ITS-PCR-RFLP Method for Distinguishing Commercial Cultivars of Edible Mushroom, *Flammulina velutipes*

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ABSTRACT: Nucleotide sequence and polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) analysis of the ribosomal RNA gene (rDNA) regions containing the internal transcribed spacers (ITSs) and the 5.8S rRNA coding sequence was used to differentiate between 7 typical *Flammulina* strains. These nucleotide sequences revealed the presence of strain-specific deletions, insertions, and substitutions. Moreover, RFLP patterns produced using restriction endonucleases *Dra*I, *Fok*I, *Hae*II, *Mbo*II, and *Nla*IV, enabled identification of specific *Flammulina* strains. Thus, PCR-RFLP analysis of the ITS regions appears to be a useful tool for the identification of *Flammulina* strains.

Keywords: *Flammulina velutipes*, PCR-RFLP, edible mushroom, rDNA, ITS

Introduction

THERE ARE MANY COMMERCIALLY CULTIvated species of mushrooms, with many cultivars of each species being bred. It is, however, generally very difficult to protect the patent for a commercial cultivar under the Seeds and Seedlings Law on a variety in Japan and also in many other countries, because it is very difficult to morphologically distinguish between an original cultivar, newly bred, or a cultivar that is essentially derived from one previously patented. This is an important problem in the mushroom industry. Development of a new method by which to genetically distinguish cultivars at a molecular level is of interest and would be a great asset to the industry.

Comparison of nucleotide sequences of the ribosomal RNA (rDNA) gene is a powerful tool for phylogenetic analysis, but the high cost of sequence analysis has, until recently, made it impractical to determine the sequences of all of the strains being bred in the mushroom industry. In recent years, however, new molecular biological tools, including polymerase chain reaction (PCR)-mediated amplification of the internal transcribed spaces (ITS) region and nuclear 5.8S rDNA, combined with restriction fragment length polymorphism (RFLP) analysis, have been used to evaluate intraspecific and interspecific variation in several fungi (Kuninaga and others 1997; Ristaino and others 1998; Nakamura and others 1998). Among rDNAs, which include those encoding the ITS as well as the large, small and 5.8S subunits, the ITS evolves very rapidly (White and others 1990). Consequently, analysis of PCR-RFLPs within nuclear rDNA repeat units may be a useful method for distinguishing different strains within a given species or genus.

Flammulina velutipes is currently the most abundantly produced commercial mushroom in Japan: annual production reached 109,324 metric tons in 1997 and earned 4,800 million Japanese yen (Magae and Hayashi 1999). Nonetheless, the genetic background of this mushroom has not yet been described. In this report, we describe nucleotide sequences of nuclear ITS I – 5.8S rDNA – ITS II regions of 7 commercially cultivated strains and wild type strains of *Flammulina*, as well as a method by which to distinguish each cultivar using RFLP analysis of ITS-PCR products. We also succeeded in recognizing differences between commercial cultivars and wild type strains. This study is part of our attempt to construct a novel system for readily distinguishing cultivars of commercially important mushrooms, including *F. velutipes*, by analyzing them at the level of their DNA.

Materials and Methods

Strains and culture conditions

Commercial cultivars (MH09201, MH09208, MH09210, MH09234, MH09286, MH09289, MH09297) and wild type (MH09209, MH09226, MH09236 MH09255) strains of *F. velutipes* dikaryon which were kept in stock by Hokuto Sangyo Co., were used (Table 1). The strains were grown in PS broth (potato extract, 2% sucrose) medium for later DNA extraction. Additional 4 fruiting body samples (ODA, TIK, OKA, HKK) which were commercially cultivated, were purchased at local supermarkets in Hiroshima, Japan. The fruiting body was used for later DNA extraction after washing with distilled water and then lyophilizing.

