

POLYMERASE CHAIN REACTION (PCR) PRIMERS FOR AMPLIFYING AND SEQUENCING NUCLEAR 18S rDNA FROM LICHENIZED FUNGI

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In our studies of ascomycete evolution we have sought to include representatives of the largest group of ascomycetes, those 13,500 described species of lichen symbionts. As is well known, a lichen is a symbiosis between up to three different organisms: the fungal mycobiont with a green algal or cyanobacterial photobiont and sometimes both an algal and a cyanobacterial symbiont. As a further complication, the purified DNA from a lichen may contain up to six different ribosomal DNA (rDNA) genomes: those of the fungal nuclei and mitochondria, the green algal nuclei, mitochondria, and chloroplasts, and that of a cyanobacterium. For sequence analysis of the fungal nuclear small subunit (18S) rDNA, the complicating sequence is the green algal 18S rDNA. With the polymerase chain reaction it is possible to sequence only the DNA of the fungal component.

To sequence the DNA of the fungal mycobiont from a lichen association we designed primers for the polymerase chain reaction (PCR) which amplify the fungal 18S rDNA but not the green algal 18S rDNA. These primers were based on a comparison of 18S rDNA from *Neurospora crassa* Shear & Dodge (GenBank code name NEURRNAS), *Saccharomyces cerevisiae* Meyen ex Hansen (GenBank code name YSCRGEA), and the green alga (Chlorophyta) *Chlamydomonas reinhardtii* Dangeard (DNA sequence from Gunderson et al., 1987). The selected primer regions are nearly identical between the two fungi (having at most one nucleotide substitution), but the green algal sequence has in all cases except one at least one different nucleotide on the 3' end of the primer. Details of primer design are presented in Innis and Gelfand (1990) and Kwok et al. (1990).

The primers, their product sizes and T_m s [the melting temperature, midpoint of the temperature range where the primer and template DNA denature, as determined with the method of Meinkoth and Wahl (1984)] are shown in TABLE

I. NS17UCB through NS24UCB are designated NS for nuclear small subunit and UCB for the University of California at Berkeley. Primers with odd numbers prime the coding strand; primers with even numbers prime the noncoding strand. Product size in basepairs (bp) is based on the sequence of *Saccharomyces cerevisiae*.

FIGURE 1 shows the sequence alignment for each primer with DNA sequences from representative organisms of the Ascomycotina [*Neurospora crassa*, *Saccharomyces cerevisiae*, *Lecidea crustulata* (Acharius) Sprengel, and *Morchella esculenta* (Linnaeus) Persoon], Myxomycetes (*Dictyostelium discoideum* Raper, GenBank code name DDIRGE), and Chlorophyta (*Chlamydomonas reinhardtii*). The *Lecidea* and *Morchella* sequences are from the authors' work, sequences from the primer sites NS17UCB and NS24UCB are not known for these two organisms. A schematic view of the nonalgal primer sites compared to *Saccharomyces*' 18S rDNA sequence is given in FIG. 1.

Representatives of the green algal genus *Trebouxia* de Puymaly (the most common lichen symbiont) and the green algal species *Chlamydomonas reinhardtii* do not amplify with these nonalgal primers when used as described. FIGURE 2 shows the PCR products from DNA extracted from cultures of *L. crustulata* (ATCC 18297) and *C. reinhardtii* (UCB Microgarden 55-002). The primer pair NS1-NS8 amplified both fungal and algal DNA [PCR amplification as in White et al. (1990); 50 C annealing temperature, 30 cycles]; the nonalgal primer pair NS17UCB-NS24UCB amplified the fungal, but not the algal DNA (52 C annealing temperature, 30 cycles). The *Lecidea* PCR product is slightly longer because of an insertion in this region.

These primers work well for amplifying and sequencing lichenized fungal nuclear DNA. We have symmetrically amplified double stranded DNA from nearly the entire small subunit (ca 1750 bp) with NS17UCB-NS24UCB and

5' → 3'	NS17UCB	NS18UCB	NS19UCB	NS20UCB
Neurospora	CATGCTAAGTTTAAGCAA	CTCATTCCAATTACAAGACC	CCGGAGAAGGAGCCTGAGAAAC	CGTCCCTATTAATCATTACG
Saccharomycesa.....g.....	t.....
Lecideag.....	t.....
Morchellag.....g.acg..g.....	t.....g.....t
Dictyosteliumg.....a.tnc...tg.....g.t	tac.....c.....t
Chlamydomonasc.....gg.....
NS21UCB	NS22UCB	NS23UCB	NS24UCB	
Neurospora	GAATAATAGAATAGGACG	AATTAAGCAGACAAATCACT	GACTCAACACGGGAAACTC	AAACCTTGTTACGACTTTTA
Saccharomycesg.....
Lecidea
Morchella
Dictyostelium	g...c.tc...ttc.....gg.....tc.c
Chlamydomonasc.cg.....tc.c.t...gg.actc.c

Primer location

Nucleotide number
(bp, based on *Saccharomyces*)

FIG. 1. Primer sequences aligned with the 18S rDNA of representatives of each genus.

