Phylogenetic Implications of Restriction Maps of the Intergenic Regions Flanking the 5S Ribosomal RNA Gene of *Lentinula* Species

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Abstract

Intergenic spacer regions (IGR-1 and 2) flanking the 5S ribosomal RNA genes (5S rDNA) of *Lentinula edodes*, *L. boryana*, *L. lateritia*, and *L. novaezelandiae* were enzymatically amplified via the polymerase chain reaction (PCR). Length heterogeneities of IGR-1 and 2, ranging from <50 to 750 base pairs, were observed at both the inter- and intra-specific levels. Amplified IGRs were subsequently digested with restriction endonucleases. Comparisons of single digests of amplicons of various sizes facilitated mapping and determination of the orientation of the maps. Appropriate pairs of endonucleases were used to effect double digestion of the IGRs to further map the spacers. Relatively consistent conservation of mapped restriction sites was observed for the different species, with the exception of *L. boryana*. This species was highly polymorphic with respect to length heterogeneity and exact mapping sites in IGR-2. Maps of restriction sites of the rDNA IGRs of *L. boryana*, *L. edodes*, *L. lateritia*, and *L. novaezelandiae* are presented. The computer programs RESTSITE and MEGA were used to analyze data derived from the restriction sites and to reconstruct a phylogeny of the species. The phylogeny indicates a close relationship between the species *L. edodes*, *L. lateritia*, and *L. novaezelandiae*.

Key words: rDNA, IGR, fungi.

Introduction

Lentinula edodes (Berk.) Pegler, or shiitake in the vernacular, is a basidiomycete of the family Tricholomataceae. Shiitake ranks second in U.S. mushroom production after the button mushroom (*Agaricus bisporus* [Lang.] Imbach) (USDA, 2008), and second in world mushroom production after the button mushroom (*Agaricus bisporus* [Lang.] Imbach) (Chang, 2005). Shiitake are sold either fresh or dried and are one of the most important fungal constituents of the cuisine of countries such as Japan and China. Shiitake continue to gain popularity in Western countries. In the United States alone, shiitake consumption increased from 1,343 metric tons in 1993 to 4,632 metric tons in 2007 (USDA, 2008). Extant relatives of *L. edodes* are also comestible and contribute to a diverse genetic pool for the genus *Lentinula* Earle.

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Shiitake has been known generically as *Lentinus* Fr. and *Collybia* (Fr.) Staude among many other names (Pegler, 1975a; 1975b). In the early 1980's, Pegler (1983) assigned shiitake to the genus *Lentinula*. Currently, there are six species that are generally recognized in the genus *Lentinula*, three (*L. edodes*, *L. lateritia* [Berk.] Pegler, and *L. novaezelandiae* [Stev.] Pegler) are of Asia-Australasian distribution, while the remaining three (*L. boryana* (Berk. & Mont.) Pegler, *L. guarapiensis* (Speg.) Pegler, and *L. raphanica* (Murrill) Mata & R.H. Petersen) are distributed in the Americas. Recent work has suggested that the Asia-Australasian-distributed species comprise a single biological species as evidenced by their ability to interbreed (Shimomura et al., 1992; Guzman et al., 1997), with the indication that the species could all be classified as *L. edodes* according to a biological species approach. However, Hibbett and others (Hibbett, 1992; Hibbett et al., 1995; Hibbett and Donohue, 1996; Hibbett et al., 1998) advise against a single Asia-Australasian biological species classification and advocate distinct phylogenetic species to delineate the unique, divergent genetic populations of shiitake possibly existing as monophyletic groups. It is clear that *L. edodes* and *L. boryana* remain separate biological species, unable to interbreed (Mata and Guzmán, 1989; Guzman et al., 1997). Unfortunately, no living specimens of *L. guarapiensis* are available for study, so our understanding of the *Lentinula* representatives distributed in the Americas is limited.

Regardless of a biological or phylogenetic classification approach, morphological characters and properties of cultivation, physiology, and biochemistry have traditionally provided the primary basis for classification of shiitake (Agricultural Production Bureau, 1980; Kwan et al., 1992a). The environment can influence such phenotypic characters and subsequently limit their use for strain typing or cataloguing, making it important, therefore, to exploit stable genetic differences between strains, or closely related species, for this purpose. Comparatively little is known about the molecular genetics of shiitake, although there exists a growing body of literature pertinent to molecular classification of *Lentinula* and *L. edodes* in particular (*e.g.*: Fukuda and Tokimoto, 1991; Hibbett, 1992; Hibbett et al., 1995; Hibbett and Donohue, 1996; Hibbett et al., 1998; Kulkarni, 1991; Kwan et al., 1992a; 1992b; Nicholson et al., 1993; Royse and May, 1987; Royse and Nicholson, 1993).

With the development and application of PCR, the ability to amplify regions of DNA has become routine (see sidebar). For example, amplification of regions of the nuclear ribosomal DNA (rDNA) repeat may be carried out with crude preparations

of total genomic DNA and subsequently utilized for further laboratory analyses. Digestion of amplified regions of rDNA with restriction endonucleases in single and double reactions facilitates the construction of maps of restriction sites (see sidebar). As large numbers of restriction fragment length polymorphism (RFLP) markers can be detected for a given genome (Landry et al., 1987; Tanksley and Hewitt, 1988), maps of exact restriction sites are attractive for genetic variation studies involving molecular marker comparisons of rapidly evolving sequences. Evolutionarily-isolated populations of *Lentinula* can be expected to have divergent rDNA genes through independent accumulation of base substitutions, length mutations, and rearrangements. Restriction maps of the rDNA repeat may lead to further understanding of the evolutionary relationships and diversity within the genus *Lentinula* and may serve as a valuable method of characterization for a given biotype. Here we present maps of restriction sites of the rDNA IGRs 1 and 2 of *L. edodes*, *L. lateritia*, *L. novaezelandiae*, and *L. boryana*. These maps facilitate direct comparisons of the structural features of these heterogeneous regions between the species. Also presented is a reconstruction of the phylogenetic relationships of the species used in this study. This reconstruction is in the form of a phylogenetic tree based on the mapped restriction sites for the isolates of the given species studied.