DNA extraction

DNA was extracted from lyophilized tissue of the seven *F. velutipes* strains using the CTAB (cetyltrimethyl ammonium bromide) procedure essentially as described previously (Zolan and Pukkila 1986). The extracted DNA was suspended in 20 µL of TE (50 mM Tris-HCl, 10 mM EDTA [pH 8.0]), and then electrophoresed in 1% agarose gel along with control lambda DNA (0.1 µg). The gels were stained with ethidium bromide (0.5 µg/mL), and photographed under UV light. The amount of each DNA present was estimated by comparing the ethidium bromide fluorescence intensity of the control lambda DNA with that of each of the *F. velutipes* DNAs. In general, about 100 ng of DNA was used as a template in PCR.

PCR reaction

The primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to am-

Table 1—Strains of Flammulina velutipes used in this study.

Strain No.	Strain Name	Source	Mating Type	Remarks	
	MH09201	Nakano	Not tested	Hokuto Sangyo Co.	Commercial
	MH09208	721B1	A3A4B3B4	Do.	Commercial
	MH09209	KUF	A11A12B11B12	Do.	Wild type
	MH09210	HokutoM-50	A1A2B1B2	Do.	Commercial
	MH09226	Myoukou	Not tested	Do.	Wild type
	MH09234	Yamase-1	Not tested	Do.	Commercial
	MH09236	Nonoumi	Not tested	Do.	Wild type
	MH09255	Sasagamine	Not tested	Do.	Wild type
	MH09286	MP-8	Not tested	Do.	Commercial
	MH09289	Hokuto M-90	A1A2B1B5	Do.	Commercial
	MH09297	Yanagihara	Not tested	Do.	Commercial
	ODA	—	Not tested	Oita	Commercial
	TIK	—	Not tested	Fukuoka	Commercial
	OKA	—	Not tested	Okayama	Commercial
	HKK	—	Not tested	Hiroshima	Commercial

plify the ITS region I, 5.8S rDNAs, the ITS region II, and a portion of the 28S rDNA. (White and others 1990). The same primers were also used for direct sequencing. PCRs were conducted in 100-µL reaction volumes. Each reaction tube contained approximately 100 ng of DNA template, 10 µL 10´PCR buffer (TaKaRa Syuzo Co, Kyoto, Japan), 200 µM each of dNTP, 0.5 pM each ITS 1 and 4 primers, and 2.5 U of TaKaRa Taq™ polymerase (TaKaRa Syuzo Co. , Kyoto, Japan). The thermal cycling parameters were initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 sec; a final extension at 72 °C for 10 min was done at the end of the amplification. The amplified ITSs were purified with a Microcon-100® filter (Millipore, Bedford, MA, USA) and used as a DNA template for direct sequencing. PCR products that could not be sequenced directly, were subcloned into pT7-Blue T-vector (Novagen, Madison WI USA), and the resultant recombinant plasmid DNAs were sequenced.

