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AMPLIFICATION AND DIRECT SEQUENCING OF FUNGAL RIBOSOMAL RNA GENES FOR PHYLOGENETICS

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Comparative studies of the nucleotide sequences of ribosomal RNA (rRNA) genes provide a means for analyzing phylogenetic relationships over a wide range of taxonomic levels (Woese and Olsen 1986; Zimmer *et al.* 1988; Medlin *et al.* 1988; Jorgensen and Cluster 1989). The nuclear small-subunit rDNA sequences (16S-like) evolve relatively slowly and are useful for studying distantly related organisms, whereas the mitochondrial rRNA genes evolve more rapidly and can be useful at the ordinal or family level. The internal transcribed spacer region and intergenic spacer of the nuclear rRNA repeat units evolve fastest and may vary among species within a genus or among populations.

Numerous sequences of rRNA genes have been obtained primarily by isolating and sequencing individual cloned genes (Medlin *et al.* 1988). Direct rRNA sequencing (Lane *et al.* 1985) has also been used to rapidly obtain sequence data. However, this method requires relatively large amounts of RNA and is prone to errors since only one strand is sequenced.

The polymerase chain reaction (PCR) and direct sequencing offer several advantages over cloning and direct rRNA sequencing: (1) the method utilizes relatively crude preparations of total DNA such as

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those from minipreps (see Chapter 34); (2) only small amounts of DNA are required, about 0.1 to 10 ng per amplification; (3) both strands of the gene can be sequenced, which reduces errors; and (4) the method is compatible with automated DNA sequencing instruments that utilize fluorescently labeled sequencing primers or dideoxynucleotide triphosphates.

Figure 1 shows the location of the primers, and Table 1 describes the sequences of primers that we have designed for amplifying various segments of the nuclear and mitochondrial rDNA genes of fungi. The specificity of the primers for amplification of the target genes is excellent using the cycling parameters indicated (Fig. 2). The range of organisms that can be studied (as described below) can be extended or restricted to some extent by altering the annealing temperature.

Primers NS1 through NS8 were based on conserved nucleotide sequences from the 18S rRNA genes from *Saccharomyces cerevisiae*, *Dictyostelium discoideum*, and *Stylonicha pustulata* (Dams *et al.* 1988). Primers NS1 and NS2 have amplified rDNA from a wide variety of fungi, protists, and red and green algae. NS3 through NS6 have amplified all fungal DNAs tested. NS7 and NS8 also amplify some plant and vertebrate rDNAs.

