Mycologia, 91(3), 1999, pp. 553–556.
© 1999 by The Mycological Society of America, Lawrence, KS 66044-8897

A method for designing primer sets for speciation studies in filamentous ascomycetes

Ignazio Carbone[1]
Linda M. Kohn
Department of Botany, University of Toronto at Mississauga, 3359 Mississauga Road North, Mississauga, Ontario, Canada L5L 1C6

Abstract: A simple method is described for designing primer sets that can amplify specific protein-encoding sequences in a wide variety of filamentous ascomycetes. Using this technique, we successfully designed primers that amplified the intergenic spacer region of the nuclear ribosomal DNA repeat, portions of the translation elongation factor 1 alpha, calmodulin, and chitin synthase 1 genes, and two other genes encoding actin and ras protein. All amplicons were sequenced and determined to amplify the target gene. Regions were successfully amplified in *Sclerotinia sclerotiorum* and other sclerotiniaceous species, *Neurospora crassa, Trichophyton rubrum, Aspergillus nidulans, Podospora anserina, Fusarium solani,* and *Ophiostoma novo-ulmi*. These regions are a potentially rich source of characters for population and speciation studies in filamentous ascomycetes. Each primer set amplified a DNA product of predicted size from *N. crassa*.

Key Words: *Aspergillus nidulans,* intergenic spacer, *Neurospora crassa, Sclerotinia sclerotiorum, Trichophyton rubrum*

Both gene genealogies and species trees provide a historical framework for studying population and species-level processes. Study of speciation processes at the species/population interface requires multiple, physically unlinked, genomic regions with both intra- and interspecific resolution (Templeton 1994). The suite of nuclear ribosomal DNA sequences accessioned in databases is not sufficient, especially at the intraspecific level. The increasing availability of protein-encoding sequences in GenBank and other genetic databases makes it possible to design primer sets that can amplify many specific genomic regions across many different fungal species. In this paper we describe a simple primer design method specifically for filamentous ascomycetes and associated mitosporic taxa, a group that includes approximately 45 000 species of parasites, pathogens, symbionts, and saprophytes (Hawksworth et al 1995) and a number of model systems, such as *Aspergillus nidulans, Neurospora crassa* and *Podospora anserina*.

Our method implements the database search tools developed by the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/). The steps involved in primer selection and design are outlined below. (i) Perform a search of GenBank for all *Neurospora crassa* protein-encoding sequences, with defined intron/exon boundaries. To do this, use the Entrez nucleotide sequence search site (http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html) and search all fields for "*Neurospora crassa* gene intron". Retrieve all inton-containing genes in *N. crassa*. The size and position of introns within a gene can be viewed by selecting the graphical view link. Within the graphical view window, select the option to view the sequence in FASTA format. Select and copy the FASTA sequence and proceed to the BLAST search page (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast?Jform=0). Paste the FASTA sequence in the sequence display buffer and submit the query. This will search all non-redundant nucleotide sequences in GenBank for all matches to the query using version 2.0.6 of BLASTN (Altschul et al 1997), a program at NCBI that produces a graphical alignment overview, with gaps, of the database sequences aligned to the query sequence (FIG. 1). (ii) Inspect the graphical alignment output for the presence of conserved segments among all sequences. Select primers that amplify regions of approximately 300–500 bp in length. If possible, target regions that span one or more introns and design primers from homologous exon sequences flanking homologous intron insertion sites (FIG. 1). This will ensure that primer sets amplify DNA products of predicted size. (iii) Perform a BLAST database search using the primer sequence as the query. Retain only those primers that yield the greatest number of matching hits to their target sequence and have no more than two mismatches with their target sequences. This is important to ensure that primer sets amplify DNA products with high specificity (TABLE I). (iv) Amplify target loci (TABLE II). The protocol used in our study was as follows: PCR mixtures (20 μL) contained 0.5 μM of each primer, 200 μM dNTPs, 1× PCR Buffer II with 1% DMSO and 2 mM $MgCl_2$ (Perkin-Elmer, Norwalk, Connecticut), 0.5 U of AmpliTaq® DNA polymerase (Perkin-Elmer), and 10 μL of a 0.2–0.5 ng/μL genomic DNA solution extracted following the small-scale protocol of Kohn et al (1991). DNA amplifications were carried out in a 9600 Thermocycler (Perkin-Elmer) programmed for an

Accepted for publication December 11, 1998.
[1] Email: icarbone@credit.erin.utoronto.ca

