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Development of Primer Sets Designed for Use with the PCR To Amplify Conserved Genes from Filamentous Ascomycetes

N. LOUISE GLASS* AND GARY C. DONALDSON

Botany Department and Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada

Received 1 August 1994/Accepted 15 November 1994

We constructed nine sets of oligonucleotide primers on the basis of the results of DNA hybridization of cloned genes from *Neurospora crassa* and *Aspergillus nidulans* to the genomes of select filamentous ascomycetes and deuteromycetes (with filamentous ascomycete affiliations). Nine sets of primers were designed to amplify segments of DNA that span one or more introns in conserved genes. PCR DNA amplification with the nine primer sets with genomic DNA from ascomycetes, deuteromycetes, basidiomycetes, and plants revealed that five of the primer sets amplified a product only from DNA of the filamentous ascomycetes and deuteromycetes. The five primer sets were constructed from the *N. crassa* genes for histone 3, histone 4, β-tubulin, and the plasma membrane ATPase. With these five primer sets, polymorphisms were observed in both the size of and restriction enzyme sites in the amplified products from the filamentous ascomycetes. The primer sets described here may provide useful tools for phylogenetic studies and genome analyses in filamentous ascomycetes and deuteromycetes (with ascomycete affiliations), as well as for the rapid differentiation of fungal species by PCR.

Current taxonomic identification of filamentous fungi is based on micro- and macromorphological characteristics, such as cultural morphologies including colony and color characteristics on specific culture media, the size, shape, and development of sexual and asexual spores and spore-forming structures, and/or physiological characteristics such as the ability to utilize various compounds as nitrogen and carbon sources. The advent of molecular methods has supplemented traditional taxonomic methods with DNA-based tools with which to examine phylogenetics and systematics of fungi (2–4). Most phylogenetic analyses are focused on the nuclear ribosomal DNA (rDNA), which contains tandem repeats of three rRNA genes (28S, 18S, and 5.8S). DNA sequence comparisons of the highly conserved nuclear 18S rRNA gene have been used to infer evolutionary relationships between different groups of fungi as well as their relationships to organisms in other kingdoms (1, 3).

DNA-based molecular methods are used to differentiate genera, species, subspecies, races, and strains of fungi as well as to identify individuals or clones within fungal populations in ecological studies. These methods include using restriction fragment length polymorphisms (RFLP) of mitochondrial and nuclear DNA (10, 20) and pattern differentiation of repetitive elements (15, 25). The advent of the PCR (27) has expedited the molecular analysis of fungal genomes for both phylogenetic and population structure studies. DNA sequence comparisons of the mitochondrial small-subunit rDNA and the nuclear rDNA region containing the two internal transcribed spacer (ITS) regions (ITS1 and ITS2) and the 5.8S rRNA gene are useful in determining relationships between fungal genera and species (3, 23, 30). Randomly amplified polymorphic DNA (46) is used to construct RFLP maps that are useful in genetic analysis and also for population studies at the intraspecific level (11, 12, 19, 32).

In addition to the study of fungal taxonomy and population structure, molecular methods based on PCR have been developed to aid in the detection and identification of fungi. Many of these studies exploit sequence variability in the rDNA region. DNA sequence variations and RFLPs within the ITS region have been shown to be associated with a particular genus or species (17, 23, 28). On the basis of rDNA sequence information, DNA probes have been designed for detection of specific fungi by hybridization to the ITS PCR product (16, 22).

The purpose of this study was to develop tools for both the detection and the identification of filamentous ascomycetes and deuteromycetes (with ascomycete affiliations) that would have the sensitivity of PCR, would be reproducible, and would provide PCR products in addition to the rDNA for genome analysis. In addition, the tools would be readily transferable to diverse genera of filamentous ascomycetes. To reach these objectives, we developed primer sets to conserved genes in filamentous ascomycetes. As described in this paper, the development of these primer sets is based on the following observations: (i) a number of genes encoding metabolic and structural functions have been cloned and sequenced from filamentous ascomycetes, specifically, *Neurospora crassa* and *Aspergillus nidulans*; (ii) the genes for metabolic and structural proteins are a conserved class of genes; (iii) the protein-coding regions of genes are generally more highly conserved in related species than intron sequences; and (iv) introns in filamentous fungi tend to be short, generally between 50 and 200 bp (8).

In this study, we examined the feasibility of this approach by determining whether 16 metabolic and structural genes from *N. crassa* and *A. nidulans* hybridized to genomic DNA from filamentous ascomycetes and deuteromycetes (with ascomycete affiliations). On the basis of DNA-DNA hybridization experiments, we constructed nine primer sets from protein-coding regions from eight conserved genes in which the PCR product would span at least one intron. We tested seven primer pairs for the ability to amplify DNA by PCR from the genomes of a number of fungi and two conifer species. We found that six of the primers amplified DNA from filamentous ascomycetes and that the PCR products from five of these primers were highly polymorphic.