Materials and Methods

Strains and DNA purification. Strains of *Lentinula* representing four species (Table 1) were individually grown in 100 ml potato dextrose yeast extract broth. After sufficient growth, about three weeks in still culture, the mycelium was filtered onto filter paper, rinsed with distilled de-ionized water, and air-dried. Care was taken between samples to ensure the filter apparatus was appropriately cleaned, so as to prevent carry-over of mycelium or cellular debris. DNA was extracted from each sample according to the procedures outlined by Yoon et al. (1991).

Enzymatic amplification of rDNA. Amplification of the IGRs flanking the 5S rDNA was performed utilizing custom primers (Table 2; Figure 1) and *Tfl* polymerase (Epicenter Technologies, Madison, WI, USA). Individual, 50 µl reactions consisted of 5 µl DNA (concentration optimized empirically by dilution), 1.75 µM dNTPs (Pharmacia Biosystems Inc., Piscataway, NJ, USA), 1.65 mM $MgCl_2$ (supplied with polymerase), 1 µM of each primer, 1x reaction buffer (supplied with polymerase), and 1 U *Tfl* polymerase. A thermal cycler (Bio Therm, Fairfax,

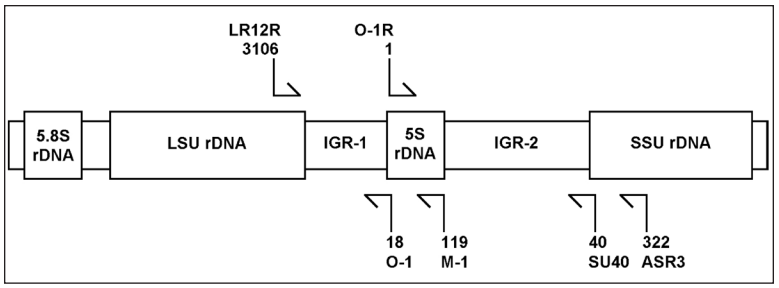

Figure 1. Structure of the rDNA repeat and locations of PCR primers used to amplify the IGRs 1 and 2 (sequences given in Table 2). The arrows represent the 5'to 3' direction of each primer. LSU rDNA refers to the large subunit ribosomal DNA, SSU rDNA refers to the small subunit ribosomal DNA.

DNA Fingerprinting in Fungi
A Primer

Copying DNA:

The process of copying DNA with the use of enzymes is called DNA amplification. The Polymerase Chain Reaction (PCR) is an automated DNA amplification process that allows for the reliable reproduction of large numbers of short pieces of DNA that are identical to a specific piece of target DNA taken from an organism under study.

PCR requires some basic ingredients and some initial understanding of the target DNA of the organism. The basic ingredients required include a heat-stable DNA polymerase, free nucleotides, a sample of target DNA, and "primers" that are specific for the flanks of the target DNA segment. The **polymerase** actually reads the target DNA and synthesizes a strand of DNA complimentary to the target. Free **nucleotides** are provided as the raw material that the polymerase links into a polymer of new DNA. The target DNA can be from any source, including small amounts of crudely prepared cellular debris or highly purified DNA. The "**primers**" are short single-stranded pieces of DNA that are known to match segments of DNA on the flanks of a specific region of the target DNA. Because a good match to the flanks is required for the primers to allow amplification of the targeted segment of DNA, some knowledge of the target DNA is required.

DNA amplification is essentially accomplished by mixing the basic ingredients discussed above and subjecting the PCR mixture to a series of temperature cycles (refer to Figure 1 as indicated below). In each cycle, the mixture is heated to break the bonds that hold together the two strands of DNA. This causes the double-helix (1a) to unwind and open up (1b), and this is the first step whenever DNA is replicated. The mixture is then cooled. As the temperature drops, the small, single-stranded primers bind to their complimentary sequences at the flanks of the target DNA, making small segments of double-stranded DNA (1c). DNA polymerase attaches to the small segments of double-stranded DNA, and uses the segments as a starting point for DNA synthesis. The polymerase incorporates free nucleotides into a polymer of DNA that is complimentary to the target DNA, ultimately making a long double stranded copy of each of the strands of the original target DNA (1d). When the first cycle (1a-1d) is complete, the number of copies of target DNA is actually doubled. With each PCR cycle the number of copies of target DNA doubles, so at the end of the second cycle, there are four copies of the target DNA (1e), and at the end of the third cycle, there are eight copies of the target DNA (1f). Thus, if there is only a single copy of target DNA to start with, it might be "amplified" to more than 1 trillion copies in only 31 cycles of PCR.

Since the copies of DNA produced by PCR are identical and in a large quantity, a small

VA, USA) was used to repeatedly extend the target sequence for 41 cycles (95°C for 30 seconds to denature the DNA, 55°C for one minute to anneal the primers, and 72°C for 2 minutes to extend the primers with a final extension of 7 minutes). Positive controls (alternative species of fungi including *Agaricus* L., *Collybia*, *Pleurotus*, *Saccharomyces cervisiae* Mayen & Hansen, and *Trichoderma* Pers.) and negative controls (no-DNA) were employed as checks. Amplification products were electrophoresed in 1.0% agarose gels to determine size (sized with 123 bp ladder [Gibco, Gaithersburg, MD, USA]) and relative quantity. Ethidium bromide was added to the molten gel (0.03-0.1 µg/ml gel) to allow staining of the DNA, and subsequent visualization and photographing under UV illumination.

Restriction mapping. Amplified products for each strain were single and double digested (Figure 2) with restriction enzymes (Table 3) following the enzyme manufacture's suggested protocols. Restriction products were electrophoresed as previously detailed to determine fragment sizes. Comparison of single digests of amplicons of different sizes facilitated mapping and determination of the orientation of the maps (Figure 2a). Restriction fragments of double enzyme digestion of rDNAs were sized between restriction fragments digested by the single enzymes that made up each double digest (Aquadro et al., 1992) (Figure 2b). The order of the fragments for each enzyme was deduced logically and the relative positions of the restriction sites were identified accordingly.

