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Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes

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Abstract

A primer able to amplify the internal transcribed spacers (ITS) of the ribosomal DNA (rDNA), having enhanced specificity for ascomycetes, was identified by reviewing fungal ribosomal DNA sequences deposited in GenBank. The specificity of the primer, named ITS4A, was tested with DNA extracted from several species of ascomycetes, basidiomycetes, zygomycetes, mastigomycetes and mitosporic fungi (formerly deuteromycetes) and also from plants. The PCR annealing temperature most specific for ascomycetes was found to be 62°C and 64°C for the primer pairs ITS5 + ITS4A and ITS1F + ITS4A, respectively. At these annealing temperatures, all ascomycetous DNA samples were amplified efficiently with the ITS4A primer. The sensitivity limit was in the range 10^{-14} g of DNA. This primer could also provide useful tools in suggesting the affinities of many mitosporic fungi with their perfect states.

Keywords: Ascomycotina; ITS-rDNA; PCR; Taxon-selective primer; Molecular probe

1. Introduction

DNA fingerprinting techniques using polymerase chain reaction (PCR) such as random amplified polymorphic DNA (RAPD) (Williams et al., 1990), DNA amplification fingerprinting (DAF) (Caetano-Anolles et al., 1991), arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990, 1991) and AFLP fingerprinting (Vos et al., 1995) are useful for identification of organisms. The sequencing of particular regions of DNA, e.g. rDNA, has proven to be a valuable tool for the development of molecular systematics, including molecular evolution and population biology, in organisms such as fungi (Boysen et al., 1996; Kuninaga et al., 1997; Takamatsu, 1998).

Nuclear rDNAs, and particularly the ITS regions, are a good target for phylogenetic analysis in fungi (Bruns et al., 1991). These regions are nested in the rDNA repeat between the highly conserved sequences of the 18 S, 5.8 S and 28 S rRNA genes. Several features make the ITS regions convenient targets for molecular identifica-

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tion of fungi: (i) the entire region is usually between 600 and 800 bp in length and can be readily amplified using universal primers that are specific for the rRNA genes (White et al., 1990); (ii) the multicopy nature of the rDNA repeat makes the ITS regions relatively easy to amplify from small, dilute or highly degraded DNA samples, and (iii) the ITS regions are often highly variable between taxonomically distinct fungal species (Gardes et al., 1991; Chen et al., 1992; Lee and Taylor, 1992) or even isolates of the same species (Gardes and Bruns, 1993; Boysen et al., 1996; Kuninaga et al., 1997).

Sequence variation in rRNA genes allows the use of these genes as targets for differential amplification. In a broader context, taxon-selective amplification of the ITS region is likely to become a common approach in molecular identification strategies. Amplification with taxon-selective ITS has already been used for detection and differentiation of the fungal pathogens *Verticillium* (Nazar et al., 1991), *Phytophthora* (Lee et al., 1993) and *Mycosphaerella* (Johanson et al., 1994), and primers based on 18 S rRNA sequences are used to specifically amplify vesicular-arbuscular endomycorrhizal fungal DNA from complex samples that include other fungal and plant DNAs (Simon et al., 1992). Properties of the ITS region, combined with the growing database of ITS are likely to make this region increasingly popular in microbial ecology.

Fungal ITS primers currently in use are ITS1 through ITS5 (White et al., 1990) and also a set of two primers has been described, ITS4B, specific for basidiomycetes, and ITS1F, that amplifies any fungal DNA (Gardes and Bruns, 1993). These primers in combination preferentially amplify the ITS regions of basidiomycetes. However, no specific primers have been identified for ascomycetes, which constitutes the largest group of fungi comprising almost 40% of the known fungal species.

On the other hand, the use as a molecular technique of the designed primer with enhanced specificity for certain groups of fungi (i.e. *Ascomycetes*) could provide useful tools in suggesting the affinities of many mitosporic fungi with their perfect states.

