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A Short Ribosomal DNA Sequence from a 115-Year-Old *Lentinula guarapiensis* Herbarium Specimen

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ABSTRACT: *Lentinula guarapiensis* is one of the five species of edible mushrooms (including shiitake) comprising the genus *Lentinula*. Currently, no live cultures or recent collections of *L. guarapiensis* are available. Only a few specimens of a single collection, dated 1879, are available at the Kew, Paris, and La Plata (Argentina) herbaria. The polymerase chain reaction was used to amplify various regions of the ribosomal DNA (rDNA) repeat from one of the Paris specimens. Only relatively short (<500bp) segments of the rDNA repeat were successfully amplified. A short segment (327 bp) of the large-subunit ribosomal RNA gene was amplified, sequenced and compared to the published *Saccharomyces cerevisiae* sequence, as well as to partial sequences of the same segment from related species and genera. Our results indicate that direct sequencing of PCR products from specimens as old as and in such poor condition as that of *L. guarapiensis* may provide unsatisfactory results. Sequencing of several cloned PCR products may be the most effective way of determining the true sequence in the presence of damage induced sequence variation.

1 INTRODUCTION

According to Pegler (1983), the economically important genus *Lentinula* Earle contains five recognized species including *Lentinula edodes* (Berk.) Pegler the commercial mushroom known in Japan and the USA as shiitake. Only four of these species are available as living cultures or as recent collections: *L. edodes*, *L. boryana* (Berk. & Mont.) Pegler, *L. lateritia* (Berk.) Pegler, and *L. novaezelandiae* (Stev.) Pegler. The fifth species, *L. guarapiensis* (Speg.) Pegler, remains unavailable except in the severely limiting form of a few mold-infested herbarium specimens dated 1879 (Pegler 1983).

Hibbett (1992) argued that to understand the phylogenetic relationships of *Lentinula*, as well as assess the monophyly of the genus, representatives of all species of *Lentinula* must be compared at a molecular level. According to Royse and Nicholson (1993), understanding of the evolutionary relationships of the species of *Lentinula* will remain limited provided *L. guarapiensis* is not included in future analyses. Efforts focusing on the evolutionary relationships, systematics, and breeding of *L. edodes* would certainly benefit from the exploitation of this and other available herbarium specimens however there are technical difficulties in including such specimens in studies involving DNA amplification by PCR and sequencing.

When herbarium specimens are included in studies involving the analysis of DNA the primary problem is degradation of DNA, making enzymatic amplification difficult. Oxidation and hydrolysis results in the modification of pyrimidines and sugars and creates baseless sites (Lindahl 1993, Paabo 1989). DNA extracted from dried tissues is usually of low molecular weight making the amplification of large fragments difficult. The condition of many specimens also means that the possibility of contamination by foreign DNA cannot be ruled out.

To investigate the possibility of including herbarium material such as that of *L. guarapiensis* into our current research projects we examined methods of amplifying and sequencing DNA from herbarium specimens.