The primers NS1 through NS8 will generally allow the sequenc-

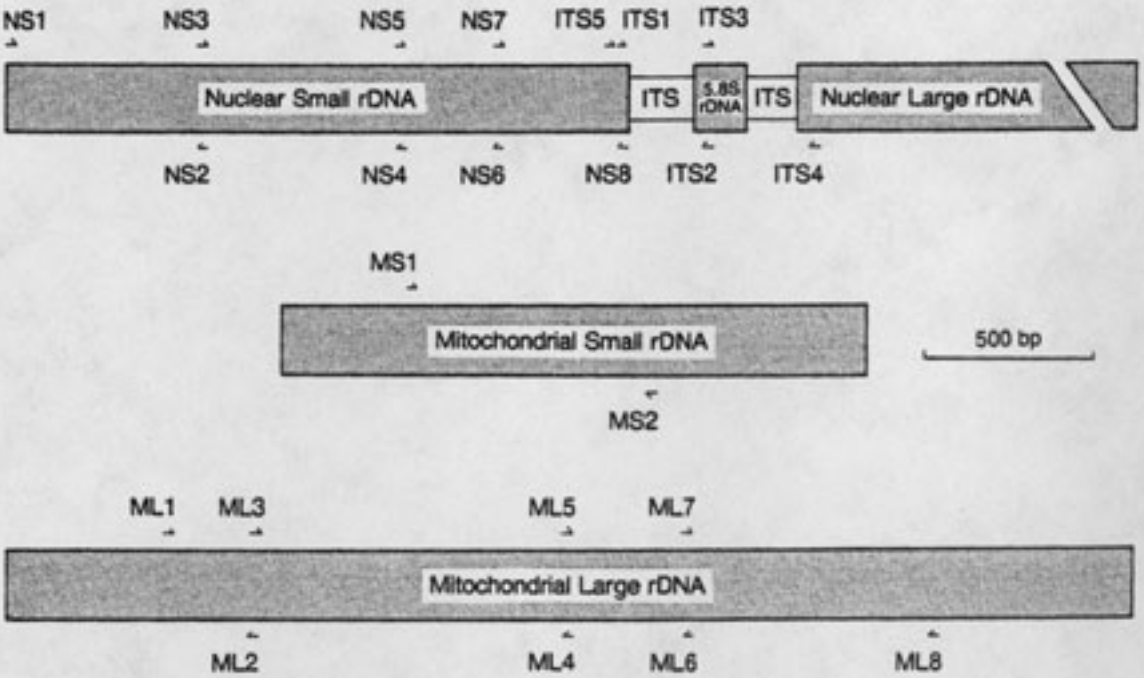

Figure 1 Locations on nuclear and mitochondrial rDNAs of PCR primers given in Table 1. The arrowheads represent the 3′ end of each primer. The nuclear large rDNA is truncated in this figure.

Table 1

Primers for Amplification of Fungal Ribosomal RNA Genes

rRNA Gene	Primer[a]	Product Size (bp)[b]	T_m (°C)[c]
Nuclear, small			
NS1	GTAGTCATATGCTTGTCTC	555	56
NS2	GGCTGCTGGCACCAGACTTGC		68
NS3	GCAAGTCTGGTGCCAGCAGCC	597	68
NS4	CTTCCGTCAATTCCTTTAAG		56
NS5	AACTTAAAGGAATTGACGGAAG	310	57
NS6	GCATCACAGACCTGTTATTGCCTC		65
NS7	GAGGCAATAACAGGTCTGTGATGC	377	65
NS8	TCCGCAGGTTCACCTACGGA		65
Nuclear, ITS			
ITS1	TCCGTAGGTGAACCTGCGG	290	65
ITS5	GGAAGTAAAAGTCGTAACAAGG	315	63
ITS2	GCTGCGTTCTTCATCGATGC	290	62
ITS3	GCATCGATGAAGAACGCAGC	330	62
ITS4	TCCTCCGCTTATTGATATGC		58
Mitochondrial, small			
MS1	CAGCAGTCAAGAATATTAGTCAATG	716	65
MS2	GCGGATTATCGAATTAAATAAC		63
Mitochondrial, large			
ML1	GTACTTTTGCATAATGGGTCAGC	253	68
ML2	TATGTTTCGTAGAAAACCAGC		63
ML3	GCTGGTTTTCTACGAAACATATTTAAG	934	67
ML4	GAGGATAATTTGCCGAGTTCC		68
ML5	CTCGGCAAATTATCCTCATAAG	359	66
ML6	CAGTAGAAGCTGCATAGGGTC		65
ML7	GACCCTATGCAGCTTCTACTG	735	63
ML8	TTATCCCTAGCGTAACTTTTATC		57

[a] All odd-numbered primers are 5′ primers; even numbers indicate 3′ primers. Sequences are written 5′-3′.

[b] Product sizes are approximate based on the rRNA genes of *S. cerevisiae*; the size of the region amplified is the product size minus the primers. Primers NS3 and NS4 amplify mitochondrial and bacterial rDNA from some organisms; expected product sizes are approximately 365 bp and 425 bp, respectively (see Fig. 2, lane 5).

[c] T_m's were calculated by the method of Meinkoth and Wahl, 1984.