TABLE I. Identification of target nuclear loci, primer sets, and resolution level within *Sclerotinia sclerotiorum* or among *S. sclerotiorum* and related species in the Sclerotiniaceae

Locus	Definition	Primer[a]	Primer DNA sequence (5′–3′)	Database hits	Optimal annealing temp (C)	Product size (bp)	Intron size (bp)	Resolution[c]
EF-1α	translation elongation factor 1 alpha	EF1-728F	CATCGAGAAGTTCGAGAAGG	101	58	350	250	intraspecific/ interspecific
		EF1-986R	TACTTGAAGGAACCCTTACC	100				
CAL	calmodulin	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC	98	55	500	350	intra/inter
		CAL-737R	CATCTTTCTGGCCATCATGG	36				
CHS-1	chitin synthase 1	CHS-79F	TGGGGCAAGGATGCTTGGAAGAAG	20	58	300	—	intra/inter
		CHS-354R	TGGAAGAACCATCTGTGAGAGTTG	30				
IGS[b]	intergenic spacer region	PTR-1a	GGCGCATGGATAAACTCTCC	15	58	300	300	intra/inter
		PTR-1b	GATTATCCATTTGCCCCCAC	18				
		IGS-12a	AGTCTGTGGATTAGTGGCCG	10	58	400	360	intra/inter
		NS1R	GAGACAAGCATATGACTAC	100				
ACT	actin	ACT-512F	ATGTGCAAGGCCGGTTTCGC	80	61	300	200	intra/inter
		ACT-783R	TACGAGTCCTTCTGGCCCAT	75				
RAS	ras protein	RAS-264F	GATGAATATGATCCTACGAT	32	53	350	70	intra/inter
		RAS-565R	AAATCACATTTGTTACCAAC	36				

[a] Primer numbers refer to the 3′-most nucleotide position in the query sequence. *N. crassa* was used as the query sequence for EF-1α (GenBank accession no. D45837), CAL (L02964) and ACT (U78026), *S. sclerotiorum* for the IGS (AF040080), *B. cinerea* for CHS-1 (X77937) and *Botryotinia fuckeliana* (de Bary) Whetzel for RAS (U79558).

[b] Primers were designed to amplify contiguous segments of the promoter region of nuclear ribosomal DNA (rDNA). PTR-1a, PTR-1b, and IGS-12a are based on sequences in *S. sclerotiorum* (AF040080–AF040085) and closely related species, not yet accessioned in GenBank (Carbone unpubl). PTR-1a is located within the highly conserved RNA polymerase I core promoter domain (Baldridge and Fallon 1992). NS1R is located within the nuclear small rDNA subunit, as described previously (White et al 1990).

[c] Level of resolution in *S. sclerotiorum*; intraspecific (=intra), DNA sequence polymorphisms detected among isolates identified as *S. sclerotiorum* acquired in extensive population samples (Carbone et al 1999); interspecific (=inter), DNA sequence polymorphisms detected among isolates determined to be *S. sclerotiorum*, other species of *Sclerotinia*, and other species in closely related genera in the Sclerotiniaceae (Carbone and Kohn 1993, Holst-Jensen et al 1997, 1998). Intra/inter designates presence of DNA sequence polymorphisms both among isolates of *S. sclerotiorum* and among *S. sclerotiorum* and other sclerotiniaceous species. Isolates are listed in Table II.

TABLE II. Results of DNA amplification of target loci

Species	Primer set						
	EF1-728F EF1-986R	CAL-228F CAL-737R	CHS-79F CHS-354R	PTR-1a PTR-1b	IGS-12a NS1R	ACT-512R ACT-783R	RAS-264F RAS-565R
Sclerotinia sclerotiorum[a] and closely related species	+	+	+	+	+	+	+
Neurospora crassa[b]	+	+	+	+	+	+	+
Trichophyton rubrum	+	+	+	+	+	+	−
Aspergillus nidulans	−	+	+	+	+	+	−
Podospora anserina	−	+	+	+	+	+	−
Fusarium solani	+	+	+	+	+	+	−
Ophiostoma novo-ulmi	+	+	+	+	+	+	+
Saccharomyces cerevisiae	−	+	−	+	+	−	+
Candida albicans	−	−	−	−	+	−	+
Agaricus bisporus	−	+	−	−	+	−	−
Armillaria gallica	−	+	−	+	+	−	−

