Assessment The Genetic Diversity of *Auricularia* Strains by Two PCR-Based Typing Methods

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Abstract: Two PCR-based methods, enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and randomly amplified polymorphic DNA (RAPD), were adopted for differentiating *Auricularia* strains. Taken the similarity coefficient as 75%, 29 strains of three *Auricularia* species were grouped into 6 and 9 clusters by RAPD and ERIC, respectively. The dendrogram from ERIC exhibited two distinct parts, one representing *A. auricula* and the other *A. polytricha*, but the dendrogram from RAPD failed to clearly distinguish between these two species. However, both methods similarly revealed high homology between *A. fuscosuccinea* and *A. auricula*. The homology relationships among the three species obtained from ERIC were validated by Southern hybridization. The analyses showed that RAPD is able to differentiate mainly at the species level, while ERIC is effective at the strain level and therefore more consistent with cultivation characteristics. The results indicate that the method of ERIC-PCR is more rapid and reliable than RAPD, and may substitute for RAPD in research related to the genetic identification and genetic diversity in *Auricularia*.

Key words: *Auricularia*, ERIC-PCR, RAPD analysis, Genetic diversity

Traditional phenotypical identification of *Auricularia*, based mainly on morphological characteristics[1,2], is still widely employed. However, since this method is frequently influenced by environmental factors, the identification result is usually not as consistent and exact as it would be. On the other hand, such method usually needs a rather long period. While molecular methods, depending on genetic information, can provide more convincible explanation. In recent years, several molecular tools have been taken in species identification and to assess genetic diversity among the genus. These molecular approaches have incorporated different techniques including RFLP-based studies of the 5'end of 28S rDNA and of rDNA intergenic spacer regions (ITS)[3,4]. In addition, differentiation within the genus *Auricularia*[5], and identification of intraspecific hybrids of *A. auricula*[6], has been achieved using the randomly amplified polymorphic DNA (RAPD) assay. The RAPD assay is based on the use of short random sequence primers, about 10 to 20 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures. However, methods such as ITS and RAPD only allowed an overall differentiation among species, and were not effective at the strain level.

Recently, a new method, called ERIC-PCR, has been successfully employed to evaluate genetic diversity among eukaryotic and prokaryotic organisms. This technique involves PCR amplification using primers that correspond to enterobacterial repetitive intergenic consensus (ERIC) sequences initially described in *Escherichia coli* and *Salmonella typhimurirm*[7~16]. ERIC sequences are 126bp elements which contain a highly conserved central inverted repeat and appear to be restricted to transcribed regions of the bacterial chromosome, either in intergenic regions of polycistronic operons or in untranslated regions upstream or downstream of open reading frames. Their position in the genome appears to be different in different species. The use of outward-facing primers complementary to each end of these repetitive sequences has been employed extensively for the characterization of bacteria. These primers have also been used for genetic diversity studies in fungi, and have successfully distinguished closely related isolates of the plant pathogens, *Drechslera avenae* and *Stemphylium solani*[13]. The present study was conducted to determine the effectiveness of ERIC-PCR for differentiating *Auricularia strains*, with RAPD method as a comparison. It is the first time to use this technique to deal with edible fungi.

1 Materials and methods

1.1 Strains and culture conditions

29 primarily cultivated strains belonging to three species

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of *Auricularia* were provided by the Edible Fungi Institute, Shanghai Academy of Agricultural Sciences (EFI-SAAS). The strains, listed in Table 1, were grown in 250mL flasks containing 100mL potato dextrose yeast medium (PDY) on a rotary shaker for 10 days at 25℃. Mycelia were harvested by filtering through sterile gauze, washed twice with sterile distilled water and stored at −20℃.