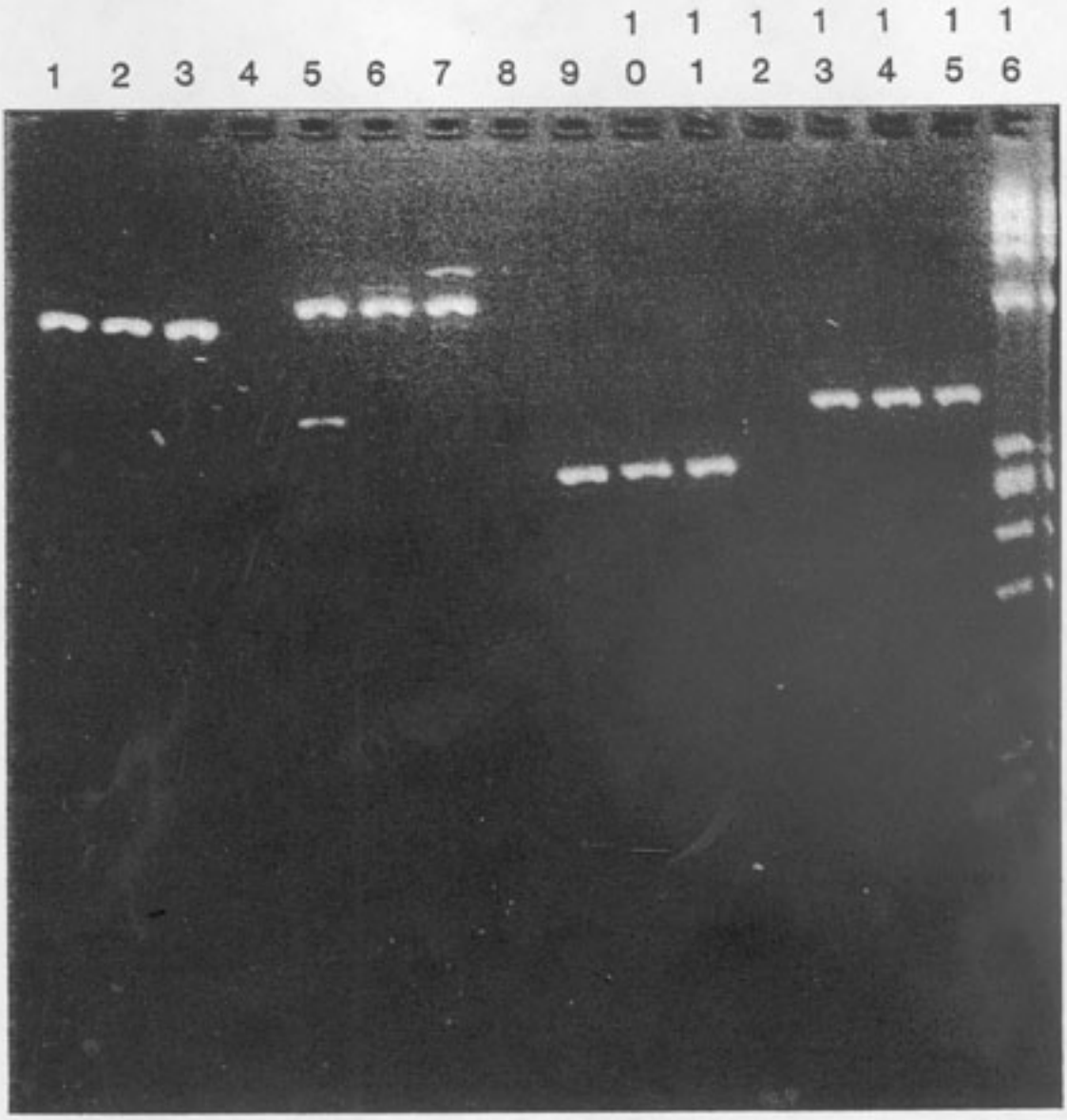

Figure 2 PCR amplification of adjacent segments of the nuclear small-subunit rRNA gene from ascomycetes: (lanes 1–4, NS1 and NS2; lanes 5–8, NS3 and NS4; lanes 9–12, NS5 and NS6; lanes 13–15, NS7 and NS8) from *Talaromyces flavus* (lanes 1, 5, 9, 13), *T. leycettanus* (lanes 2, 6, 10, 14), and *Byssochlamys nivea* (lanes 3, 7, 11, 15). Negative controls (no DNA) in lanes 4, 8, 12. Molecular weight standards: ΦX174RF *Hae*III digest in lane 16.

ing of most of the nuclear small rRNA gene excluding the areas of the primer sequences. NS2 and NS3, NS4 and NS5, and NS6 and NS7 are complementary, and a better design would have offset them to permit sequencing of the primer regions. NS1 and NS8 will amplify nearly the entire 18S gene in all fungi tested, but we have not yet devised optimal conditions for obtaining a good yield of single-strand template from an amplified product of this size.