[a] Accession nos. and source information (in parentheses) for *S. sclerotiorum* (Lib.) de Bary (LMK-211 and W1-A1-1, L. M. Kohn) and closely related species: *Sclerotinia* sp. (T2-3 and Q5-1-2, I. Carbone), *Sclerotinia* sp. (1358, T. Schumacher & A. Holst-Jensen, Dept. of Biology, Univ. of Oslo), *S. minor* Jagger (LMK-115, P. Adams, no. Ss-42, available from L. M. Kohn), *S. trifoliorum* Erikss. (LMK-36, ATCC no. 34327, American Type Culture Collection), *S. tetraspora* Holst-Jensen & Schumacher (642, T. Schumacher & A. Holst-Jensen), *S. glacialis* Graf & Schumacher (1128, F. Graf, Swiss Federal Institute for Forest, Snow and Landscape Research), *Sclerotium cepivorum* Berk. (LMK-1, no. 83-11, W. Gams, Centraalbureau voor Schimmelcultures, Baarn), *Dumontinia tuberosa* (Hedw.) Kohn (901, T. Schumacher), *Cristulariella moricola* (Hino) Redhead (LMK-161, L. M. Kohn), and *Botrytis cinerea* Pers. (LMK-18, B. Jarvis, no. 11, available from L. M. Kohn). Isolates of *S. sclerotiorum* were determined to be conspecific based on DNA fingerprints, morphological and epidemiological characters (Kohn et al 1991). Relatedness of isolates representing other taxa to those of *S. sclerotiorum* was based on data reviewed or reported in Carbone and Kohn (1993), Holst-Jensen et al (1997, 1998).

[b] Accession nos. and source information for other fungal species: *N. crassa* Shear & Dodge (74-OR23-1A, R. A. Collins, Dept. of Medical Genetics, Univ. of Toronto), *T. rubrum* (Castellani) Sabouraud (98F-1005, Y. Kohli, Ontario Ministry of Health), *A. nidulans* (Eidam) Winter (FGSC no. 222, Fungal Genetics Stock Center, Kansas City), *P. anserina* (Cesati) Niessl (JS162, J. A. Scott, Dept. of Botany, Univ. of Toronto), *F. solani* f.sp. *xanthoxyli* Sakurai & Matuo (NRRL 22163, K. O'Donnell, USDA, ARS, Microbial Properties Research Laboratory, Peoria, Ill), *Ophiostoma novo-ulmi* Brasier (MH75, M. Hubbes, Dept. of Forestry, Univ. of Toronto), *Saccharomyces cerevisiae* Hansen (Martina Celeri, Univ. of Indiana), *Candida albicans* (Robin) Berkhout (P1, S. Richardson, Hospital for Sick Children), *Agaricus bisporus* (Lange) Imbach (U-1, P. A. Horgen, Dept. of Botany, Univ. of Toronto), and *Armillaria gallica* Marxm. & Romagn. (no. 671, J. B. Anderson, Dept. of Botany, Univ. of Toronto).

initial denaturation at 95 C for 8 min, followed by 35 cycles of 95, 55, and 72 C for 15, 20, and 60 s, respectively, with a 5 min extension at 72 C on the final cycle. Amplifications for each primer set were also performed at an annealing temperature of 50, 58, and 61 C.

Each primer set amplified a DNA product of predicted size from *Neurospora crassa*. All amplicons were sequenced as described by Carbone et al (1999). The sequences were then compared, using BLAST, to the accessioned sequences for the target gene. All amplicons were determined to amplify the sequence of the target genes. All primer sets produced a single amplicon of approximately the same size from *Sclerotinia sclerotiorum* and closely related fungal species (TABLE I). The only exception was the CHS-1 region in *S. trifoliorum* which amplified a 550 bp product. Primer sets for the CHS-1 and ACT regions amplified discrete DNA products in all filamentous ascomycetes, but failed to amplify a product in the ascomycetous yeasts, *Candida albicans* and *Saccharomyces cerevisiae,* and in the filamentous basidiomycetes, *Agaricus bisporus* and *Armillaria gallica* (TABLE II). At an annealing temperature of 55 C, the CAL primer set amplified a single DNA product in all fungal species, except *C. albicans.* At an annealing temperature of 58 C, at least one of the two contiguous segments of the promoter region of the IGS could be amplified in all fungal strains (TABLE II). At an annealing temperature of 53 C, the RAS primer set failed to amplify in *Trichophyton rubrum, Aspergillus nidulans, Podospora anserina, Fusarium solani* and the basidiomycetes. Intraspecific DNA sequence variation in *Sclerotinia sclerotiorum,* verified by sequencing, was observed in the intergenic spacer region of nuclear rDNA and in gene fragments from five other nuclear loci: translation elongation factor 1 alpha, calmodulin (Carbone et al 1999), chitin synthase 1,