Phylogenetic reconstruction. Phylogenetic information was inferred from the mapped restriction sites to reconstruct a basic evolutionary history of the species involved in the study (see Appendix I for data). The computer program RESTSITE (Miller, 1991; Nei and Miller, 1990) was used to make pairwise comparisons between restriction sites of the different map types from the species studied and subsequently yielded nucleotide substitutions per site as distance estimates between each pair of map types. The distance data from the pair-wise comparisons were analyzed using the computer program MEGA (Kumar et al., 1993) to reconstruct the phylogenetic relationships of the species. The resulting phylogenetic tree was assembled using the Unweighted Pair-Group Method with Arithmatic Mean (UPGMA) (Sneath and Sokal, 1973).

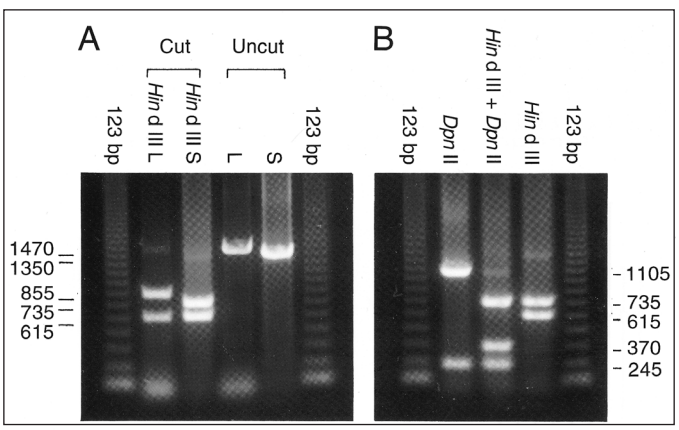

Figure 2. Products of endonuclease restriction of IGR-1 of *Lentinula edodes*. Sizes are in base pairs and restriction endonucleases are specified per lane. A) Single endonuclease restriction of differentially sized products using *Hin*d III. S and L refer to small and large amplification products from primer pairs LR12R & O-1 and LR12R & M-1, respectively. B) Single endonuclease restrictions using *Hin*d III or *Dpn* II flanking a double restriction with both endonucleases.

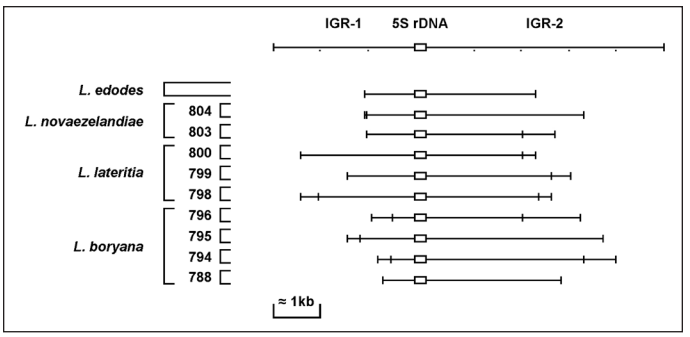

Figure 3. Relative sizes of intergenic spacers compared among isolates and species. Vertical bars at either end denote the to-scale lengths of the IGRs, with length heterogeneity represented by more than one bar per individual IGR. The 5S rDNA is represented by an open box. Species and isolates are noted with brackets. Arrangements of spacers within a tandem repeat or as pertaining to a specific nucleus within an isolate are not implied. Approximate sizes of the different spacers are summarized in Table 3.4.

Results

***Location and direction of transcription of 5S rRNA gene in* Lentinula.** Identification of the amplified DNA, beyond assumptions based on primer specificity, was ascertained by partial sequencing of the 5S rDNA (unpublished data). In all species studied, the 5S rRNA gene was oriented in the same transcriptional direction, based on primer sequence position, as the 18S, 5.8S, and 26S rRNA genes. In all species, the 5S rRNA gene is located in the non-transcribed spacer between repeats of the 18S to 26S rRNA gene transcript.

Intergenic spacer length heterogeneity. Length heterogeneities of IGR-1 and 2 were observed between the species, between strains of each species, and within given strains. These length heterogeneities varied from about 100 base pairs to several hundred base pairs and are summarized graphically in Figure 3. Approximate lengths of the IGRs are summarized in Table 4. For either IGR, additional products were observed at times, some of which were of constant size but of inconsistant yield. As these products were not consistent and generally endured restriction digestion intact, they were considered artifactual and not included in the analysis. Adjustment of PCR parameters had a significant effect

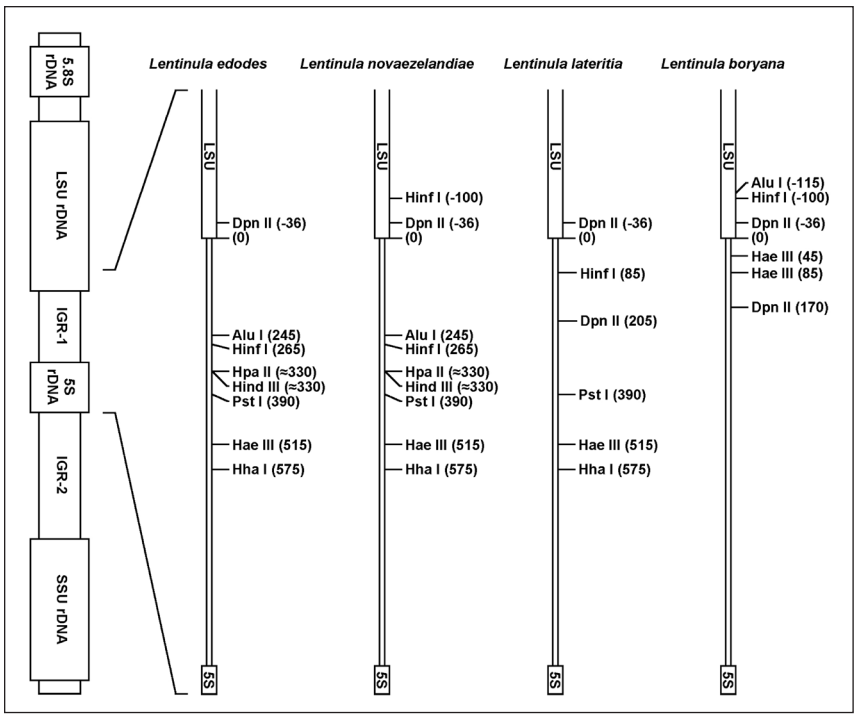

Figure 4. Restriction site map of IGR-1 of *Lentinula* species. All maps are aligned relative to the LSU rDNA. Distances are in base pairs from the 3' end (with respect to transcription) of the LSU rDNA. Map sites of *L. boryana* are characteristic of strain 788. The *L. novaezelandiae* map site (*Hin*f I) is an additional site characteristic of strain 803.