* Corresponding author. Phone: (604) 822-3155. Fax: (604) 822-6097. Electronic mail address: glass@unixg.ubc.ca.

TABLE 1. Genes used for the development of primer sets

Plasmid	Gene	Protein	Reference
pFB6	*pyr-4*	Orotidine 5′-monophosphate decarboxylase	29
pBC1[a]	*Bml*^{r}	β-Tubulin	31
pNT4	*trp-4*	Anthranilate phosphoribosyl transferase	44
pCYT20	*un-3*	Valyl-tRNA synthetase	21
pNC2[a]	*trp-1*	Phosphoribosyl-anthranilate isomerase	39
pMO31[a]	*cpc-1*	Cross-pathway regulatory protein	33
pDB1[a]	*trp-3*	Tryptophan synthetase	5
pKH14[a]	*pma-1*	Plasma membrane ATPase	14
pAN5-22[b]	*gdp-A*	Glyceraldehyde-3-phosphate dehydrogenase	36
pBJ005[a]	*pho-4*	Phosphate permease	24
pNcH3	*H3*	Histone 3	47
pNcH4	*H4*	Histone 4	47
pMP1[b]	*ald-A*	Aldehyde dehydrogenase	35
palcA2EB	*alc-A*	Alcohol dehydrogenase	13
pQa2[a]	*qa-2*	Quinic acid dehydrogenase	40
9-4-7cos[a]	rbs DNA	rDNA repeat	44

[a] Obtainable as plasmids from the Fungal Genetics Stock Center.
[b] *A. nidulans* plasmids; remaining plasmids were isolated from *N. crassa.*

TABLE 2. List of species

Species	Origin or strain	Source
Neurospora crassa Shear and Dodge	2225	Fungal Genetics Stock Center
Sclerotinia sclerotiorum (Libert) de Bary	Bean	R. J. Copeman[a]
Sclerotinia sclerotiorum	Chicory	R. J. Copeman
Sclerotinia sclerotiorum	Bean	R. J. Copeman
Botrytis cinerea Persoon: Fries	Carrot	R. J. Copeman
Trichoderma reesei Simmons	Soil	R. J. Copeman
Fusarium oxysporum-Schlechtendahl	Conifer seedling	P. E. Axelrood[b]
Fusarium moniliforme Sheldon	Conifer seedlot	P. E. Axelrood
Ustilago hordei (Persoon) Lagerhans	4854-10	J. Kronstad
Schizophyllum commune Fries	V4-5	L. Guisson
Saccharomyces cerevisiae Meyen ex Hansen	EG 123	I. Sadowski
Pseudotsuga menziesii (Mirb.) Franco	British Columbia	J. Carlson
Picea glauca (Moench) Boss	British Columbia	J. Carlson

[a] University of British Columbia.
[b] B.C. Research Inc.

MATERIALS AND METHODS

Cloned genes. The GenBank and EMBL DNA databases were initially screened for genes cloned from filamentous ascomycetes. The genes selected for analysis were isolated from either *N. crassa* or *A. nidulans*. The plasmids were requested from either the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City, Kans.) or individual research investigators listed in Table 1.

DNA isolation. The eight fungal and two plant species that were tested are listed in Table 2. The various fungal strains were grown in liquid Vogel's medium (43) at 25°C in stationary cultures for 5 to 10 days. The hyphal mats were recovered by vacuum filtration, and genomic DNA isolation from fungal strains was done according to the method of Stevens and Metzenberg (42). DNA from *Saccharomyces cerevisiae* was a gift from I. Sadowski, Biochemistry Department, University of British Columbia. DNA from *Ustilago hordei* and *Schizophyllum commune* were gifts from J. Kronstad and L. Giasson, respectively, Biotechnology Laboratory, University of British Columbia. DNA from *Pseudotsuga menziesii* and *Picea glauca* was a gift from J. Carlson, Biotechnology Laboratory, University of British Columbia.

DNA hybridization. Genomic DNA from fungal strains was digested with appropriate restriction enzymes (Boehringer-Mannheim, Laval, Quebec), subjected to electrophoresis in an 0.8% agarose gel, and transferred to a Hybond membrane (Amersham, Oakville, Ontario, Canada) according to the manufacturer's specifications. For DNA hybridizations to the PCR amplification products, 10 μl of PCR mix was subjected to electrophoresis in a 2% agarose gel and transferred to a Hybond membrane (Amersham) as described above. DNA probes from the digested plasmids were labelled with [^{32}P]dCTP (Amersham) by the random priming method (T7 Quick Prime; Pharmacia Corp., Baie d'Urfe, Quebec) and hybridized to membranes at 65°C as described by Sambrook et al. (38). Membranes were washed at 60°C in 0.1% sodium dodecyl sulfate–0.1% SSC (0.15 M NaCl plus 0.015 M sodium citrate) and exposed to Kodak X-OMAT film at −70°C for 1 to several days.

Oligonucleotide synthesis and PCR. Primers used to amplify the ITS region, ITS1 and ITS4, are based on published composite sequences (45). The ITS primers have been used previously to examine relationships between fungal species (23) and were included in this study as an internal control for the development of the primer sets.