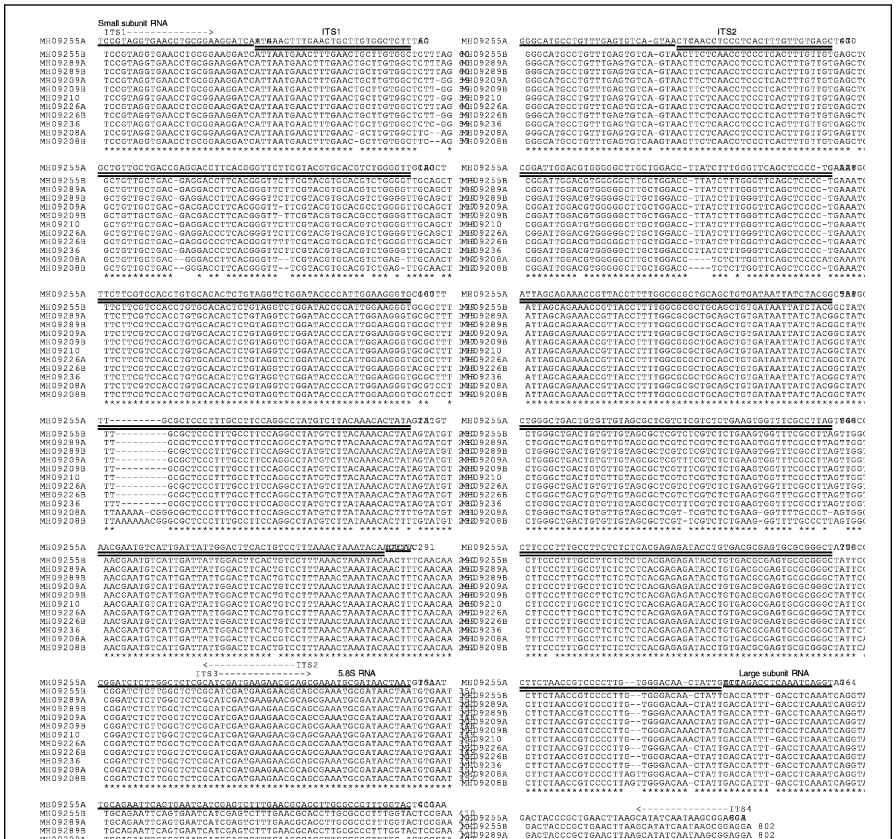

Figure 1—Comparison of the nucleotide sequences of ITS regions from commercial cultivars of F. velutipes dikaryon. Asterisks indicate a nucleotide sequence identical to that in all the strains. Arrows indicate position of ITS primers. rRNA coding regions are underlined and ITS regions are underlined twice. Each nucleotide sequence of cloned PCR fragment is represented as follows; MH09208A and B, MH09209A and B, MH09226A and B, MH09255A and B and MH09289A and B. The nucleotide sequences from MH09210 and MH09236 were determined directly. In this figure, the nucleotide sequence from only MH09210 is shown, because the nucleotide sequence from MH09210 was identical with that from MH09201, MH09234, MH09286, MH09297, ODA, TIK, OKA, and HKK.

DNA sequencing

DNA sequencing was carried out in an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems Japan Co., Tokyo Japan) using the chain-termination procedure with a BigDye Terminator™ Cycle Sequencing kit (Applied Biosystems Japan Co., Tokyo, Japan) with ITS1, ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'), ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 primers. Recombinant plasmid were sequenced with M13 universal primers. Multiple sequence alignment was constructed with the CLUSTAL W version 1.81 multiple sequence alignment program on the world wide web (www) server at European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/).

RFLP analysis

Purified PCR products were digested with the restriction enzymes *Dra*I, *Fok*I, *Hae*II, *Mbo*II, and *Nla*IV. restriction diges-

Table 2—Restriction fragment size of PCR products.

Restriction endonuclease	Fragment size (bp)	
	MH09210	MH09236
*Dra*I	536	536
	266	267
*Fok*I	488	
	314	No cutting position
*Hae*II	609	611
	193	192
	475	475

tion mixture (15 µL) containing 1 µL enzyme, 1.5 µL 10 ´ restriction enzyme buffer and 0.5 µg of PCR product were incubated at 37 °C for 5 h. The digestion products were then subjected to 2% NuSieve® 3:1 agarose gel electrophoresis, and stained with ethidium bromide (0.5 µg/mL) and visualized under UV light. The gels were photographed using an AE-6913 CCD digital camera (ATTO Co., Tokyo, Japan).

Results and Discussion

PCR AMPLIFICATION OF ALL STRAINS OF *Flammulina* using primers ITS1 and ITS4 yielded an estimated 800-bp ITS-PCR product containing ITS region I, the 5.8S rDNAs, the ITS region II and a portion of the 28S rDNA. Moreover, electrophoresis confirmed that the sizes of the amplified products were similar among the different strains (Figure 1, panel A, data not shown).