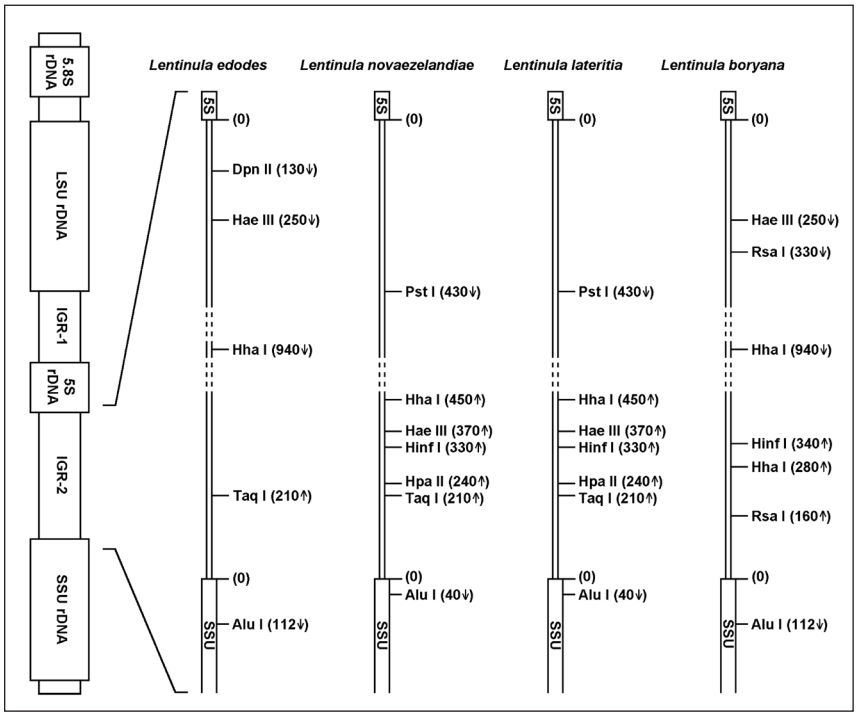

Figure 5. Restriction site map of IGR-2 of *Lentinula* species. Distances are in base pairs from either end of the intergenic spacer, as denoted by the arrow (arrow points away from origin).

on the occurrence of these artifactual products, possibly indicating non-specific binding of the primers to pseudogene sequences or to degenerate DNA within the repeat. Contamination by exogenous DNA from other fungi was not a problem.

amount of starting material (*i.e.*, single cells or scant tissue samples) can be efficiently used to facilitate a wide array of simple molecular analyses.

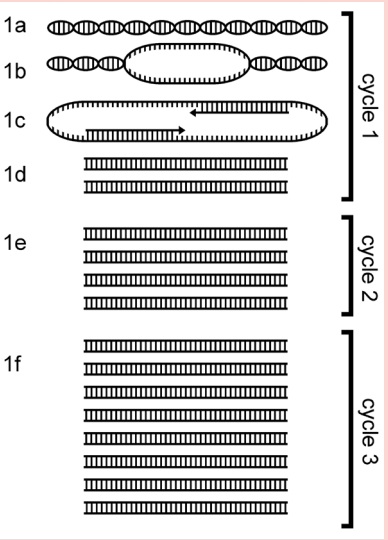

Figure 1. Diagrammatic overview of PCR for three cycles.

Making a DNA Fingerprint

The amplification product from PCR can be analyzed in many ways. A simple way to analyze the product is to produce Restriction Fragment Length Polymorphisms, or RFLPs. RFLPs can be further analyzed to produce genetic maps or perhaps to isolate differences between forms of specific genes.

Purified or amplified DNA from related species or strains of fungi can be cut (digested) with enzymes called restriction endonucleases to produce fragments that are different lengths. RFLPs yield specific banding patterns, or "**fingerprints**," when they are separated with gel electrophoresis. Gel electrophoresis separates different sized pieces of DNA with an electrical charge. DNA samples are placed at one end of a gel slab and a current is applied to the gel. Since DNA is negatively charged, it moves towards the positive end of the gel, but the larger molecules of DNA move slower than the smaller molecules of DNA – resulting in the separation of RFLPs in the electrophoresis gel. Standards of known size can be run with the samples, and a photograph can be taken of the separated DNA products. RFLP patterns, while sometimes very similar, are often unique for different strains or species of organisms being studied. RFLP fingerprint analysis, therefore, may lead to further understanding of the evolutionary relationships and diversity within a group or organisms and may serve as a valuable method of characterization for a given biotype.

Restriction Mapping with Single and Double Digests:

Restriction fragments of DNA that has been digested with two enzymes simultaneously (double enzyme digestion) can be sized between restriction fragments of DNA that has been digested with the two individual enzymes respectively. The relative

positions of the restriction sites for each enzyme on the DNA can then be deduced in a logical manner. This can be done as in the following example: Suppose that single and double digestion of PCR-amplified DNA is performed. The original piece of DNA is 12.3 kb in size, and it is digested by *Taq*I, *Taq*I+*Hin*fI, and *Hin*fI, yielding 3, 4, and 2 fragments respectively. The sizes of the fragments are illustrated in Figure 2. The double digestion with *Taq*I+*Hin*fI produced four fragments with the sizes: 5.1, 3.2, 1.2, and 2.8 kb.

The possible relative positions of the fragments from each enzyme are illustrated in Figure 3; it can be seen that there are three possible orders, or maps, to the fragments from *Taq*I and two possible orders to the fragments from *Hin*fI. From this, various combinations of the possible *Taq*I and *Hin*fI maps must be examined and compared to the data of the double digest pattern. Only the map combination A & D is workable, since it allows segments of the sizes specified by the *Taq*I+*Hin*fI double digest, as shown in F. No other combination of the individual *Taq*I and *Hin*fI maps can yield fragments equal in size and number to the double digest. Several combinations of enzymes can be considered and used to produce maps of value. With each new map, compatibility with previous maps would need to be ascertained, and so assumptions regarding working maps should be flexible and modified as new information is accumulated.