Oligonucleotide primers were designed from protein-coding regions which were both conserved and adjacent to introns. Potential primers for PCR were subsequently analyzed for suitability, using the OLIGO program (37), the GCG programs FOLD, SQUIGGLES, and STEMLOOP (6), and standard primer analysis methods (18). The 14 primer sequences used are shown in Table 4. The oligonucleotide length for all of the *N. crassa* primers ranged from 18 to 24 bases. The optimal length for each primer was not determined. Oligonucleotide primers were synthesized by an Applied BioSystems 380A DNA synthesizer (Nucleic Acid-Protein Service Unit, Biotechnology Laboratory, University of British Columbia).

DNA amplifications were performed with a GeneAmp kit (Perkin-Elmer Cetus, Norwalk, Conn.) with a model 480 Perkin-Elmer DNA thermal cycler. Amplifications were performed in a 100-μl reaction volume, using 0.2 μM (each) primer and approximately 200 ng of template DNA. The *N. crassa* amplifications were used as positive controls. Additional control amplifications using primers only were performed to ensure that reagents used were not contaminated with extraneous template DNA. The standard PCR cycling protocol consisted of the following: 94°C for 1 min; 58 or 68°C for 1 min; 72°C for 1 min; repeat protocol for 32 cycles, with a 5- to 10-s extension time per cycle. PCR products were digested with 4- and 6-base recognition restriction enzymes (Boehringer-Mannheim) and subjected to electrophoresis in either a 2% agarose gel or 0.5% agarose with 2% Synergel (Diversified Biotech, Boston, Mass.). The PCR products were visualized with UV illumination and photographed with type 55 Kodak film.

RESULTS

Hybridization of cloned metabolic and structural genes. Cloned and sequenced genes from *N. crassa* and *A. nidulans* that encoded structural or metabolic proteins were obtained from various sources (Table 1). The 16 genes from Table 1 were hybridized to *Sclerotinia sclerotiorum* and *N. crassa* genomic DNA under conditions of high stringency (Table 3). *S. sclerotiorum* was chosen for the initial DNA hybridizations as a representative outgroup to both *N. crassa* and *A. nidulans*. Five of the 16 genes assayed failed to hybridize to *S. sclerotiorum* genomic DNA under these conditions (Table 3). All 16 genes hybridized to genomic DNA from *N. crassa.*

To extend the initial hybridization results, probes from 7 of the 16 genes (*ald-A*, *H4*, *H3*, *Bml*^{r}, *pma-1*, *trp-4*, and *un-3*) were hybridized to genomic DNA from *Fusarium oxysporum*, *Fusarium moniliforme*, and *Trichoderma reesei* (Table 3). Four of the seven genes tested, encoding histone 4, histone 3, β-tubulin, and the plasma membrane ATPase, hybridized to genomic DNA from all of the filamentous ascomycete species surveyed. Although the *ald-A* gene from *A. nidulans* hybridized to genomic DNA from *F. oxysporum* and *T. reesei*, it was rejected for further analysis because it failed to hybridize to the genome of *F. moniliforme.*

Primer construction and amplification. Eight of the genes which tested positive for hybridization to *S. sclerotiorum*

TABLE 3. Hybridization of *N. crassa* and *A. nidulans* genes to genomic DNA from filamentous ascomycetes[a]

Gene	Hybridization to DNA from:				
	S. sclerotiorum[b]	*F. oxysporum*	*F. moniliforme*	*T. reesei*	*N. crassa*
ald-A	+	+	−	+	+
H3	+	+	+	+	+
H4	+	+	+	+	+
Bml^r	+	+	+	+	+
pma-1	+	+	+	+	+
trp-4	−	−	−	−	+
un-3	−	+	−	−	+
rDNA	+	NT	NT	NT	+
alc-A	+	NT	NT	NT	+
cpc-1	−	NT	NT	NT	+
gpd-A	+	NT	NT	NT	+
pho-4	+	NT	NT	NT	+
pyr-4	+	NT	NT	NT	+
qa-2	−	NT	NT	NT	+
trp-1	−	NT	NT	NT	+
trp-3	+	NT	NT	NT	+

[a] Hybridizations were conducted under conditions of high stringency. +, hybridization; −, no hybridization; NT, not tested.
[b] The three strains of *S. sclerotiorum* assayed were isolated from three different hosts (bean, chicory, and carrot).

genomic DNA were selected for the development of the primer sets (encoding histone 3, histone 4, β-tubulin, plasma membrane ATPase, alcohol dehydrogenase, glyceraldehyde 3-P dehydrogenase, phosphate permease, and tryptophan synthetase [Table 1]). Nine oligonucleotide primer sets were synthesized and tested for amplification with genomic DNA from *N. crassa*. Difficulty in obtaining consistent PCR products in control amplifications from three sets of primers (*gpd-A*, *alc-A*, and *trp-3* primers) resulted in their rejection; six primer sets from five genes have been used in this study. The DNA sequences of the oligonucleotide primer pairs are shown in Table 4. The five genes used for the development of the primer sets (*H3*, *H4*, *Bml*^{r}, *pma-1*, and *pho-4*) were isolated from *N. crassa*. The remaining primer set spans the ITS region in the nuclear ribosomal repeat, which includes the 5.8S rDNA gene (Fig. 1C) (45).