Table 1 *Auricularia* strains used in this study

Species	Strain	Number	Origin
A. polytrica	0960	1	EFI-SAAS
A. polytrica	Hongdaer	2	EFI-SAAS
A. polytrica	Zimuer	3	EFI-SAAS
A. auricula	0093	4	EFI-SAAS(Mutated from 1606)
A. auricula	0104	5	EFI-SAAS (Hybrid from 17)
A. auricula	0959	6	Hunan Province Edible Fungi Institute
A. auricula	0965	7	Hebei Province (AuGu 1)
A. fuscosuccinea	0120	8	USA
A. polytrica	0122	9	Guilin, Guangxi Province
A. auricula	0108	10	Zhejiang Province Microbiology Institute (793-Hebei Province, Fang County)
A. auricula	0957	11	Dept. Biology, Shanxi University
A. auricula	0100	12	Shanxi Province 10
A. auricula	Junxianheimuer	13	EFI-SAAS
A. auricula	0951	14	Hunan Province Edible Fungi Institute (Au14)
A. polytrica	0966	15	Zhangjiajie, Hunan province
A. polytrica	Maomuer No.3	16	EFI-SAAS
A. auricula	2×30	17	EFI-SAAS
A. polytrica	0732	18	Qiqihaer Vegetable Institute
A. polytrica	Maomuer AP4	19	EFI-SAAS
A. auricula	Huer No.3	20	EFI-SAAS
A. auricula	2×20	21	EFI-SAAS
A. auricula	0961	22	Yanbian Fungi Institute, Jilin Province (Au 1)
A. auricula	Danpianmuer	23	EFI-SAAS
A. auricula	Changbaishangheimuer	24	EFI-SAAS
A. polytrica	8401	25	EFI-SAAS
A. auricula	0955	26	Dept. Biology, Shanxi University
A. auricula	0097	27	Baoding, Hebei Province (No.7)
A. auricula	0962	28	Yanbian Fungi Institute, Jilin Province (Au 2)
A. auricula	0701	29	USA

1.2 Preparation of genomic DNA

Genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method described previously[17]. The concentration of each DNA template was determined by the Hoefer DNA Quant 200 Fluorometer (Amersham Biosciences) and adjusted to 40ng/μL. DNA preparations from each of the 29 strains were size separated by electrophoresis to determine the integrity of the extracted DNA and then stored at −20℃.

1.3 RAPD assay

Preliminary studies were undertaken with sixty 10-mer primers from Shanghai Sangon Ltd. Of these, 14 primers were retained for subsequent studies because of the clear and distinct banding patterns obtained (Table 2). Amplifications were carried out using a PCR Express thermal cycler (HYBAID) in 25μL reaction volumes containing 25pmol primer (Sangon, Shanghai), 1.0U *Taq* DNA polymerase (Promega), 40ng genomic DNA, together with all necessary buffer components and nucleotides. The cycling parameters were as described previously[7]. RAPD products were analyzed after electrophoresis in 1% agarose gels. Amplification conditions (i.e. dNTP, Mg^{2+} and DNA concentration) were optimized in order to obtain a good reproducibility of banding patterns.

Table 2 The random primers used in RAPD studies

Random primers	Sequences	Random primers	Sequences
S5	TGCGCCCTTC	S42	GGACCCAACC
S10	CTGCTGGGAC	S44	TCTGGTGAGG
S15	GGAGGGTGTT	S47	TTGGCACGGG
S23	AGTCAGCCAC	S52	CACCGTATCC
S24	AATCGGGCTG	S53	GGGGTGACGA
S31	CAATCGCCGT	S55	CATCCGTGCT
S35	TTCCGAACCC	S60	ACCCGGTCAC

1.4 ERIC-PCR

The sequences of the primers were: ERIC1R 5′-ATGTAAGCTCCTGGGGATTCAC-3′; ERIC2 5′-AAGTAAGTGACT-GGGGTGAGCG-3′[7]. PCR amplification was performed in 25 μL reaction volumes containing 10 mmol/L Tris-HCl (pH 8.3), 50mmol/L KCl, 2mmol/L $MgCl_2$, 200μmol/L dNTP, 50 pmol of each primer, 80 ng of genomic DNA, and 1U of *Taq* polymerase (Promega). The PCR cycle programme was: 95℃ for 7 min, followed by a short period at 75℃ for addition of *Taq* polymerase; 30 cycles of 94℃ for 1min, 52℃ for 1min and 65℃ for 8min; and a final extension at 65℃ for 16min. After electrophoresis on 1% (W/V) agarose gel, the PCR products were stained with ethidium bromide (0.5μg/mL) and visualized under UV light. Amplification conditions were optimized in order to obtain a good reproducibility of banding patterns.

1.5 Analysis of PCR products

Products of ERIC-PCR and RAPD assay were evaluated using the Hoefer DNA Quant 200. Approximately 400 ng of each product was analyzed by agarose gel electrophoresis (1% agarose gels run in 0.5 × Tris-borate-EDTA [TBE] buffer) using DNA molecular weight markers as standards.