2. Materials and methods

2.1. Design of ITS4A primer

The 28 S rDNA sequences compared in this paper were retrieved from EMBL and GenBank databases. The sequences were aligned using the CLUSTAL W 1.7 Program (Thompson et al., 1994), and visually checked for regions which have homologies among ascomycetes but not with other fungi and plants. A taxon-selective primer for the ITS region intented to be specific for ascomycetes (ITS4A) was designed by comparing fungal and plant sequences in the 5′ terminal region of the 28 S rRNA (Fig. 1). The primer corresponds to positions 30–52 from the 5′ end of the ITS4 primer. This region is shown in Fig. 2 for some representatives of fungal and plant species. The ITS4A primer has the nucleotide sequence 5′-CGCCGTTACTGGGGCAATCCCT-G-3′. This 23-mer primer was synthesized by ISOGEN Bioscience BV (Maarssen, The Netherlands).

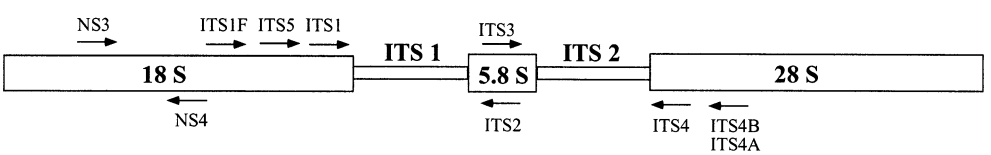

Fig. 1. Schematic representation of the rDNA region of fungi. The open boxes represent the ribosomal genes. The arrows represent the positions of the primers previously reported and the new ascomycetes-specific primer (ITS4A) described in this work. ITS 1 and ITS 2 regions are also indicated.

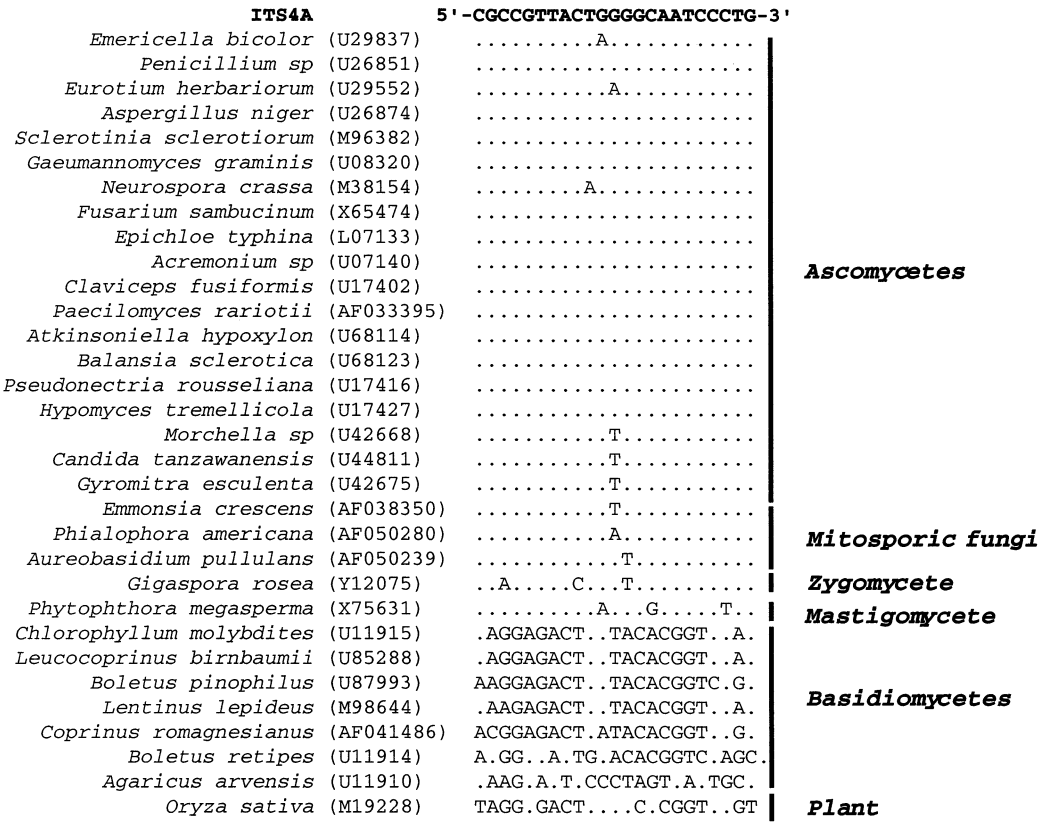

Fig. 2. Alignment of known nucleotide sequences that were used to design the ITS4A primer with specificity for ascomycetes. Next to the latin names are indicated the GenBank accession numbers. Dots indicate identity of rDNA and ITS4A primer sequences.