Primer selection for the five *N. crassa* genes was based on obtaining a DNA amplification product of approximately 500 bp that would span at least one intron. DNA amplification with each primer set was initially tested with *N. crassa* genomic DNA at an annealing temperature of 68°C. All of the primer sets from the five *N. crassa* genes gave amplification products of the predicted sizes (Fig. 1 and Table 5). The primer placement within the *H3* and *H4* genes (47) is shown in Fig. 1A. Figure 1B shows the primer placement within *pma-1* (14) and the *pho-4* gene (24). Figure 1C shows the primer location within the *N. crassa Bml*^{r} gene (31), which contains six introns. Two sets of primers were therefore devised to amplify alternate segments of the *Bml*^{r} gene that contained different introns. Primers used to amplify the ITS region, ITS1 and ITS4 (45) (Fig. 1C), did not amplify a segment of DNA from *N. crassa* at an annealing temperature of 68°C; however, DNA amplification from *N. crassa* genomic DNA with ITS primers gave a product of approximately 600 bp at an annealing temperature of 58°C (Table 5).

Amplification of target DNA. To test the feasibility of using the various primer pairs to amplify segments of DNA from fungal species other than *N. crassa*, we assayed a discomycete, *S. sclerotiorum*; three deuteromycetes, *Botrytis cinerea*, *T. reesei*, and *F. oxysporum*; and one ascomycete yeast, *Saccharomyces cerevisiae*. Two basidiomycetes, *Schizophyllum commune* and *Ustilago hordei*, and two conifer species, *Pseudotsuga menziesii* and *Picea glauca*, were included in this study (Table 2). At an annealing temperature of 68°C, DNA amplification products could not be detected in the two plant species, the basidiomycete species, or *Saccharomyces cerevisiae* and *S. sclerotiorum* with any of six *N. crassa* primer pairs. The ITS1-ITS4 primer set also failed to amplify a discrete segment of DNA from any of the organisms tested at an annealing temperature of 68°C. However, primer pairs from four of the five *N. crassa* genes (*H3*, *H4*, *Bml*^{r}, and *pma-1*) gave discrete DNA amplification products at the annealing temperature of 68°C from the genomes of one or more of the three remaining filamentous ascomycetes, *B. cinerea*, *T. reesei*, and *F. oxysporum* (Table 5).

At an annealing temperature of 58°C, discrete amplification products were observed with the ITS1 and ITS4 primers in all of the fungal species (Table 5). The ITS amplification product varied in size from approximately 600 bp in *N. crassa*, *T. reesei*, and *S. sclerotiorum* to 550 bp in *B. cinerea* and *F. oxysporum.*

Five of the primer sets (H3-1, H4-1, Bt1, Bt2, and A1) amplified discrete DNA products only from filamentous ascomycete DNA at the annealing temperature of 58°C. The H3-1 amplification product was identical in size (approximately 450 bp) in *N. crassa*, *T. reesei*, *B. cinerea*, and *S. sclerotiorum*. In *F. oxysporum*, however, the size of the H3-1 amplification product

TABLE 4. Primer sets and corresponding amplification targets[a]

Target gene	Primer	Primer DNA sequence
Histone 3	H3-1a	5′ ACTAAGCAGACCGCCCGCAGG 3′
	H3-1b	5′ GCGGGCGAGCTGGATGTCCTT 3′
Histone 4	H4-1a	5′ GCTATCCGCCGTCTCGCT 3′
	H4-1b	5′ GGTACGGCCCTGGCGCTT 3′
β-Tubulin	Bt1a	5′ TTCCCCCGTCTCCACTTCTTCATG 3′
	Bt1b	5′ GACGAGATCGTTCATGTTGAACTC 3′
	Bt2a	5′ GGTAACCAAATCGGTGCTGCTTTC 3′
	Bt2b	5′ ACCCTCAGTGTAGTGACCCTTGGC 3′
ITS[b]	ITS1	5′ TCCGTAGGTGAACCTGCGG 3′
	ITS4	5′ TCCTCCGCTTATTGATATGC 3′
Plasma membrane ATPase	A1a	5′ TTCCTCGGTTTCTTCGTCGGTCCC 3′
	A1b	5′ CTAGTCAGACGAGAATGGCCGCTC 3′
Phosphate permease	pho4-1a	5′ GCTGCCCTTGATGCTTGG 3′
	pho4-1b	5′ CAGGGACCAGCAGGGCGA 3′

[a] See Fig. 1 for details of primer location.
[b] Described previously (45).