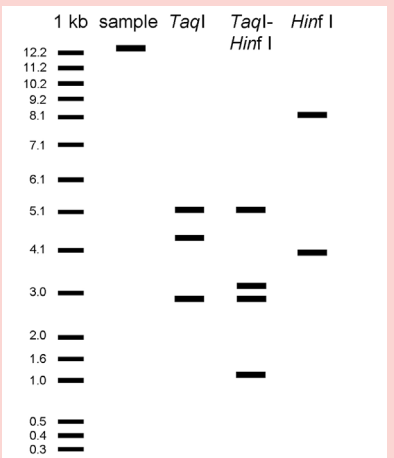

Figure 2. Diagrammatic single and double digests next to 1 kb standard and original, undigested DNA sample.

Figure 3. Possible restriction maps from the single digests shown in Figure 2.

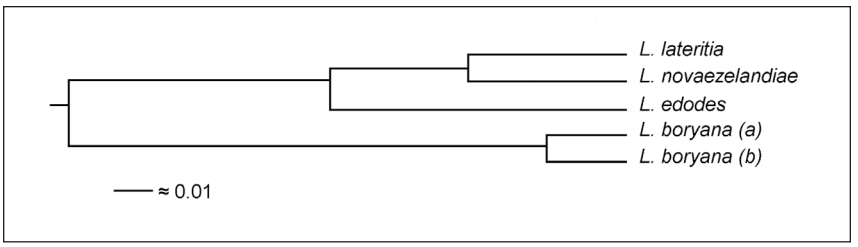

Figure 6. Phylogenetic relationships of *Lentinula* spp. based on data resulting from restriction site analysis of rDNA IGRs. Dissimilarity was calculated using RESTSITE V1.1 (Miller, 1991) and the tree assembled with MEGA V1.02 (Kumar et al. 1993) using the UPGMA method. Species are identified at each branch. The scale approximates dissimilarity as a proportion of estimated nucleotide substitutions per site.

lem as shown by comparison of amplification products to those from *Agaricus*, *Collybia*, *Pleurotus*, *S. cervisiae*, and *Trichoderma*.

Restriction map of rDNA IGR-1 and 2 of* Lentinula *species. Mapped restriction sites of the IGRs- 1 and 2 for each species of *Lentinula* are shown in Figures 4 and 5, respectively. Conservation of restriction sites for a species was relatively consistent, except for *L. boryana*. This species proved highly polymorphic in both length and exact mapping sites of the IGR-2. The IGR-1s of *L. edodes* and *L. novaezelandiae* were strikingly similar. *Lentinula lateritia* shared only one putative IGR-1 site and had two similar sites compared to *L. novaezelandiae* and *L. edodes* respectively. *Lentinula boryana* shared one similar IGR-1 site with *L. lateritia*. Since the maps for IGR-2 were developed by comparing restriction products of differential lengths, the central portion of the spacers of each species were not mapped.

Phylogenetic relationships of* Lentinula *species. The phylogenetic relationships of the species of *Lentinula* based on IGR restriction map sites were determined. According to the UPGMA tree (Figure 6) produced by MEGA from the RESTSITE distance matrix (Table 5), the species *L. lateritia* and *L. novaezelandiae* are the closest relatives, and *L. edodes* is indicated as a closer relative to this pair than is *L. boryana*. The two types of *L. boryana*, A (isolate 788) and B (remaining isolates studied) as differentiated by IGR-1 maps, appear very closely related.

Discussion

Substantial variation was observed in the IGRs of *Lentinula* species. This variation was evident by direct comparison of restriction maps of the regions flanking the 5S rRNA gene as well as by differences in the lengths of each spacer. Because the IGRs are not transcribed, and therefore not highly conserved, this result is not surprising. Length heterogeneity in the IGRs of rDNA has been observed in numerous fungal groups and species (Buchko and Klassen, 1990; Garber et al., 1988; Henrion et al., 1994; Hibbett and Vilgalys, 1991; Kim et al., 1992; Royse and Nicholson, 1993; Tan et al., 1994), and in other organisms (Rocheford et al., 1990; Rogers et al., 1986; Saghai-Maroof et al., 1984). Such heterogeneity has been attributed to unequal recombination, transposition, and gene conversion (Appels and Dvorak, 1982; Metzenberg et al., 1985; Rogers et al., 1986; Selker et al., 1981). Each of these mechanisms would be expected to result in homogeneity of the rDNA within a population or species and heterogeneity between populations or species. Subrepeated arrays within the spacers are a possible result of the proposed mechanisms of heterogeneity and have been putatively implicat-

ed as contributing to length heterogeneity of intergenic regions (Buchko and Klassen, 1990; Rocheford et al., 1990; Rogers et al., 1986). A lack of length heterogeneity, however, may not necessarily imply a lack of subrepeats (Klassen and Buchko, 1990), so caution is needed in the possible assignment of a mechanism to explain this difference.

The intra- and interspecific length heterogeneity of IGRs within the Asian biotypes, *L. edodes*, *L. lateritia*, and *L. novaezelandiae*, indicates a large pool of genetic diversity in these species. Such heterogeneity, however, may be due to unmappable, yet simple, subrepeated arrays within the 3' end of IGR-1 and the central portion of IGR-2. Comparable IGR length and restriction polymorphisms have been observed in *Pleurotus cornucopiae* (Paulet :Fr.) Rolland (Iraçabal and Labarère, 1994). In this study, seven strains of *P. cornucopiae* were found to possess two types (I and II) of IGR-1 based on restriction site maps. These two IGR types were further broken into a total of six subgroups due to length variations. With this in mind, the near-identical maps between *L. edodes* and *L. novaezelandiae* for IGR-1 and *L. lateritia* and *L. novaezelandiae* for IGR-2 suggest great commonality between these biotypes. Other shared or similar restriction sites, particularly those in IGR-1 of *L. lateritia* also suggest a close relationship between these biotypes. The variation in mappable IGR-1 restriction sites in *L. boryana* may indicate a high degree of genetic variation within that species or within a discrete population of the species. A basic understanding of the complexity of these spacers and an indication of their gross structure as provided by these maps may be used to direct future investigations of fungal rDNAs.