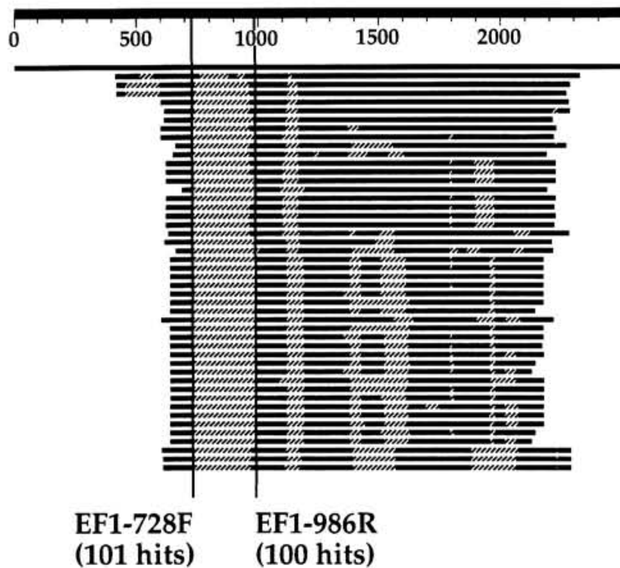

FIG. 1. BLASTN 2.0.6 graphical alignment for EF-1α with *N. crassa* as the query sequence (see scale at top). Intron positions are indicated with striped lines in the alignment. The first match or hit in the alignment is the actual query sequence. Only the first 47 sequences producing significant alignments out of a total of 630 hits are shown. The position of the 3′-most end of each primer sequence that yields the greatest number of database hits (TABLE I) is indicated with a vertical line in the alignment.

actin and ras (TABLE I). Both intraspecific and interspecific variation (TABLE I) was due primarily to base substitutions and some insertion/deletion motifs that could be scored like base substitutions by coding the entire motif as a single character. With the exception of the CHS-1 region, length polymorphisms were not detected.

This primer design strategy has two advantages over other methods. First, designing primers in exon sequences that are homologous among many filamentous ascomycetes increases the chance of amplifying the region, even among distantly related species. Second, in selecting genes one can choose regions containing introns of tractable size (ca. 250 bp) whose presence is conserved. Intraspecific variation is notably more abundant in such noncoding regions (O'Donnell et al 1998, Carbone et al 1999). Users of our method of primer design might wish to try using less conserved genes with smaller introns, with the risk of amplification failure, or less intron-rich genes, with the risk of insufficient sequence variation.

Acknowledgments.—This research was supported by a Postgraduate Scholarship to I. C. and a Research Grant to L. M. K. from the Natural Sciences and Engineering Research Council of Canada.

LITERATURE CITED

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402.

Baldridge GD, Fallon AM. 1992. Primary structure of the ribosomal DNA intergenic spacer from the mosquito, *Aedes albopictus*. DNA Cell Biol 11:51–59.

Carbone I, Anderson JB, Kohn LM. 1999. Patterns of descent in clonal lineages and their multilocus fingerprints are resolved with combined gene genealogies. Evolution 53:11–21.

———, Kohn LM. 1993. Ribosomal DNA sequence divergence within internal transcribed spacer 1 of the Sclerotiniaceae. Mycologia 85:415–427.

Hawksworth DL, Kirk PM, Sutton BC, Pegler DN. 1995. Ainsworth & Bisby's dictionary of the fungi. 8th ed. Wallingford, Oxon, UK: CAB International. 616 p.

Holst-Jensen A, Kohn LM, Schumacher T. 1997. Nuclear rDNA phylogeny of the Sclerotiniaceae. Mycologia 89: 885–899.

———, Vaage M, Schumacher T. 1998. An approximation to the phylogeny of *Sclerotinia* and related genera. Nordic J Bot 18 (In press).

Kohn LM, Stasovski E, Carbone I, Royer J, Anderson JB. 1991. Mycelial incompatibility and molecular markers identify genetic variability in field populations of *Sclerotinia sclerotiorum*. Phytopathology 81:480–485.

O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC. 1998. Multiple evolutionary origins of the fungus causing panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proc Natl Acad Sci USA 95:2044–2049.

Templeton AR. 1994. The role of molecular genetics in speciation studies. In: Schierwater B, Streit B, Wagner GP, DeSalle R, eds. Molecular ecology and evolution: Approaches and applications. Basel, Switzerland: Birkhäuser Verlag. p 455–477.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: A guide to methods and applications. San Diego: Academic Press. p 315–322.