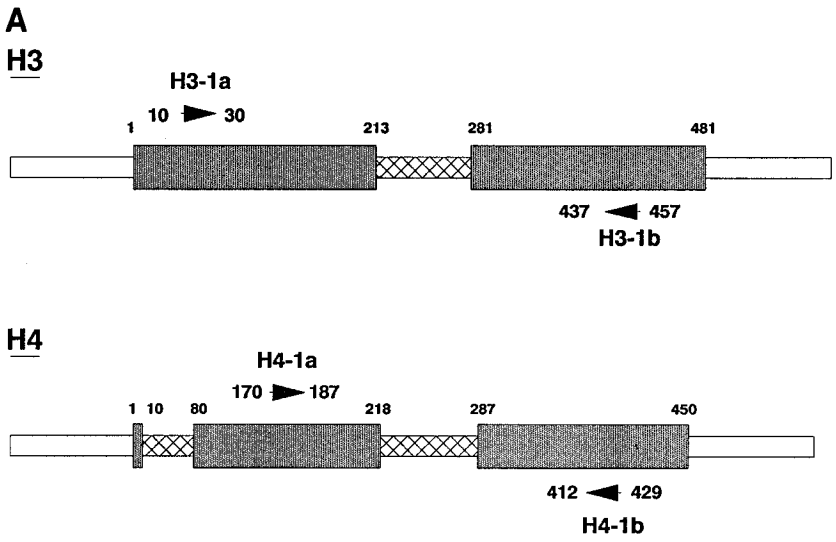

FIG. 1. Genomic structure of the genes used for the development of primer sets. Shaded boxes denote protein-coding sequences (exons), and cross-hatched boxes denote introns. Open boxes denote sequences surrounding the protein-coding sequence of each gene. Numbers above and below correspond to DNA sequence position, with the number 1 denoting the first base of the protein-coding sequence. Arrows are identified as given in Table 4 and denote the position of the primers. (A) Structure of *N. crassa* histone 3 (*H3*) and histone 4 (*H4*) genes (47). H3-1a and H3-1b primers amplify a 447-bp fragment from *N. crassa* and H4-1a and H4-1b amplify a 259-bp fragment. (B) Structure of *N. crassa* plasma membrane ATPase (*pma-1*) (14) and phosphate permease (*pho-4*) (24) genes. The A1a and A1b primers amplify a 546-bp fragment and the pho4-1a and pho4-1b primers amplify a 1,128-bp fragment from *N. crassa*. (C) Structure of *N. crassa* β-tubulin gene (31) and nuclear rDNA region (45). The Bt1a and Bt1b primers amplify a 537-bp fragment and the Bt2a and Bt2b primers amplify a 495-bp fragment from *N. crassa*. The ITS1 and ITS4 primers are based on composite sequences and amplify a 600-bp fragment from *N. crassa.*

was approximately 540 bp. All of the H3-1 PCR products from the filamentous ascomycetes hybridized to a probe from the *N. crassa H3* gene. The size polymorphisms observed in H3-1 amplification products presumably reflect differences in the sizes of the introns because the H3 protein is highly conserved (47). The size of the H4-1 DNA amplification product from all of the filamentous ascomycetes was approximately 260 bp, although minor variations in the size of the product are apparent (Fig. 2B); the H4-1 amplification products hybridized to a probe from the *N. crassa H4* gene.

The Bt1 primer set also amplified a similar-sized 540-bp fragment from genomic DNA from all of the filamentous ascomycetes which hybridized to a probe from the β-tubulin gene. The fourth primer pair, A1a and A1b, amplified a DNA segment of approximately 550 bp from all of the filamentous ascomycetes with the exception of *T. reesei*, in which it failed to amplify a discrete band (Table 5). The A1 amplification products all hybridized to a probe from the plasma membrane ATPase gene.

The greatest degree of size polymorphism in the PCR product from the filamentous ascomycetes was observed in the Bt2 amplification product. The Bt2 primer set amplified a 495-bp fragment from *N. crassa*, but only a 475-bp segment was amplified from *B. cinerea* and *S. sclerotiorum*. In *F. oxysporum* and *T. reesei*, the Bt2 primer set amplified smaller fragments of 340 and 360 bp, respectively. In *N. crassa*, the Bt2 primers amplified a segment that spans three introns. The Bt2 size polymorphism observed in the amplified products from the different filamentous ascomycetes presumably reflects variability in the number of introns in the β-tubulin gene. The Bt1 and Bt2 PCR products from the five fungi all hybridized to a probe from the *N. crassa Bml*^{r} gene.

Distinct PCR products were not observed with the H3-1, H4-1, Bt1, Bt2, or A1 primer pairs at the annealing temperature of 58°C from conifer DNA, basidiomycete DNA, or *Saccharomyces cerevisiae* DNA (Table 5). The sixth primer pair, pho4-1a and pho4-1b, amplified a discrete band of approximately 1 kbp from DNA of *N. crassa*, *B. cinerea*, and *Saccharomyces cerevisiae*, but PCR products were not detected from *T. reesei*, *F. oxysporum*, and *S. sclerotiorum* DNA (Table 5).