The similarity coefficient (Cs) of ERIC-PCR or RAPD fingerprinting among the 29 strains was calculated according to Jaccard's formula $Cs = 2j/(a + b)$, in which a and b are the number of bands in each fingerprint of two strains, and j is the number of bands shared by both strains. Two fingerprints were adjudged to be different if they varied by the presence or absence of at least one band. The very faint bands and the variations in band intensity were overlooked in the calculations. Subsequently, all data from two PCR methods were analyzed with the NTSYS-pc software (Version 1.70, Applied Biostatistics, Inc). The overall available patterns were used to construct dendrograms using the UPGMA (unweighted pair group methods using arithmetic averages).

1.6 Hybridization

Southern blot was employed to confirm the veracity of the results from ERIC-PCR according to Wei *et al*. (submitted). Eighteen of the total 29 strains were selected on the basis that they represented every identifiable type according to the dendrogram obtained from ERIC-PCR. The amplified products obtained from all 18 isolates using the ERIC primers were transferred to nylon membranes and used in Southern hybridization. Fragments from one *A. auricula* strain which was widely cultivated, were labelled with digoxigenin (DNA labeling kit, Roche) and used as a probe. The process of hybridization was followed according to instructions in the reference manual for the kit.

2 Results

2.1 Fingerprinting of RAPD analysis

The RAPD assay produced distinguishable patterns for the 29 strains when 14 primers were used. DNA fragments ranged from 0.1kb to 3.0kb in size, and an average of 5 bands per isolate were obtained. The similarity coefficient values range from 55.16% to 90.62%. When the similarity coefficient was taken as 75%, the strains could be grouped into six clusters, designated as R1-R6 (Fig.1). Five strains of *A. polytricha* (8401, 0966, 0732, Maomuer AP4 and Maomuer No.3) represented five different types (R1, R2, R4, R5 and R6), while the remain 24 strains were clustered into the R3 group. It is noteworthy that a further four *A. polytricha* isolates and one strain of *A. fuscosuccinea* (that showed 81% homology to *A. auricula*) could not be differentiated from *A. auricula* at this level. Another noticeable feature was the high homology observed among some of the examined strains. For example, similarity coefficients for *A. auricula* strains 0959 and 0965, and for strains 2 × 20 and 0961, were 91% and 90%, respectively, indicating that these paired strains should belong to the same strain.

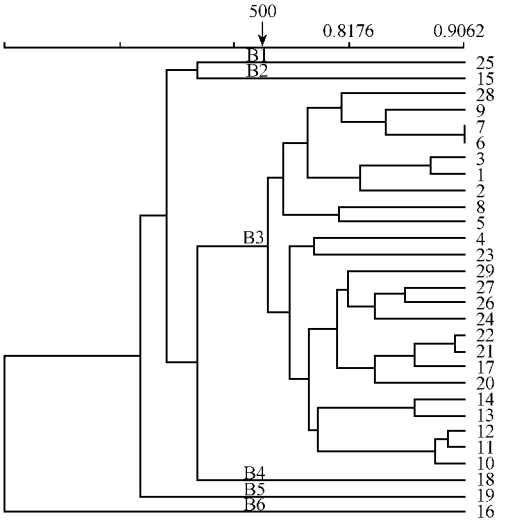

Fig.1 Dendrogram of cluster analysis based on RAPD fingerprinting of 29 *Auricularia* strains

R1 ~ R6 represent six different groups, the arrow denotes the cutting level for separation clusters

2.2 Fingerprinting of ERIC-PCR

PCR amplification of DNA from the 29 isolates of *A. auricula*, *A. polytricha* and *A. fuscosuccinea* using the

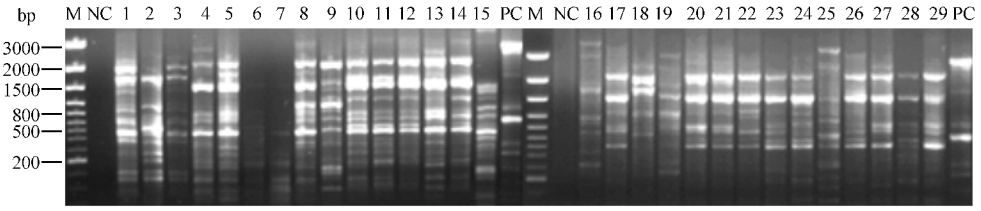

Fig. 2 ERIC fingerprints of 29 *Auricularia* strains

The lane numbers correspond to the isolate numbers shown in Table 1. Lanes M and PC contained the reference marker and a positive control, respectively.