The phylogenetic reconstruction made possible by the restriction sites from the restriction site data indicates that the Asia-Australasian-distributed species of *Lentinula* are evolutionarily divergent from the American-distributed *L. boryana*, as noted by previous researchers (Guzman et al., 1997; Hibbett, 1992; Hibbett et al., 1995; Hibbett and Donohue, 1996; Hibbett et al., 1998; Hibbet, 2001; Mata et al., 2001). In addition, the species *L. lateritia* and *L. novaezelandiae* were found to be closer relatives to each other than either is to *L. edodes*. These results correspond to results from Nicholson et al. (1995), who used ITS and IGR-1 and -2 restriction fragment length polymorphism data to elucidate the phylogenetic relationships within the genus *Lentinula*. Although their analysis violated certain assumptions of homogeneity, individual isolates could be distinguished among each species and the Asian-Australasian species were found to be monophyletic.

Restriction maps of rRNA genes have been published for several fungal genera. Garber et al. (1988) have compiled a considerable number of such maps for comparison purposes. They found that the organization of the 17S - 5.8S - 25S genes was conserved in all 18 species of fungi studied by other independent researchers. The rDNA repeat length ranges from 7.7kb to 12.0kb between the species and some conservation of restriction sites is evident when the maps are adjacently compared; however, many restriction sites, even within the genes, do not appear to be highly conserved. The IGR was found to be the most variable in both size and restriction sites. In addition, RFLP maps of rDNA have been constructed to elucidate five species of *Candida* Berkhout (Magee et al., 1987) and differentiate 13 genotypic classes of *Cryptococcus* Kützig (Vilgalys and Hester, 1990). More recently, phylogenetic relationships among *Coprinus* Pers. species and relatives were determined using restriction maps (Hopple and Vilgalys, 1994). This work further demonstrates the ability of RFLPs to "fingerprint" given isolates and elucidate phylogenetic relationships between species. Aside from being phylogenetically informative, intergenic spacers have been associated with economic traits of importance in barley and maize (Allard, 1988; Powell et al., 1992; Rocheford, 1990), thereby increasing the significance of these non-coding regions. Of interest are the extent, structure, and inheritance of IGRs of diverse fungi and the possibility of the association of important economic traits with length heterogeneity due to variable subrepeat arrays.

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References

Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries, Japan. 1980. The Standards of Judgement of Classification; Shiitake mushroom; *Lentinus edodes* (Berk.) Sing.

Allard, R. 1988. Genetic changes associated with the evolution of adaptedness in cultivated plants and their wild progenitors. *Journal of Heredity* 79: 225–38.

Appels, R., and J. Dvorák. 1982. Relative rates of divergence of spacer and gene sequences within the rDNA region of species in the Triticeae: implications for the maintenance of homogeneity of a repeated gene family. *Theoretical Applications of Genetics* 63: 361–65.

Aquadro, C., W. Noon, and D. Begun. 1992. RFLP analysis using heterologous probes. Pp. 115-57 In: A. Hoelzel (ed.) *Molecular Genetic Analysis of Populations.* Oxford University Press, NY.

Buchko, J., and G. Klassen. 1990. Detection of length heterogeneity in the ribosomal DNA of *Pythium ultimum* by PCR amplification of the intergenic region. *Current Genetics* 18: 203–05.

Chang, S. 1993. Mushrooms biology: the impact on mushroom production and mushroom products. In: S. Chang, J. Buswell, and S. Chiu (eds.), *Mushroom Biology and Mushroom Products.* The Chinese University of Hong Kong, Hong Kong.

Chang, S. 2005. Witnessing the development of the mushroom industry in China. In: Tan et al. (eds.) Proceedings of the 5th International Conference on Mushroom Biology and Mushroom Products. *Acta Edulis Fungi* 12: 3–19.

Garber, R., B. Turgeon, E. Selker, and O. Yoder. 1988. Organization of ribosomal RNA genes in the fungus *Cochliobolus heterostrophus*. *Current Genetics* 14: 573–82.

Georgiev, O., N. Nikolaev, A. Hadjiolov, K. Skryabin, V. Zaeharyev, and A. Bayev. 1981. The structure of the yeast ribosomal RNA genes. 4. Complete sequence of the 25S rRNA gene from *Saccharomyces cerevisiae*. *Nucleic Acids Research* 9: 6953–58.

Guzman, G., D. Salmones, and F. Tapia. 1997. *Lentinula boryana*: morphological variations, taxonomic position, distribution and relationships with *Lentinula edodes* and related species. *Reports of the Tottori Mycological Institute* 35: 1–28.

Henrion, B., G. Chevalier, and F. Martin. 1994. Typing truffle species by PCR amplification of the ribosomal DNA spacers. *Mycological Research* 98: 37–43.

Hibbett, D., and R. Vilgalys. 1991. Evolutionary relationships of *Lentinus* to the Polyporaceae: evidence from restriction analysis of enzymatically amplified ribosomal DNA. *Mycologia* 83: 425–39.

Hibbett, D. 1992. Towards a phylogenetic classification for shiitake: taxonomic history and molecular perspectives. *Reports of the Tottori Mycological Institute* 30: 30–42.

Hibbett, D., Y. Fukumasa-Nakai, A. Tsuneda, and M. Donoghue. 1995. Phylogenetic diversity in shiitake inferred from nuclear ribosomal DNA sequences. *Mycologia* 87: 618–38.

Hibbett, D., and M. Donoghue. 1996. Implications of phylogenetic studies for conservation of genetic diversity in shiitake mushrooms. *Conservation Biology* 10: 1321–27.

Hibbett, D., K. Hansen, and M. Donoghue. 1998. Phylogeny and biogeography of *Lentinula* inferred from an expanded rDNA dataset. *Mycological Research* 102: 1041–49.

