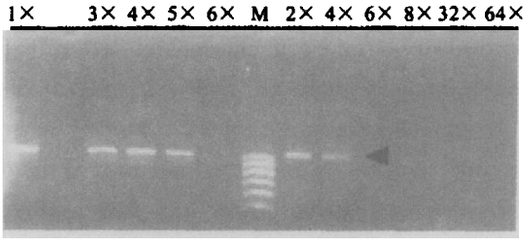

Fig. 1 ITS-PCR products from diluted upper liquid
1×~64×. Diluted multiples of extraction liquid;
M. 100 bp Ladder(800,700,600,500,400,300)

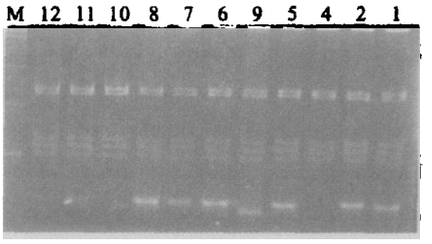

Fig. 2 ITS digested by *Hinf* I enzyme
1~12. isolation samples; M. marker (2 000, 1 600, 1 200, 800, 600, 400, 200 bp)

RAPD amplification shows good results with higher dilution multiples, amplification of 1× to 3× diluted substrate is inferior to the higher diluted multiples, the 16× diluted substrate has the best amplified result (Fig. 3), RAPD amplification seems to need fewer substrate, and lower consistent substrate has lower impurity, which may affect the activity of Taqpolymerase.

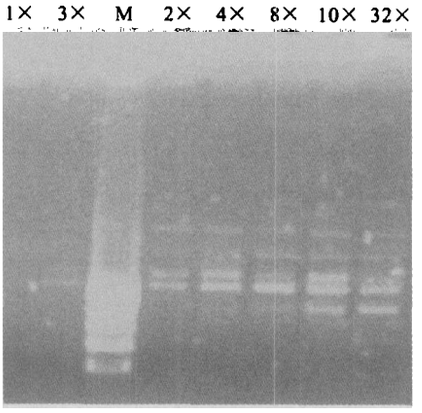

Fig. 3　RAPD products from diluted upper liquid
1～32×. Diluted multiples; M. 100 bp ladder

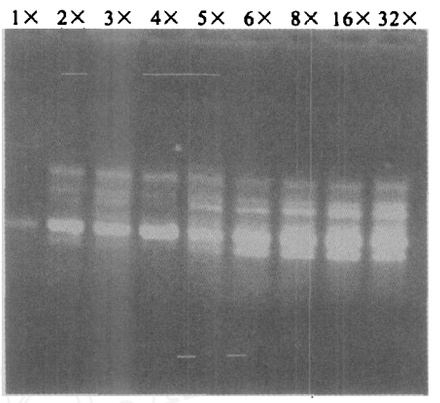

Fig. 4　SSR amplification from diluted upper liquid
1～32×. Diluted multiples

SSR amplification shows the same results as that of RAPD. The higher (over 8×) diluted multiples of upper extraction liquid has more significant products (Fig. 4).

All these results infest the DNA extracted by bi-ball method fitted well for the molecular study of *Melampsora larici-populina*. The 16× diluting substrates has good random primer PCR products.

3　Discuss

The past study shows bi-ball method can get enough DNA for PCR operation from a single uredinia[8], while in practice, more DNA are ready for using, more success can be achieved. DNA achieved by this way may be getting worse if the extraction mixture is put in 4 C room for a long time, and can not be used continuously. It is best to put the extraction mixture under −20 C. DNA from 0.01 g urediniospores extraction mixture can be achieved by adding Tris-HCl (pH 8.0) over and over, at least more than 6-fold, the liquid still can get good ITS-PCR results. Even with 32× diluted liquid, the random PCR reaction also has productive results, which means enough DNA template can be achieved for future study.

DNA got from bi-ball method often has fewer chemical and proteins remains, only Tris-HCl and KOH are added to the extraction liquid and proteins are erased by hatching in the 95 C water for 10 min, although many studies report adding Proteinase K has good effect on erasing proteins[3,10～11]. This study manifests adding KOH instead of NaOH to the extraction buffer can improve the Taqpolymerase activity, and using a sheathed tube to receive the tossed DNA mixture can improve the DNA production ration.

PCR amplification of ITS, RAPD and SSR shows the DNA got by this way is quite fit for using as amplifying substrate. There are no facts about how the substrate DNA is on the formation and completion, but the electrophoresis results showed the weight of DNA from bi-ball method was near to that of DNA from the other traditional extraction protocols[8]. So the bi-ball approach for DNA extraction is a good way for molecular study of this kind of rust fungi.

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落叶松-杨栅锈菌DNA提取新方法研究

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[摘 要] 对提取DNA"二球法"的一些步骤和提取成分进行了更新，使得PCR反应灵敏性显著增加。改进的内容包括：(1)在有二钢珠和1.6 g/L Chelex的离心管中加入夏孢子，夏孢子可以来自带有风干寄主叶肉组织的夏孢子堆；(2)在离心管中加入KOH而不是NaOH，然后剧烈斡旋30 min；(3)将离心管管底穿一小孔，套上一新离心管，短暂离心甩出内容物。用该方法得到的DNA可用作ITS-nrDNA-PCR，RAPD和SSR的底物。同时，分析了PCR扩增中提取上液的稀释倍数，发现RAPD和SSR扩增稀释倍数达到16倍以上有较好的反应结果。

[关键词] 落叶松-杨栅锈菌；DNA提取；二球法；聚合酶链反应