RFLP analyses. The primer sets were designed to amplify DNA segments that span introns. From the results reported in the previous section, it is probable that the size polymorphisms in the conserved genes are due to differences in the number and/or size of introns. In addition to size differences, the PCR products from the different genera should contain polymorphisms that are detectable by RFLP analysis. Figure 2 shows restriction enzyme digestion analysis of the H4-1 and Bt1 PCR products from the filamentous ascomycetes. An undigested H4-1 PCR product is approximately 260 bp in all of the filamentous ascomycete species, although minor variations are observable (Fig. 2B). In Fig. 2A, lanes 2, 3, and 4, similar-sized bands are observed in an *Nde*II digestion of the *N. crassa*, *S. sclerotiorum*, and *B. cinerea* H4-1 PCR products, while the *T. reesei* and *F. oxysporum* products show unique RFLP patterns. The intron placement in the *N. crassa H4* gene is 48 bp from the H4-1a primer and 142 bp from the H4-1b primer (Fig. 1A). From the patterns produced with *Nde*II digestion of the H4-1 PCR products in the five species, it is probable that DNA polymorphisms exist in both the coding regions of the *H4* gene and the intron sequences.

In *N. crassa*, amplification of the β-tubulin gene with the Bt1 primers produces a 537-bp fragment. A similar-sized PCR product is observed in all five of the filamentous ascomycetes surveyed when the Bt1 primers are used. In Fig. 2C and D, the Bt1 product from the five species was digested with *Alu*I and *Cfo*I. Similar-sized *AluI* digestion products are observed in *N. crassa* and *T. reesei* Bt1 PCR products. However, the Bt1 PCR products from these two species are easily distinguished in *Cfo*I digestions (Fig. 2D, lanes 2 and 5). *Cfo*I digestion of the Bt1 PCR products resulted in fragment patterns that are similar in *S. sclerotiorum* and *B. cinerea* but that differ when digested with