Hibbett, D. 2001. Shiitake mushrooms and molecular clocks: historical biogeography of *Lentinula*. *Journal of Biogeography* 28 no. 2 (1 p.1/4): 231–41.

Hopple, J., and R. Vilgalys. 1994. Phylogenetic relationships among coprinoid taxa and allies based on data from restriction site mapping of nuclear rDNA. *Mycologia* 86: 96–107.

Iraçabal, B., and J. Labarère. 1994. Restriction site and length polymorphism of the rDNA unit in the cultivated basidiomycete *Pleurotus cornucopiae*. *Theoretical Applications of Genetics* 88: 824–30.

Kim, W., T. Zerucha, and G. Klassen. 1992. A region of heterogeneity adjacent to the 5S ribosomal RNA gene of cereal rusts. *Current Genetics* 22: 101–05.

Klassen, G., and J. Buchko. 1990. Subrepeat structure of the intergenic region in the ribosomal DNA of the oomycetous fungus *Pythium ultimum*. *Current Genetics* 17: 125–27.

Kulkarni, R. 1991. DNA polymorphisms in *Lentinula edodes*, the shiitake mushroom. *Applied and Environmental Microbiology* 57: 1735–39.

Kumar, S., K. Tamura, and M. Nei. 1993. MEGA: Molecular Evolutionary Genetics Analysis, version 1.0. The Pennsylvania State University, University Park, PA, 16802.

Kwan., H., S. Chiu, K. Pang, and S. Cheng. 1992a. Strain typing in *Lentinula edodes* by polymerase chain reaction. *Experimental Mycology* 16: 163–66.

Kwan, H., K. Pang, S. Chiu, and S. Cheng. 1992b. Nucleotide sequence of the 5.8S ribosomal RNA gene of *Lentinula edodes*. *Nucleic Acids Research* 23: 610.

Landry, B., R. Kesseli, B. Farrara, and R. Michelmore. 1987. A genetic map of lettuce with a restriction fragment length polymorphism, isozyme, disease resistance and morphological markers. *Genetics* 112: 331–37.

Lee, S., and J. Taylor. 1990. Isolation of DNA from fungal mycelia and single spores. Pp. 282–287 In: M. Innis, D. Gelfand, J. Sninsky, T. White (eds) *PCR Protocols: A Guide to Methods and Applications.* Academic Press, New York.

Magee, B., T. D'Souza, and P. Magee. 1987. Strain and species identification by restriction fragment length polymorphisms in the ribosomal DNA repeat of *Candida* species. *Journal of Bacteriology* 169: 1639–43.

Mata, G., and G. Guzmán. 1989. Hibridación entre una cepa Mexicana de *Lentinus boryanus* y una Asiática de *Lentinus edodes*. *Revista Mexicana de Micologia* 5: 77–80.

Mata, J., R. Petersen, and K. Hughes. 2001. The genus *Lentinula* in the Americas. *Mycologia* 93(6): 1102–12.

Metzenberg, R., J. Stevens, E. Selker, and E. Morzycka-Wroblewska. 1985. Identification and chromosomal distriction of 5S rRNA genes in *Neurospora crassa*. *Proceedings of the National Academy of Sciences USA* 82: 2067–71.

Miller, J. 1991. RESTSITE V1.1. MIT, Cambridge, MA, 02139.

Nei, M., and J. Miller. 1990. A simple method for estimating average numbers of nucleotide substitutions within and between populations from restriction data. *Genetics* 125: 873–79.

Nicholson, M., D. Royse, and B. Bunyard. 1993. Restriction fragment length polymorphisms (RFLPs) of ribosomal DNA (rDNA) of shiitake, *Lentinula edodes*. *Inoculum* 44: 51 (abstr.)

Nicholson, M., M. Thon, and D. Royse. 1995. Phylogeny of *Lentinula* spp. based on restriction fragment length polymorphism analysis of internal transcribed spacers and intergenic regions of ribosomal DNA. Pp. 153–60 In: T. J. Elliott (ed.) *Science and Cultivation of Edible Fungi.* Balkema, Rotterdam.

Pegler, D. 1975a. The classification of the genus *Lentinus* Fr. (Basidiomycota). *Kavaka* 3: 11–20.

Pegler, D. 1975b. The genus *Lentinus*: a world monograph. *Kew Bulletin Additional Series* 10: 1–281.

Pegler, D. 1983. The genus *Lentinula* (Tricholomataceae tribe Collybieae). *Sydowia* 36: 227–39.

Powell, W., W. Thomas, D. Thompson, J. Swanston, and R. Waugh. 1992. Association between rDNA alleles and quantitative traits in doubled haploid population of barley. *Genetics* 130: 187–94.

Rocheford, T., J. Osterman, and C. Gardner. 1990. Variation in the ribosomal DNA intergenic spacer of a maize population mass-selected for high grain yield. *Theoretical and Applied Genetics* 79: 793–800.

Rogers, S., S. Honda, and A. Bendich. 1986. Variation in the ribosomal RNA genes among individuals of *Vicia faba*. *Plant Molecular Biology* 6: 339–45.

Royse, D., and B. May. 1982. Isozyme variation: identification of genotypic classes and confirmation of crosses between lines of *Agaricus brunnescens*. *Phytopathology* 72: 265.

Royse, D., and M. Nicholson. 1993. Allozymes, ribosomal DNA and breeding in *Lentinula*. *Reports of the Tottori Mycological Institute* 31: 162–67.

Saghai-Maroof, M., K. Soliman, R. Jorgensen, and R. Allard. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Science USA* 81: 8014–18.

Selker, E., C. Yanofsky, K. Driftmier, R. Metzenber, B. Alzner-DeWeerd, and U. RajBhandary. 1981. Dispersed 5S RNA genes in *N. crassa*: structure, expression and evolution. *Cell* 24: 819–28.

Shimomura, N., K. Hasebe, Y. Nakai-Fukumasa, and M. Komathu. 1992. Intercompatibility between geographically distant strains of shiitake. *Reports of the Tottori Mycological Institute* 30: 26–29.

Sneath, P., and R. Sokal. 1973. Pp. 230-231. In: *Numerical taxonomy.* W. H. Freeman, San Francisco.