Initially, in order to estimate the most appropriate restriction endonuclease analysis of RFLP, all PCR products were directly sequenced using ITS1, ITS2, ITS3 and ITS4 as primers. The nucleotide sequences of the PCR products from MH09201, MH09210, MH09234, MH09236, MH09286, MH09297, ODA, TIK, OKA and HKK could be obtained directly with ITS1, ITS2, ITS3 and ITS4 primers. The nucleotide sequence from MH09210 strain which was a typical commercial strain in Japan, and MH09236 strain have appeared in the DDBJ Data Libraries under the accession number AB064957 and AB064958, respectively. The nucleotide sequence from MH09210 showed a 99% identity with that from *F. velutipes* DH 97-080 strain (accession number of GenBank/EMBL/DDBJ Data Libraries is AF159426) isolated in China. The nucleotide sequence from MH09236 strain showed a 98% identity with that from *F. velutipes* Collection DAOM 197553 strain isolated in Canada (accession number of GenBank/EMBL/DDBJ Data Libraries is AF141133) (Hughes and others 1999). Surprisingly, the nucleotide sequences from MH09210 strain were perfectly identical with the nucleotide sequences from the other 8 commercially cultivated strains (MH09201, MH09234, MH09286, MH09297, ODA, TIK, OKA and HKK) except for MH09236 strain. From this result, it suggests that the almost commercially cultivated strains in Japan might be bred from the same original strains from a long time ago and the MH09210 strain may be a typical *F. velutipes* strain in Japan (Hughes and others 1999); however, direct sequencing of the PCR products from the other 5 strains (MH09208, MH09209, MH09226, MH09255 and MH09289) was unsuccessful. The amplified DNA fragments from MH09208, MH09209, MH09226, MH09255 and MH09289 strain were therefore cloned into a pT7-Blue T-vector using the TA cloning method; after which the resulting plasmids were sequenced. We sequenced two clones from each strain, and the results showed that deletion or insertion of nucleotides had occurred in the ITS regions, and that it was those changes that made direct sequencing impossible (Figure 1). The aligned nucleotide sequences of the ITS region from the commercial cultivars and similar wild type strains entered in GenBank/EMBL/DDBJ Data Libraries (data not shown). The nucleotide sequence of the ITS-PCR product from MH09208 was notable in that it was markedly different from the sequence of the