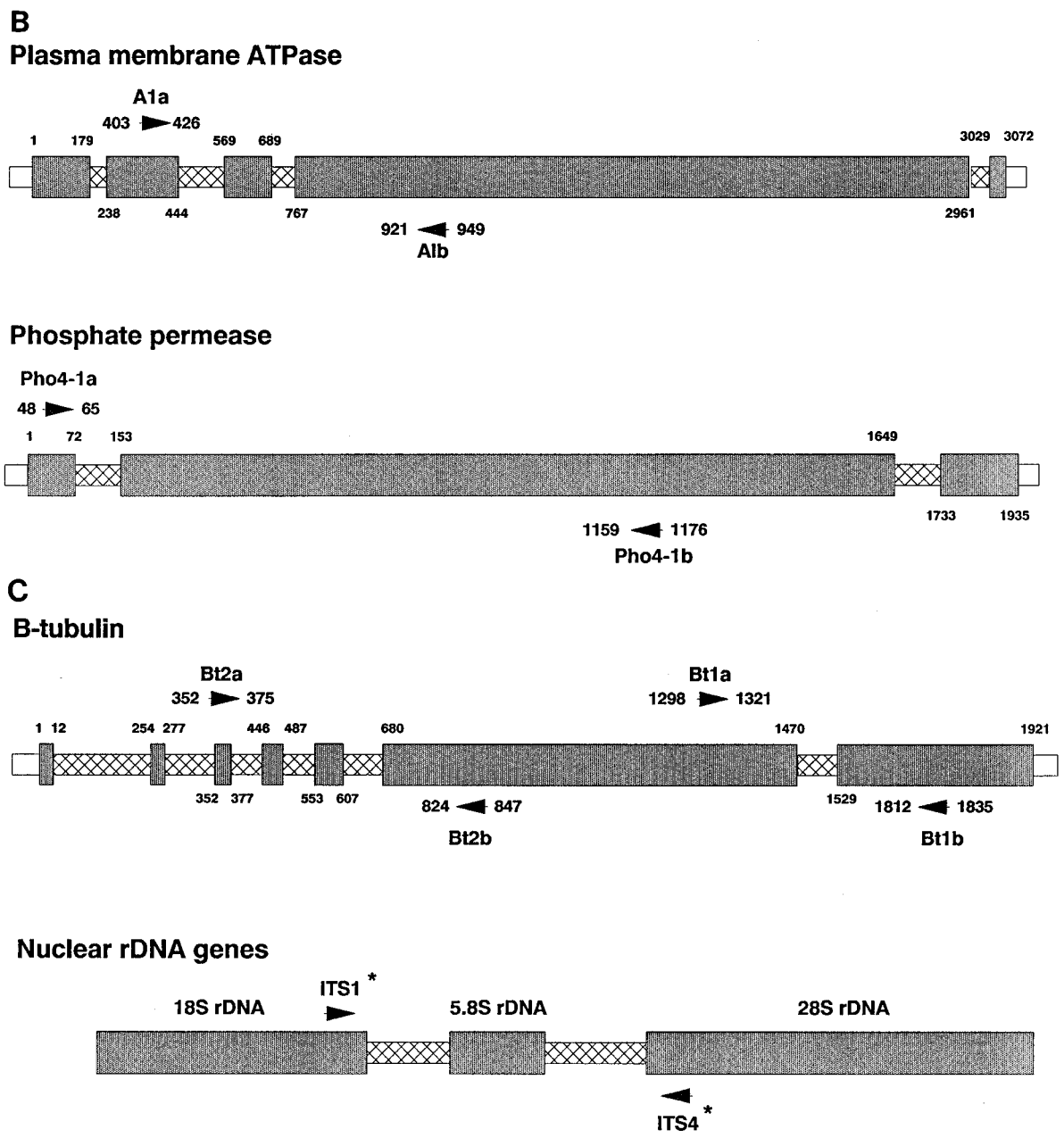

FIG. 1—*Continued.*

*Alu*I enzyme (Fig. 2C and D, lanes 3 and 4). In *N. crassa*, the intron in the Bt1 PCR product is 172 bp from the Bt1a primer and 306 bp from the Bt1b primer (Fig. 1C). As with the H4-1 PCR product, it is probable that there are DNA polymorphisms in both the protein-coding regions and intron sequences of the Bt1 PCR product among the five ascomycetes. The RFLP variation in the Bt1 and H4-1 PCR products observed in Fig. 2 is similar to that observed when the H3-1, Bt2, and ITS amplification products from the five ascomycete species were digested with 4-bp recognition restriction enzymes. The amplified DNA products from the different filamentous ascomycetes contained variable restriction sites and could be differentiated by a number of restriction enzyme analyses.

DISCUSSION

We constructed nine primer sets to eight genes and tested their ability to amplify segments of DNA from ascomycetes, deuteromycetes (with filamentous ascomycete affiliations), basidiomycetes, and conifer species. We identified five primer sets that we believe to be filamentous ascomycete specific in that they did not amplify segments of DNA from *Saccharomyces cerevisiae*, two basidiomycete species, or conifers. The PCR products from the filamentous ascomycetes determined with these five primer sets contained both size polymorphisms and RFLPs that were detectable by restriction enzyme digestion. Thus, we have achieved our objective of developing tools that have the sensitivity of PCR and detect DNA sequence poly-

TABLE 5. Results of DNA amplification with primer sets[a]

Organism	Amplification with primer:													
	H3		H4		Bt1		Bt2		ITS		pma-1		pho-4	
	58°C[b]	68°C	58°C	68°C	58°C	68°C	58°C	68°C	58°C	68°C	58°C	68°C	58°C	68°C
Neurospora crassa	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Botrytis cinerea	+	s	+	+	+	+	+	+	+	−	+	+	+	−
Trichoderma reesei	+	s	+	+	+	+	+	s	+	s	s	s	−	−
Fusarium oxysporum	+	+	+	+	+	+	+	+	+	s	+	s	−	−
Sclerotinia sclerotiorum	+	s	+	s	+	s	+	s	+	s	+	s	−	−
Ustilago hordei	s	−	s	−	s	s	−	s	+	s	s	s	−	−
Schizophyllum commune	s	−	s	−	s	s	−	−	+	−	s	s	−	−
Saccharomyces cerevisiae	−	−	−	−	−	−	−	−	+	−	−	−	+	−
Pseudotsuga menziesii	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Picea glauca	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] All PCR amplifications were repeated at least twice. Genomic DNA from the filamentous ascomycetes was re-isolated and retested. PCR amplifications resulting in smears were repeated several times. +, DNA amplification; −, no DNA amplification; s, smear present.
[b] Annealing temperature for PCR protocol.

morphisms. We believe that an advantage of these primer sets is that they can be used with diverse genera of filamentous ascomycetes without the need for extensive screening of suitable primer pairs, as is required in randomly amplified polymorphic DNA analyses (9, 26, 46).

Although only six primers were examined in this study, it is possible that primer sets constructed to alternate regions of the *alc-A*, *trp-3*, and *gpd-A* genes could be useful. Weak DNA amplification products were observed with the *alc-A* and *trp-3* primers from *N. crassa* genomic DNA. Initial PCR amplifications with the *gpd-A* primers gave faint products from genomic DNA of *N. crassa*, *B. cinerea*, *F. oxysporum*, *U. hordei*, and *Schizophyllum commune* but were not pursued further in this study. Two of the primer sets gave variable PCR amplification results. The pho4-1 primer set amplified a discrete segment only from *B. cinerea* and *Saccharomyces cerevisiae*. The lack of amplification from the remaining ascomycetes may reflect the fact that the pho4-1 primer set amplifies a 1.1-kbp product rather than the 300- to 500-bp segments obtained with the other primer sets. Another primer pair, pma-1a–pma-1b, amplified a discrete DNA segment from all of the filamentous ascomycetes with the exception of *T. reesei*. It is possible that the *pma-1* primers will be useful tools for genome analysis and population studies only with select filamentous ascomycetes.