TABLE I
THE NUCLEOTIDE SEQUENCES OF THE 18S rDNA PRIMERS NS17UCB-NS24UCB

5' to 3' sequence	Product size (bp)	T _m (C)
NS17UCB: CATGT CTAAG TTAA GCAA	463	55
NS18UCB: CTCAT TCAA TTACA AGACC		60
NS19UCB: CCGGA GAAGG AGCCT GAGAA AC	491	74
NS20UCB: CGTCC CTATT AATCA TTACG		61
NS21UCB: GAATA ATAGA ATAGG ACG	510	50
NS22UCB: AATTA AGCAG ACAA TCACT		57
NS23UCB: GACTC AACAC GGGGA AACTC	586	51
NS24UCB: AAACC TTGTT ACGAC TTTTA		58

then used this double stranded product for asymmetric amplifications. For single stranded amplifications we used these pairs of primers: NS17UCB-NS18UCB, NS19UCB-NS20UCB, NS21UCB-NS22UCB and NS23UCB-

NS24UCB. Less specific universal primers (NS1-NS8; White et al., 1990), may be substituted for one of the primers or used in tandem for a second amplification. We have also used primers NS17UCB-NS24UCB for direct sequencing of double stranded product (Kusukawa et al., 1990).

When used as described in White et al. (1990) the primers NS17UCB through NS24UCB amplify fungi from the subdivisions Ascomycotina, Basidiomycotina, and Oomycotina. However, these primers are by no means fungal specific. Several have been reported to amplify vascular plant DNA (Tom Bruns, pers. comm.) and at least one amplifies insect DNA (Mary Berbee, pers. comm.) at annealing temperatures as high as 53 C.

The polymerase chain reaction creates new opportunities for research on slow-growing, difficult-to-culture, or obligately symbiotic organisms, such as rusts, endophytes, and mycorrhizal, lichenicolous or endolithic fungi, and the PCR has already been used on rDNA of a lichen chimera (Armaleo and Clerc, 1991). Fungal DNA sequences show promise as systematic characters to elucidate questions of evolution and phylogeny raised by their morphology (Brunns et al., 1991). As more fungi are sequenced it will be possible to design primers with greater specificity.

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Key Words: fungal DNA, lichen DNA, 18S rDNA, PCR primers

LITERATURE CITED

Armaleo, D., and P. Clerc. 1991. Lichen chimeras: DNA analysis suggests that one fungus forms two morphotypes. *Exp. Mycol.* 15: 1-10.

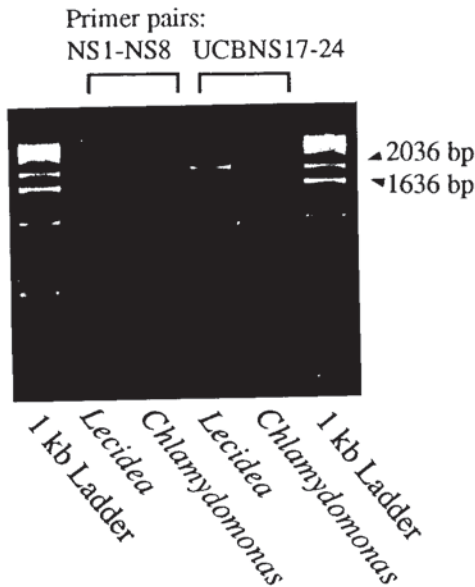


FIG. 2. Results of PCR amplifications of the 18S rDNA region comparing a universal primer pair with a nonalgal primer pair. Electrophoresis of PCR products is shown in a 3% agarose gel, lanes 1 and 6 each contain a BRL 1 kb ladder. Lanes 2 and 3 contain the PCR products from universal primer pair NS1-NS8 showing that this primer pair amplifies fungal and algal DNA; lane 2 is amplified *Lecidea* DNA; lane 3 is amplified *Chlamydomonas* DNA. Lanes 4 and 5 contain the PCR products from the nonalgal primer pair NS17UCB-NS24UCB. The presence of a band in lane 4 demonstrates that this primer pair amplifies fungal DNA; the absence of a band in lane 5 shows that this primer pair does not amplify algal DNA under defined conditions. Lane 4 is from *Lecidea* DNA; lane 5 is from *Chlamydomonas* DNA.

- Bruns, T., J. W. Taylor, and T. J. White. 1991. Fungal molecular systematics. *Annual Rev. Ecol. Syst.* **22**: 525-564.
- Gunderson, J. H., H. Elwood, A. Ingold, K. Kindle, and M. L. Sogin. 1987. Phylogenetic relationships between Chlorophytes, Chrysophytes and Oomycetes. *Proc. Natl. Acad. USA* **84**: 5823-5827.
- Innis, M. A., and D. H. Gelfand. 1990. Optimization of PCRs. Pp. 3-12. In: *PCR protocols: a guide to methods and applications*. Eds., M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, New York.
- Kusukawa, N., T. Uemori, K. Asada, and I. Kato. 1990. Rapid, reliable protocol for direct sequencing of material amplified by the PCR. *Biotechniques* **9**: 66-72.
- Kwok, S., D. E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J. J. Sninsky. 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. *Nucl. Acids Res.* **18**: 999-1005.
- Meinkoth, J., and G. Wahl. 1984. Hybridization of nucleic acids immobilized on solid supports. *Anal. Biochem.* **138**: 267-284.
- White, T. J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322. In: *PCR protocols: a guide to methods and applications*. Eds., M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, New York.
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