Tan, M., P. Wong, and M. Holley. 1994. Characterization of nuclear ribosomal DNA (rDNA) in *Gaeumannomyces graminis* and correlation of rDNA variation with *G. graminis* varieties. *Mycological Research* 98: 553–61.

Tanksley, S., and J. Hewitt. 1988. Use of molecular markers in breeding for solids content in tomato -- a re-examination. *Theoretical and Applied Genetics* 75: 811–23.

Thon, M., and D. Royse. 1999. Evidence for two independent lineages of shiitake of the Americas (*Lentinula boryana*) based on rDNA and β-tubulin gene sequences. *Molecular Phylogenetics and Evolution* 13(3): 520–24.

USDA (United States Department of Agriculture). 2008. Mushroom Production. *Keystone Ag Digest*, United States Department of Agriculture, National Agricultural Statistics Service, Early September, Vol. 7, No. 17 (see "Pennsylvania Publications" at www.nass.usda.gov/pa).

Vilgalys, R., and M. Hester. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* 172: 4238–46.

Yoon, C., D. Glawe, and P. Shaw. 1991. A method for rapid small-scale preparation of fungal DNA. *Mycologia* 83: 835–38.

Table 1. Strains and sources of *Lentinula* spp.

Species	Strains and Sources
L. edodes	R6, WC376, WC380, WC385, WC471 (from the PSUMCC[a])
L. lateritia	798, 799, 800 (from David Hibbett, Harvard Univ. Herbarium)
L. novaezelandiae	803, 804 (from David Hibbett, Harvard Univ. Herbarium)

a. The Pennsylvania State University Mushroom Culture Collection

Table 2. Custom primers[a] and relative locations in *Saccharomyces cerevisiae* rDNA used to amplify IGRs 1 and 2 flanking the 5S ribosomal RNA gene in *Lentinula* spp.

Primer name	Primer[a] sequence (5→3′)	Location[b]/direction[c] of primer extension
ASR3	CGAAAGTTGATAGGGC	322-307/18S rDNA→5S rDNA
LR12R	CTGAACGCCTCTAAGTCAGAA	3106-3126/25S rDNA→5S rDNA
M-1	AACCACAGCACCCAGGATTCCC	119-97/5S rDNA→LSU rDNA
O-1	AGTCCTATGGCCGTGGAT	18-1/5S rDNA→LSU rDNA
O-1R	ATCCACGGCCATAGGACT	1-18/5S rDNA→SSU rDNA
SU40	TTGAGACAAGCATATGAC	40-23/SSU rDNA→5S rDNA

a. Supplied by Integrated DNA Technologies, Coralville, IA, USA.
b. Georgiev et al. 1981; Vilgalys, personal communication.
c. LSU and SSU rDNA refer to large subunit and small subunit ribosomal DNAs respectively.

Table 3. Endonucleases and recognition sites used to restrict ribosomal DNA amplicons for mapping purposes.

Endonuclease	Recognition site	Endonuclease	Recognition site
Alu I[a]	AG/CT	Hinf I[a]	G/ANTC*
Dpn II[b]	/GATC	Hpa II[a]	C/CGG
Hae III[a]	GG/CC	Pst I[a]	CTGCA/G
Hha I[a]	GCG/C	Rsa I[a]	GT/AC
Hind III[a]	A/AGCTT	Taq I[b]	T/CGA

* N = variable site
a. Supplied by United States Biochemical Corp, Cleveland, OH, USA.
b. Supplied by Boehringer Mannheim, Indianapolis, IN, USA.

Table 4. Approximate lengths of IGR-1 and IGR-2 of the rDNA within *Lentinula* spp. Sizes are in base pairs.

Species	Strain	IGR-1	IGR-2
L. edodes	all	1065	2250
L. novaezelandiae	803	1010	2000, 2600
	804	1010, 1065	3260
L. lateritia	798	2035, 2400	2230,2600
	799	1420	2600, 3000
	800	2400	2000, 2260
L. boryana	788	690	2790
	794	560, 805	3260, 4000
	795	1175, 1420	3660
	796	500, 890	2030, 3210

Table 5. Distance matrix (lower left) and bootstrapped standard errors (200 repetitions) (upper right) computed by RESTSITE (Nei & Li method) based on data resulting from restriction site analysis of IGR-1 and IGR-2 rDNA regions of *Lentinula* spp.

	L.e	L.n	L.l	L.b (a)	L.b (b)
L. edodes (L.e)		0.035365	0.054977	0.044371	0.043292
L. novaezelandiae (L.n)	0.104861		0.030168	0.084382	0.084853
L. lateritia (L.l)	0.173937	0.075207		0.085756	0.074593
L. boryana (L.b)(a)	0.148852	0.330286	0.297627		0.019083
L. boryana (L.b)(b)	0.148852	0.330286	0.297627	0.036939	
	L.e	L n	L.l	L.b (a)	L.b (b)

Appendix I Data matrices of scored restriction sites. Restriction site data are across in columns. The OTU data are listed by row.

	On LSU			In IGR-1											
Taxonomic unit	Dpn II	Alu I	Hinf I	Hinf I	Hae III	Hae III	Dpn II	Dpn II	Alu I	Hinf I	Hpa II	Hind III	Pst I	Hae III	Hha I
L. edodes	1	0	0	0	0	0	0	0	1	1	1	1	0	1	1
L. novaezelandiae	1	0	0	0	0	0	0	0	1	1	1	1	0	1	1
L. lateritia	1	0	0	1	0	0	1	0	0	0	0	0	1	1	1
L. boryana (a)	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0
L. boryana (b)	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0

	In IGR-2											On SSU	
Taxonomic unit	Dpn II	Hae III	Pst I	Hha I	Hha I	Hae III	Hinf I	Hha I	Hpa II	Taq I	Rsa I	Alu I	Hae I
L. edodes	1	1	0	1	0	0	0	0	0	1	0	0	1
L. novaezelandiae	0	0	1	0	1	1	1	0	1	1	0	1	0
L. lateritia	0	0	1	0	1	1	1	0	1	1	0	1	0
L. boryana (a)	0	1	0	1	0	0	0	1	0	0	1	0	1
L. boryana (b)	0	1	0	1	0	0	0	1	0	0	1	0	1