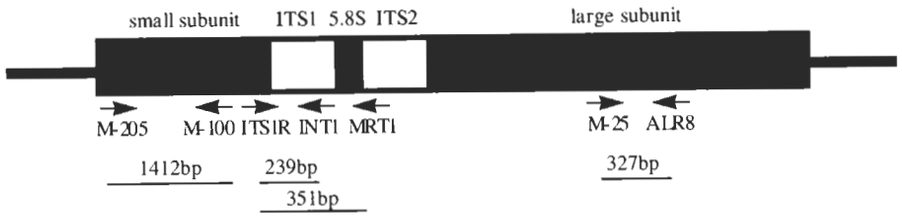
2 MATERIALS AND METHODS

2.1 *DNA extraction*

A small portion of the herbarium specimen was crushed in liquid nitrogen with a mortar and pestle to yield approximately 8 mg of fine powder. Strains of *Lentinula boryana* WC794, *L. edodes* R-60, *L. lateritia* WC798, *L. novaezelandiae* WC804, *Collybia dryophila* (Bull.:Fr.) Kummer WC37, and *Pleurotus ostreatus* (Jacqu.:Fr.) Kummer WC597, (from the Pennsylvania State University Mushroom Culture Collection) were grown in potato dextrose broth. The cultures were dried and ground to a powder in liquid nitrogen. DNA was extracted according to procedures described by Bruns *et al.* (1990), using a modified lysis buffer consisting of 50mM Tris-HCl pH 7.2; 50mM EDTA, 3% sodium dodecyl sulfate, and 1% 2-mercaptoethanol. After extraction and precipitation, extracts were dissolved in 50 µl of TE buffer.

2.2 *Polymerase chain reaction*

Amplification of portions of the ribosomal DNA repeat was performed for all isolates utilizing primer pairs listed in Fig. 1 to produce amplicons of varying sizes. DNA extracts were diluted 10 to 100 fold prior to their use in PCR. Reactions consisted of 5 µl diluted DNA extract, 1.65 mM $MgCl_2$, 1 µM each primer, 200 µM each dNTP, 25 µg BSA V (heat shock fractionated, Sigma Chemical Co., St. Louis, MO), *Tfl* buffer, and 1.25 U *Tfl* polymerase (Epicenter Technologies, Madison, WI) in a total reaction volume of 50 µl. Thermal cycling parameters varied, depending on the primer pair used but generally consisted of 40 cycles at 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 2 minutes with a final extension of 7 minutes and were performed in a Bio Therm (Fairfax, VA) thermal cycler. Monitoring for false positives was accomplished with an array of negative controls containing all requisite ingredients for PCR except one. Most frequently, no-DNA negative controls were used, though negative controls containing DNA with only one of each primer were also employed. A blind control (which had undergone all extraction procedures but contained no tissue or source DNA) served as the negative control during amplification attempts. The other specimens studied provided positive control DNA for all PCRs.



Primer	Primer sequence
M-25	GATGGGGAAGCTCCGTTT
ALR8‡	CACCTTGGAGACCTGCTGCG
M-205	CAGTGAAACTGCGAATGGC
M-100	CGGCCAAGGTTAGACTCGC
ITS1R*	GTTTCCGTAGTGAACCTGCGG
MRT1	CCAGAGGGCGCAAGGTGCG
INT1**	TCATTCTATAACATACTTCAA

‡Modified from primer sequences kindly provided by R. Vilgalys (pers comm).
*Modified from primer sequence of White *et al.* (1990).

Figure 1. Primer pairs used for amplification of DNA from *L. guarapiensis*.

2.3 *Sequencing*

PCR products were prepared for sequencing by gel electrophoresis in low melting agarose, phenol extraction and ethanol precipitation. Sequencing of the amplified PCR products were accomplished using a Sequenase Version 2.0 DNA Sequencing Kit.

3 RESULTS

Fragments less than 500 bp were successfully amplified from all specimens investigated. While the amplification of products as large as 1400 bp were possible from culture extracted DNA, attempts to amplify these products from herbarium material were unsuccessful. The addition of BSA type V was a vital ingredient for efficient amplification from the herbarium specimens.

Problems were encountered when certain PCR products were sequenced. Attempts to directly sequence PCR products from some of the herbarium material were only marginally successful and resulted in many questionable bases although the same PCR products derived from culture grown specimens provided adequate results. The best results obtained for the *L. guarapiensis* specimen were with the 327 bp LSU rDNA amplification product (Fig. 2). Partial sequences were determined for the remaining *Lentinula* species and for *C. dryophila* and *P. ostreatus*. The full *L. guarapiensis* sequence shows 74.9% similarity to the corresponding published *S. cerevisiae* sequence (Georgiev *et al.* 1981).