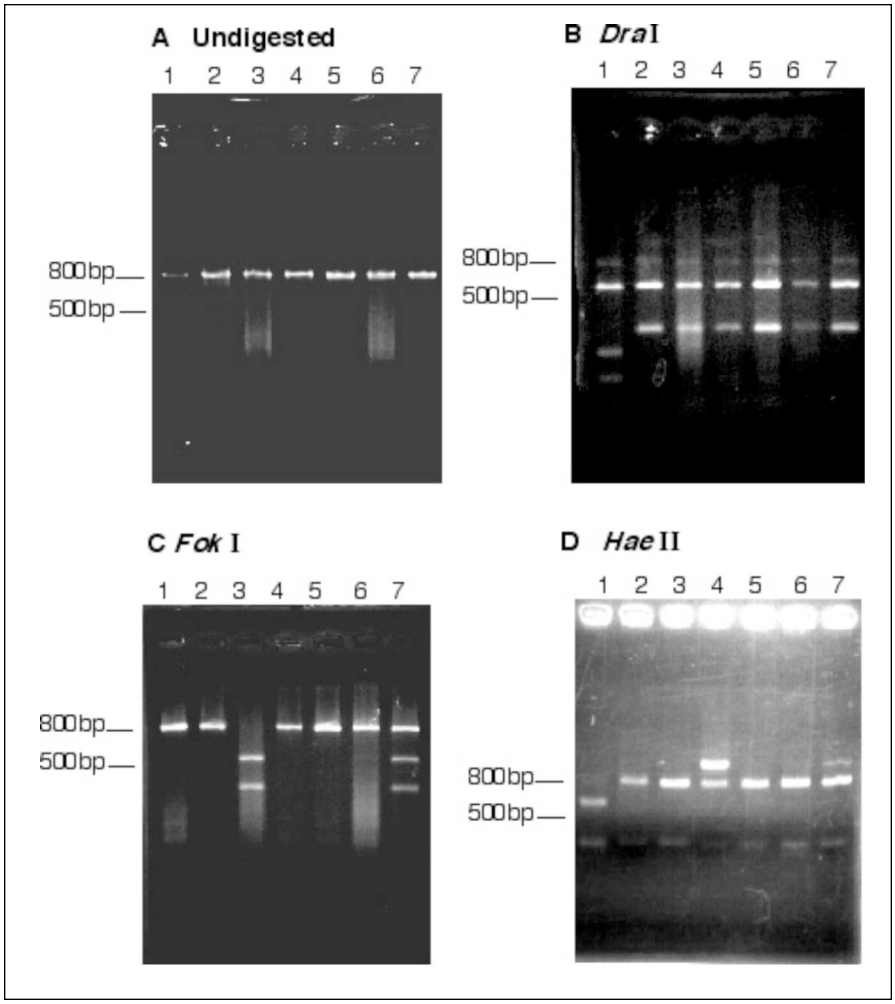

Figure 2—Agarose gel electrophoresis of amplified DNA fragments of the ITS region and digestion with several restriction endonucleases. Panel A: undigested; Panel B: DraI; panel C: FokI; panel D: HaeII; panel E: MboII; panel F: NlaIV. In each panel, Lane 1, MH09208; lane 2, MH09209; lane 3, MH09210; lane 4, MH09226; lane 5, MH09236; lane 6, MH09255; lane 7, MH09289. The size markers correspond to 100-bp DNA ladder (Takara Syuzo Co.).

other strains, being most similar to those of the ITS I, 5.8S rDNA and ITS II regions from *F. populicola* (accession number of GenBank/EMBL/DDBJ Data Libraries is AF044193). The identity was shown to be 99% homologous. These results suggested that MH09208 is a variety of *F. populicola*, while the other cultivars are varieties of *F. velutipes*. We found that *Flammulina* strains display a sequence variation in their rDNA ITS regions and in their 5.8S rRNA coding sequences. By contrast, the nucleotide sequences of the ITS-PCR products from the other cultivars showed the highest homology with those of the ITS I, 5.8S rDNA and ITS II regions from *F. velutipes* DH 97-080 strain isolated in China (accession number of GenBank/EMBL/DDBJ Data Libraries is AF159426). However variations of nucleotide sequence were appearing among isolates in Asia (Hughes and others 1999). More deletion, insertion and substitution were observed in the former rather than the latter, which is not surprising as the spacer regions are functionally less constrained than the rRNA coding sequences, and the 5.8S rRNA coding sequence. This study thus indicates that knowledge of the nucleotide sequences of the ITS regions may assist in detecting differences among commercially cultivated *Flammulina* strains. Initially, we identified a strain of *F. populicola* by recognizing differences between the nucleotide sequence of its ITS regions and that of *F. velutipes*. Furthermore, we were also able to detect differences in nucleotide sequences among several strains of *F. velutipes*. Apparently then, comparison of the nucleotide sequence of the ITS regions should enable identification of differences among strains of a species or genus that could not be detected morphologically. In the future, in order to construct an identification method at the DNA level, we must test mating ability to reveal the relationships between morphological characteristics and DNA sequence .