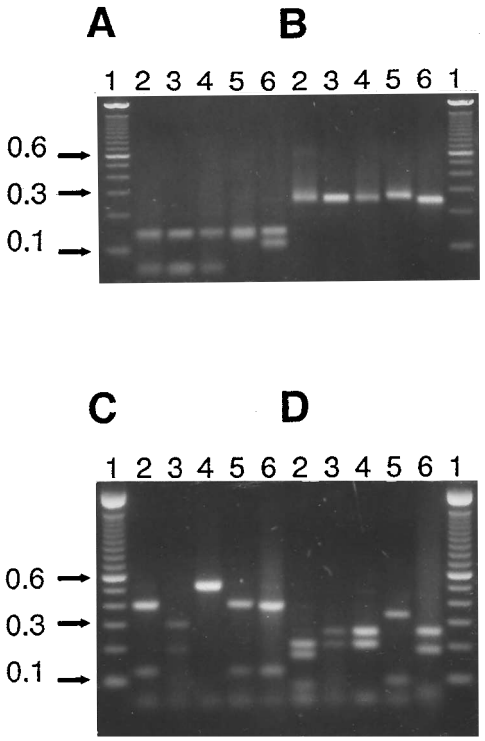

FIG. 2. Restriction enzyme digestion of PCR products, using primer sets. (A and B) PCR amplification products from the genomes of filamentous ascomycetes, using primers H4-1a and H4-1b. (A) H4-1 PCR products digested with *Nde*II; (B) H4-1 PCR products showing slight size variation. (C and D) PCR amplification products from the genomes of filamentous fungi, using primers Bt1a and Bt1b. (C) Bt1 PCR products digested with *Alu*I; (D) Bt1 PCR products digested with *Cfo*I. Lanes 1, DNA molecular mass markers, in kilobase pairs; lanes 2, *N. crassa*; lanes 3, *S. sclerotiorum*; lanes 4, *B. cinerea*; lanes 5, *T. reesei*; lanes 6, *F. oxysporum.*

Size polymorphisms were detectable in PCR amplification products when the five primer pairs with DNA from the filamentous ascomycetes were used. The sizes of the amplified product varied among the filamentous ascomycetes with the Bt2, H3-1, H4-1, and ITS primer pairs. This observation is most apparent in the DNA amplifications that use the Bt2 primers. The size polymorphisms observed are presumably due to variations in the size and/or number of introns present in the amplified segments in these conserved genes. The filamentous fungal species examined here are diverse, and therefore variability in the size of the PCR amplification products is perhaps not surprising. In a study with six species in the genus *Fusarium*, size polymorphisms in the PCR products were not observed with these five primer sets among 35 isolates (7).

In addition to size polymorphisms, RFLPs were detected in the PCR amplification products from the various filamentous ascomycetes upon digestion with restriction enzymes. It is possible that the restriction enzyme site differences observed in the PCR products are present in both the introns and protein-coding regions of the genes. The filamentous ascomycetes used in this study are not closely related, and therefore the DNA sequences of both the protein-coding regions and the introns may have diverged, although not to the extent that DNA hybridization to the cloned genes is not apparent. With these primer sets, species-specific or perhaps even clone-specific RFLP patterns could be obtained that would be useful for the identification and detection of filamentous ascomycetes. We have used these five primer sets to examine RFLPS among

species of *Fusarium* and have observed species-specific patterns in six species (7).

The primer sets constructed from these conserved genes should be useful in amplifying polymorphic regions of DNA from ascomycete fungi and may be phylogenetically informative at both the species and genus levels. The level of polymorphism within these genes may be similar to that of the ITS region, which has been used in fungi for both taxonomic and population studies (23, 30). Although we have not surveyed a large number of filamentous ascomycetes, we believe that the primer sets will successfully amplify DNA products from members of the pyrenomycete, discomycete, and deuteromycete (with filamentous ascomycete affiliations) groups and may provide useful tools for phylogenetic analyses. PCR amplification products from *Penicillium* sp. (40a) and *Cylindrocarpon* sp. (unpublished results), using the primer sets described in this study, have been reported. Two recently published studies used a similar exon-primed, intron-crossing method for phylogenetic analysis of conserved nuclear genes in whales (34) and pinnipeds (41) and compared it with a phylogenetic analysis based on mitochondrial DNA sequences.

In this study, we have achieved our objective of identifying polymorphic PCR products that may be useful in phylogenetic analyses as well as tools that may aid in the identification and detection of filamentous ascomycetes. It is unlikely that a number of different fungi would have identical RFLP patterns for all amplification products, and therefore, this test could be used as a diagnostic tool for the detection and identification of filamentous ascomycetes. Our future objective in the development of these primer sets is to devise a method to identify and differentiate pathogenic and nonpathogenic *Fusarium* species found in association with conifers and thereby devise an effective strategy for disease control in conifer bare-root nurseries.

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