4 DISCUSSION

A molecular archaeological approach to extract and sequence DNA from preserved specimens of herbarium, museum, fossiliferous amber, or other origin, though uniquely useful, is not without specific limitations or problems (Handt *et al.* 1994, Pääbo *et al.* 1989). Such problems include characterization and quantitation of the DNA, contamination from non-target DNA, inhibition of PCR, and reproducibility of results.

Microscopic inspection of the herbarium specimen revealed fungal contamination tentatively identified as *Aspergillus* sp. Since it was impossible to physically remove the mold, it is assumed to be co-purified in the DNA extraction. A comparison of the primer sequences of several fungal rDNA sequences, including that of *Aspergillus* spp. (Borsuk *et al.* 1994) suggests that at least some primer pairs may be used to selectively amplify the desired DNA.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GATGGGGAAG  CTCCGTTTCA  AAGTGCAGTA  TTTTTAGAGC  TGCCTG-ATCA  L.g.
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GAAA-GGAATC CGGTTAAAAT  TCCGGAAC-CA GAATGTGGAT- CTTTAACGGC  L.g.
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Figure 2. Ribosomal DNA sequence of a 115-year-old herbarium specimen of *Lentinula guarapiensis* corresponding to positions 1536 to 1861 in the 25S rRNA gene of *Saccharomyces cerevisiae* (Georgiev *et al.* 1981) The complete *L. guarapiensis* (L.g.) sequence with *L. edodes* (L.e.), *L. lateritia* (L.l), *L. novaezelandiae* (L.n.), *L. boryana* (L.b.), *C. dryophila* (C.d.), *P. ostreatus* (P.o.), and the published *S. cerevisiae* (S.c.) sequences. Underlined portions indicate primer sequences. K = T or G; M = A or C; R = A or G. Deletions or insertions are indicated by a (-). Identical bases in alligned sequences are indicated by a (•). Questionable bases are indicated by a (?).

The use of bovine serum albumin along with increased amounts of polymerase has been reported several times as being helpful for amplification from ancient tissue extracts (Pääbo *et al.* 1988, Stone and Stoneking 1993). We have found that its inclusion in reactions consistently provides better yield when used in conjunction with *Tfl* and *Taq* (Promega Corp. Madison, WI) polymerases.

The results presented here suggest that while amplification products may be obtained from material as old as, and in such poor condition as *L. guarapiensis*, direct sequencing of the products may be difficult. Difficulties may arise from the presence of contaminating DNA and damage to the genomic template because of the age of the specimen. Under the same conditions, PCR products amplified from culture grown sources proved to be good sequencing templates. Several attempts to sequence the more informative ITS regions of the rDNA repeat using various template preparation and sequencing protocols gave unsatisfactory results. Damage induced template variation and/or contamination from foreign sequences may be great enough to hinder the determination of the true sequence. Cloning and subsequent sequencing of several clones may be necessary to determine a complete sequence with confidence. Efforts are under way in this lab to accomplish this task.

Successful direct sequencing of PCR products from herbarium specimens has been reported by several authors in spite of damage induced sequence variation. In a comprehensive study, Bruns *et al.* (1990) sequenced 415 bp mitochondrial DNA products amplified from basidiomycete herbarium specimens to facilitate incorporation of such tropical or rare fungi in phylogenetic analyses of the Boletales. The oldest DNA amplified successfully was from a specimen of *Boletus edulis* Bull.:Fr. from 1939, while the oldest DNA sequenced was from *Truncocolumella citrina* Zeller, a specimen dating from 1948.

We have shown that DNA can be successfully extracted from a 115-year-old basidiomycete herbarium specimen, specifically that of *L. guarapiensis*, and that subsequent enzymatic amplification and sequencing of the DNA is possible. It should be known, however, that while *L. guarapiensis* herbarium specimens may facilitate and contribute to a greater understanding of the relationships within the genus *Lentinula*, more recent collections or live cultures of the species would greatly broaden the scope of possible research with the genus.

ACKNOWLEDGMENTS

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