MH09210 which was a typical commercial strain, and MH09236 were containing homologous nuclei in each cell, and the other 5 strains might be containing heterogeneous nuclei in each cell or heterozygosity with in the ribosomal repeats. Therefore, the direct sequencing method of PCR products was not suitable for differentiating the commercial cultivars of edible mushrooms, especially *F. velutipes*. Thus, we tried to differentiate/identify the commercial cultivars and wild type strains by using PCR-RFLP methods. In order to construct a method with which to rapidly and easily recognize different *F. velutipes* cultivars including wild type strains, RFLP analysis was carried out by digesting the ITS-PCR products using 5 restriction endonucleases. The ITS-PCR products from 7 *F. velutipes* cultivars and wild type strains were digested with *Dra*I, *Fok*I, *Hae*II, *Mbo*II and *Nla*IV, and the resultant DNA fragments were separated by 2% agarose gel electrophoresis. The RFLP patterns of the strains are shown in Figure 2, and estimated restriction fragment length which was based on the nucleotide sequences from MH09210 and MH09236 Table 2. All the fragments restriction fragment length corresponded to RFLP data. In all restriction digestions, polymorphism was apparent. Particularly, in the digestion of ITS-PCR product from MH09226 strain with *Hae*II (Figure 2, panel D, lane 4) and in the digestion of ITS-PCR product from MH09289 with *Fok*I (Figure 2, panel C, lane 7), the 3 bands which are undigested to about 800-bp fragment in size, and 2 digested fragments smaller than 800-bp were apparent. These results did not correspond to nucleotide sequence data, because the nucleotide sequence from MH09226 and MH09289 were sequenced by TA-cloning method. Therefore we could sequence only one allele. However these results indicated that at least 2 dikaryon strains, MH09226 and MH09289, were containing heterogeneous nuclei carrying different ITS sequences in each cell. It shows that the ITS-PCR-RFLP method is better suited for differentiating/recognizing cultivars of dikaryon strains than direct sequencing. The frequency of polymorphism appearing between cultivars was not high, because all the cultivars belong to the same species. Nevertheless, we found that each of the strains could be differentiated based on combining the respective RFLP patterns; a flow chart of the scheme of differentiation is shown in Figure 3. By cutting the ITSs with *Nla*IV, PCR