The regions used for the mitochondrial primers were selected by comparison of sequences from the ascomycetes *S. cerevisiae* and

Aspergillus nidulans with the unpublished sequence of the basidiomycete *Suillus sinuspaulianus* (T. Bruns, personal communication). Potential primer sequences were then compared to nuclear 28S and 18S sequences to ensure that they were specific for mitochondrial genes. Where the three mitochondrial sequences differ within the primer regions, the *S. sinuspaulianus* sequences were chosen. Thus some of the primers are better matched to *Suillus* and related basidiomycetes than to ascomycetes. However, the primers were designed such that mismatches are few and central to the regions of homology. All primers have been tested with the ascomycete *Neurospora crassa* and found to amplify the correct fragment. Primers ML1, ML2, ML4, MS1, and MS2 will also amplify specific fragments from the oomycete *Phytophthora cinnamomi*. The region

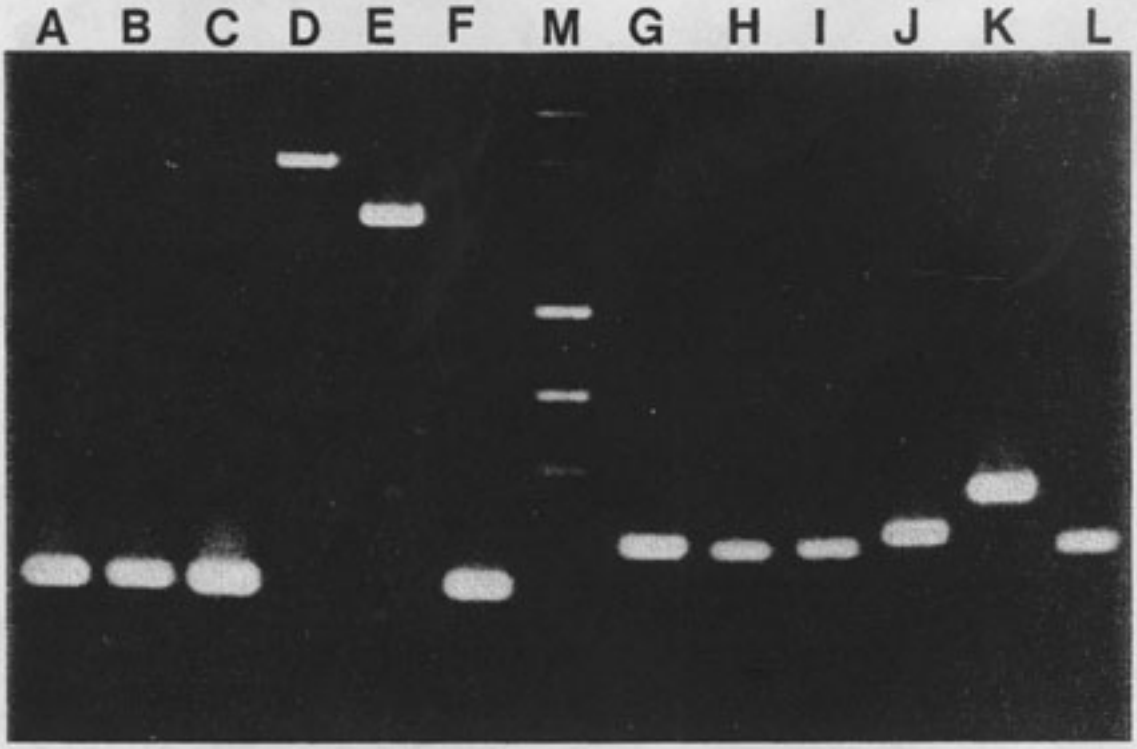

Figure 3 Length differences in the mitochondrial LrRNA gene (A–F) and nuclear ITS region (G–L). A portion of the mitochondrial LrRNA gene was amplified from six species of *Suillus* using the ML7 primer and one that is 3' to ML8 (MLID, CGGAGGATAGGATAAGTCG) and specific to the *Boletaceae* and related basidiomycetes. Four of the species yielded a fragment of approximately 610 bp, but two (D,E), which are known from previous mapping studies (Bruns and Palmer, in press) to contain an intron in the region, yielded fragments of approximately 2000 and 1700 bp. The ITS1 and ITS4 primers were used to amplify the ITS region from four species of *Suillus* and two species from the closely related genus *Rhizopogon*. Sizes of the region were found to be approximately 710 bp in the four *Suillus* species (G–I, L), but 740 and 850 bp in the two *Rhizopogon* species (J, K). The size markers (M, middle) are Phage λ *Hin*dIII and ΦX *Hae*III fragments of 2322, 2027, 1360, 1078, 878, 606, and 310 bp.