ERIC primers revealed a total of 29 PCR products. Although the major bands were less intense in some cases than in others, and the minor bands were occasionally difficult to visualize, the overall band positions, and whether they were present or absent, were highly consistent. The number of bands vary from 8 to 16 per isolate, and the fragment size from 0.15 to 3.2 kb (Fig. 2). When the similarity coefficient was set at 75%, the dendrogram obtained from the ERIC fingerprints of the 29 tested strains revealed nine different clusters, designated E1-E9 (Fig. 3). As observed with the RAPD-based

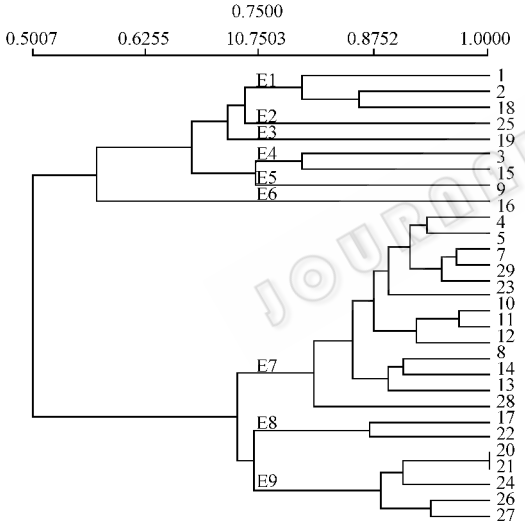

Fig. 3 Dendrogram of cluster analysis based on ERIC fingerprinting of 29 *Auricularia* strains

E1 ~ E9 represent nine different groups, the arrow denotes the cutting level for separation clusters.

cluster analysis (Fig. 1), *A. auricula* and *A. polytricha* strains were clearly separated into two different cluster groups (Fig. 3). The nine *A. polytricha* strains accounted for six clusters (E1-E6), while the 19 strains of *A. auricula* and the single *A. fuscosuccinea* strain together formed the remained three clusters (E7-E9). A very high homology was observed among the 19 strains of *A. auricula*, and contrasted sharply with the high diversity observed among the *A. polytricha* isolates. There was also high homology between the *A. auricula* isolates and the strain of *A. fuscosuccinea*, repeating the results obtained by RAPD. Furthermore, the ERIC-derived dendrogram also revealed very high similarity coefficients for *A. auricula* strains 0959 and 0965 (97%), and for strains 2X20 and 0961 (100%), also confirming data obtained from RAPD analysis. On the basis of the ERIC profiles, the 18 strains shown in Fig. 4 were selected for hybridization experiments.

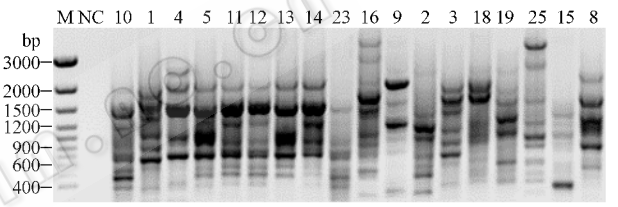

Fig. 4 ERIC fingerprints of the 18 *Auricularia* strains selected for hybridization

The lane numbers correspond to the isolate numbers shown in Table 1. Lane M contained a reference marker.

2.3 Southern Hybridization

ERIC-PCR products of strain No. 10, originating from *A. auricula*, were selected and used as probe in Southern hybridization experiment. A high degree of homology to the fragments was obtained among the 29 test strains that corresponded to the source of the ERIC probe used (Fig. 5). With probe No. 10, no hybridization signal was detected with bands originating from any of the *A. polytricha* isolates. However, the probe did hybridize with bands from the only strain of *A. fuscosuccinea* used in this study, along with the data obtained from RAPD and ERIC analyses, further indicating that this species had a closer relationship to *A. auricula* in genome evolution than to *A. polytricha*. The hybridization profiles observed among the *A. auricula* strains could be classified into 3 groups, namely EH1 (strains No. 10, No. 11, No. 12 and No. 14), EH2 (strains No. 4, No. 13 and No. 23), and EH3 (only strain No. 5). Interestingly, strain 8 (*A. fuscosuccinea*) showed closest similarity to EH1 types. These results were consistent with analyses based on the dendrograms

above.