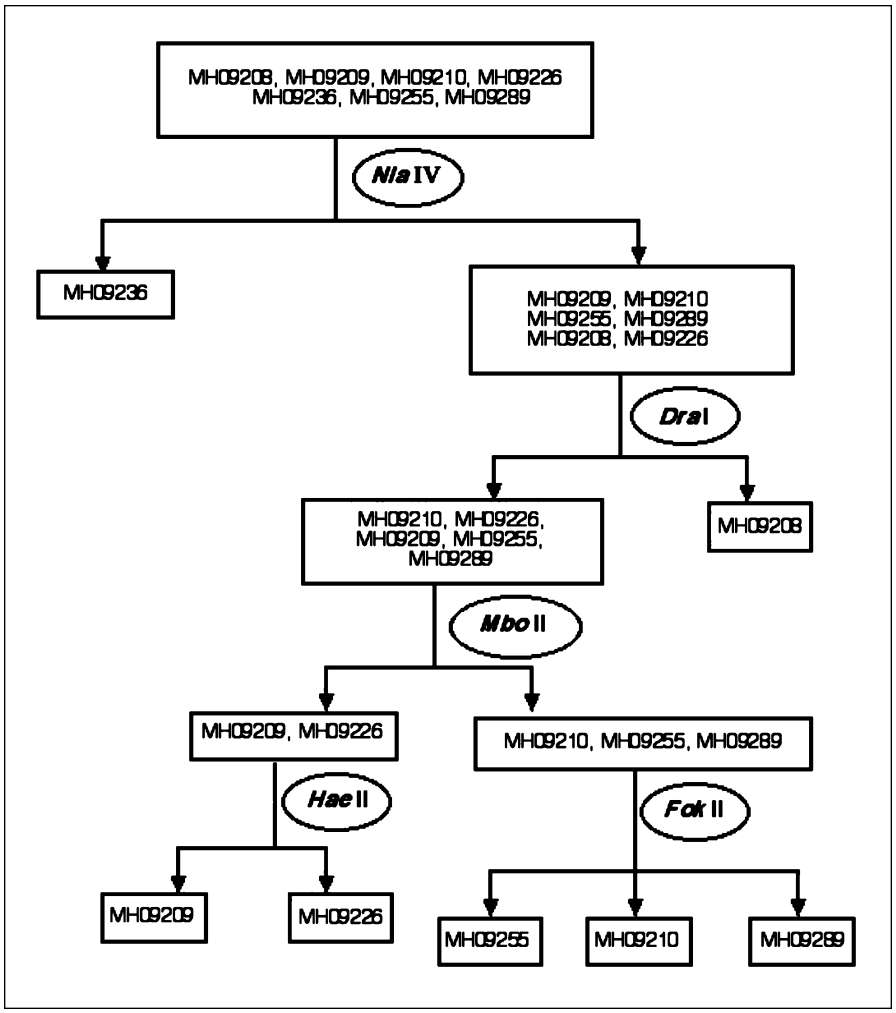

Figure 3—Flow chart for recognizing differences in the RFLP patterns among various *F. velutipes* strains.

fragment of the strain MH09236 was digested into 2 fragments whose size were about 400 and 300-bp, respectively, and smallest bands disappeared because of low resolution of agarose gel. However the RFLP pattern was different from other strains, thus MH09236 was separated; by cutting with *Dra*I and HaeII, the PCR fragment from strain MH09208 was digested into 2 and 3 fragments, respectively. Thus strain MH09208 was recognized; by cutting with *Mbo*II, the remaining strains were classified into 2 groups; strains MH09209 and MH09226 were isolated from another group containing strains MH09210, MH09255 and MH09289; by cutting with *Hae*II, the differences between strains MH09209 and MH09226 were detected; and by cutting with *Fok*I the differences among strains MH09210, MH09255 and MH09289 were recognized, because 2 fragments were undigested and 3 fragments appeared, respectively. Thus, all of the strains could be distinguished based on the RFLP patterns produced by cutting with 5 restriction endonucleases. We were unable to resolve the nucleotide sequences of all the *F. velutipes* strains used in this study by direct sequencing of the amplified PCR products, because the sequences of the ITS region might differ among the paired nuclei of some dikaryon strains. Therefore PCR-RFLP analysis using 5 endonucleases was carried out, which enabled independent identification of the *Flammulina* strains containing heterogeneous sequences in their rDNA ITS regions. However, if more strains should be tested, digestion with additional restriction enzymes will be needed, because we tested only 7 cultivar of *Flammulina.* In a future study, we will attempt to develop some molecular markers to distinguish differences in shape, size and taste among the edible mushroom strains. Moreover, we will have to try to distinguish typical commercial strains which were not able to be distinguished by the ITS-PCR-RFLP method, by using other methods such as RAPD (random amplified polymorphic DNA) or tRNA typing.

Conclusions

OUR RESULTS INDICATE THAT COMPARIsons of PCR-RFLPs of the rDNA ITS regions by cutting with 5 restriction endonucleases including *Dra*I, *Fok*I, *Hae*II, *Mbo*II and *Nla*IV, differences among edible mushroom, *Flammulina* cultivars, can be rapidly and easily distinguished. On the other hand, direct sequencing of PCR fragments is not suitable for distinguishing *F. velutipes* cultivars, because some deletion or insertion had occurred in the rDNA ITS region. Moreover, compared to alternative techniques, such as direct sequencing of PCR products, ITS-PCR-RFLP offers the advantage of being simpler, cheaper, and especially useful for the routine analysis of large numbers of strains (Cocolin and others 2000). Further analysis should enable us to develop RFLP markers, RAPD markers or PCR primers for identifying genes or chromosomes in cultivated strains, which then could be used commercially as a basis for patenting.

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