amplified by ML6 and ML5 contains an intron in some species of basidiomycetes (Fig. 3, Bruns and Palmer 1989), and in species in which this intron is large, the fragment is not amplified efficiently. Length mutations are common in these mitochondrial genes, and the length of the regions amplified may vary considerably from those listed in Table 1.

The ITS primers make use of conserved regions of the 18S, 5.8S, and 28S rRNA genes to amplify the noncoding regions between them. ITS1 is the complement of NS8 and was designed as described above. Comparisons among 5.8S rRNA sequences of *N. crassa*, *Schizosaccharomyces pombe*, *S. cerevisiae*, Broad bean (*Vicia faba*), and mouse (*Mus musculus*) were used to select ITS2 and ITS3. The 28S sequences of *S. pombe*, *S. cerevisiae*, and rice (*Oryza sativa*) were compared to select ITS4. The conserved region chosen overlaps the 28A primer of Zimmer *et al.* (1988), which has been previously used for direct sequencing of the 28S rRNA. ITS5 is identical in sequence to the *N. crassa* sequence in the 18S rDNA region (Kelly and Cox 1982) that is 25 base pairs 5′ to ITS1.

Protocol

Reagents

10× amplification buffer
- 15 m*M* $MgCl_2$
- 500 m*M* KCl
- 100 m*M* Tris HCl (pH 8.3) at 23°C
- 0.1% gelatin

10×dNTP stock mixture
- 2 m*M* each of dATP, dGTP, dTTP, and dCTP

Procedure

1. Using positive displacement pipets for steps 1–4, prepare a working reaction mixture sufficient for 10 amplifications, which consists of:
   - 275 μl sterile-distilled water
   - 100 μl 10× amplification buffer

100 μl	dNTP stock mixture (2 m*M* each dNTP)
10 μl	excess primer (50 μ*M* stock)
10 μl	limiting primer (1 μ*M* stock)
5 μl	*Taq* polymerase (5 units/μl)

2. Add 50 μl of the working mixture to each tube.
3. Dilute the DNA samples in water or TE (10 m*M* Tris-HCl [pH 8.0], 0.1 m*M* EDTA) to 0.1 to 10 ng per 50 μl.
4. Add 50 μl of the diluted sample DNA to each tube followed by two drops of mineral oil.
5. Spin briefly in a microcentrifuge.
6. Cycling parameters

Initial denaturation	2 to 3 minutes at 95°C
Annealing	30 seconds at 50° to 60°C
Extension	0.5 to 2 minutes (depending on product size) at 72°C
Denaturation	30 seconds at 95°C
Final extension	10 minutes at 72°C

7. Number of cycles
   25 for double-stranded product, using 50 pmol of each primer.
   35 for single-stranded product, using a primer ratio of 50 : 1 or 50 : 2.5 pmoles.
8. When the amplification is completed, briefly centrifuge the tubes and take 5-μl samples for analysis by minigels. Before sequencing the single-stranded PCR product, remove the oil by extraction in chloroform and remove the unincorporated nucleotides while concentrating the DNA by Centricon-30 centrifugal filtration (W. R. Grace, Danvers, Massachusetts).
9. We routinely use 7 μl of the Centricon-30 retentate for sequencing reactions. If this DNA proves too dilute, concentrate the retentate in a Speed Vac. Use 1 pmole of the limiting primer (or an internal primer) as the sequencing primer. Do not denature the double-stranded product prior to primer annealing, i.e., anneal at 65°C instead of 90 to 95°C.
10. Primers NS1 through NS8 and ITS1 through ITS4 have also been used with lower dNTP concentrations (32 μ*M* each dNTP, instead of 200 μ*M*, in the reaction).
11. These conditions have not been optimized for enzyme and magnesium ion concentrations.

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