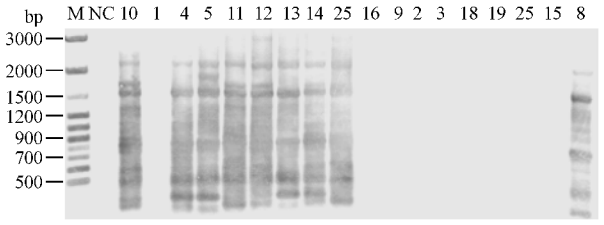

Fig. 5 Amplification products of the 18 *Auricularia* strains hybridized with probe 10

3 Discussion

In this study, a new PCR-based method, ERIC-PCR, was applied for the first time to differentiate species of *Auricularia*. In the first part of this study, ERIC-and RAPD-PCR were optimized for differentiating *A. auricula*, *A. polytricha* and *A. fuscosuccinea* strains. The effectiveness of the two typing methods was determined by adopting the three main criteria used for evaluating typing schemes, namely reproducibility, and discriminatory power. For each strain tested, reproducible profiles were obtained using ERIC-PCR.

RAPD analysis is based on low-stringency amplification and a low annealing temperature. Thus, the patterns obtained can vary greatly in response to minimal changes in the amplification conditions and, moreover, reproducibility is relatively low[13]. However, the patterns revealed with the ERIC primers are highly reproducible. This increased reproducibility may be due to the longer length of the primers used in ERIC-PCR (20 ~ 22mer as opposed to the 10mer primers in RAPD). On the other hand, the dendrogram from RAPD was constructed by fingerprints obtained from 14 random primers, which were selected from 60 ones. As for ERIC-PCR, only one pair of primers was used which was greatly timesaving, laborsaving and cost-saving. Therefore, ERIC-PCR has specific advantages compared to RAPD.

As revealed from the dendrograms, the two typing methods did not result in the same genotype grouping. That is, RAPD grouped three species into a single cluster: R3, as showed in Fig. 1, while ERIC could separate *A. auricula* from *A. polytricha*. It was effectively illuminated that the sensitivity of ERIC was more outstanding to RAPD.

In addition, the affinity observed between *A. fuscosuccinea* and *A. auricula* might also be obtained from cultivation data. The two species have very similar morphological characteristics but differ mainly in color: the former is amber and fuscous while the latter is black and brown.

In order to confirm whether bands of the same size in an ERIC-PCR fingerprinting contain the same DNA fragments, Southern blot was carried out. The results further indicated, along with the data obtained from RAPD and ERIC analyses, that *A. fuscosuccinea* had a closer relationship to *A. auricula* in genome evolution than to *A. polytricha* and a higher diversity level was revealed in *A. polytricha* than that in *A. auricula*. A potential explanation might be that *A. auricula* strains have been cultivated more widely and had a more important significance than *A. polytricha*.

ERIC-PCR is a highly reproducible method for the characterization of both bacterial and fungal species[13]. The ERIC-PCR approach has become a powerful tool for assessing the genetic diversity of isolates from different geographic locations and represents a valid alternative to the RAPD technique in permitting fingerprinting of genomes by using fewer primers.

In conclusion, ERIC-PCR is a valuable technique for characterizing members of the genus *Auricularia*. Fingerprints generated with ERIC-PCR were more reproducible and reliable than those generated with the RAPD assay. Furthermore, the genotypes obtained using ERIC-PCR were basically in accordance with the results of cultivation studies, while the genotypes from RAPD analysis displayed certain discrepancies in the clustering patterns. Finally, the results of this study provide further support for the proposal that ERIC-PCR may be broadly applicable to fingerprinting fungi.

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两种 PCR 方法对木耳属菌株的遗传多样性评价

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摘 要：应用 ERIC 和 RAPD 两种 PCR 方法对木耳属 3 种 29 个菌株进行遗传鉴别，其中 ERIC 方法是首次运用于食用菌的研究领域。在相似系数 75% 的水平上，ERIC 和 RAPD 分别将供试菌株分为 9 组和 6 组。由 ERIC 所得的聚类图可将黑木耳和毛木耳两个种区分开，而 RAPD 则不能完全区分两个种，但两种方法得到了一个相似的结果，即琥珀木耳与黑木耳的亲缘关系极其相近。Southern 杂交实验进一步证明了 ERIC 所得到的 29 个菌株的同源性关系。分析表明，RAPD 方法主要在种的水平上进行鉴别，而 ERIC 则可以在菌株水平上进行鉴别，结果与菌株栽培性状更为一致。研究结果表明 ERIC-PCR 是一种比 RAPD 更快捷可靠的分子标记方法，可以替代 RAPD 应用于木耳属的遗传多样性及遗传分类的研究。

关键词：木耳属，ERIC-PCR，RAPD，遗传多样性

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