2.2. Fungal strains and DNA isolation

A total of 53 fungal isolates, representing anamorph and teleomorph of 30 ascomycetes and 12 basidiomycetes, four mitosporic fungi, three mastigomycetes and four zygomycetes were used in this analysis (Table 1). The 53 fungal isolates used were chosen so that they represent a broad fungal diversity. Also two plant species were tested. Each fungal isolate was grown on potato dextrose agar (Difco) plates for 3–5 days at 28°C. Mycelia were transferred to liquid complete media (CM media: malt extract, yeast extract and glucose, 5.0 g l^{-1} each) and incubated for 4–5 days at 28°C at 250 rpm. Mycelial mats were harvested by filtration, blotted dry, lyophilized and ground to a fine powder prior to DNA isolation. Mycelial powder (60 mg) was suspended in extraction buffer (50 mM EDTA (pH 8.5), 0.2% SDS (dodecyl sulphate sodium salt)), incubated at 65°C for 30 min, cooled to room temperature, and centrifuged at 12 000 rpm for 15 min. The supernatant was transferred to a new tube. After the addition of RNase A to a final concentration of 50 mg ml^{-1}, the mixture was incubated for 1 h at 30°C, centrifuged for 15 min at 10 000 rpm, and the supernatant was transferred to a fresh tube on ice. One tenth vol of 5 M potassium acetate (pH 5.2) was added and the solution incubated on ice for at least 1 h. The supernatant was transferred to another tube after centrifugation at 13 000 rpm 15 min, and then was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (49/49/2). The DNA was precipitated by adding 2 vol of ethanol. After a 30 min incubation at − 20°C, and centrifugation at 12 000 rpm

Table 1
Amplified ITS fragment from fungi and plants using several primer pairs[a]

	Primer pair			
	ITS5+ITS4A	ITS1F+ITS4A	ITS5+ITS4	ITS1F+ITS4
Ascomycetes				
Aspergillus nidulans	++	++	++	+
A. awamori	++	++	++	++
A. fumigatus	++	++	++	++
A. niger	++	++	++	+
Penicillium roquefortii	++	++	+	+
P. chrysogenun	++	++	++	++
P. purpurogenum	++	++	++	++
Alternaria sp.	++	++	+	+
Ophiostoma ulmi	++	++	++	++
Cryphonectria parasitica	++	++	+	+
C. parasitica	++	+	+	+
Trichoderma harzianum	++	−	++	++
Diplodia sp.	++	++	++	++
Fusarium oxysporum f.sp. *lycopersici*	++	(+)	++	+
F. oxysporum f.sp. *radicis-lycopersici*	++	+	++	+
Neurospora crassa	+	++	++	++
Monilinia laxa	+	++	++	+
Cryptosphaeria pullmanensis	++	++	++	++
Diatrype disciformis	++	++	++	++
Diatrypella quercina	++	++	++	++
Eutypa consobrina	++	++	++	++
Eutypella cerviculata	++	++	++	++
Hypoxylon fragiforme	++	++	++	++
Xylaria hypoxylon	++	++	++	++
Biscogniauxia mediterranea	++	++	++	++
Nemania aenea	++	++	++	++
Camillea tinctor	++	++	++	++
Rosellinia buxi	++	++	++	++
Ustulina deusta	++	++	++	++
Botrytis cinerea	+	++	++	++
Mitosporic fungi				
Verticillium dahliae	−	++	++	+
Aureobasidium pullulans	++	++	++	++
Cladophialophora sp.	++	++	++	++
Helminthosporium victoriae	++	+	++	+
Zygomycetes				
Gigaspora rosea	−	−	++	+
Scutellospora castanea	−	−	++	+
Spiromyces minutus	−	−	++	+
Mucor circinelloides	+	−	++	+
Mastigomycetes				
Phytophthora infestans	(+)	−	++	++
Phytophthora megasperma	(+)	−	++	++
Pythium sp.	(+)	−	++	++
Basidiomycetes				
Macrolepiota rhacodes	−	−	++	+
Russula mairei	−	−	++	+
Rhizopogon luteolus	−	−	++	+
Coprinus micaceus	−	−	++	+
Rhizoctonia solani AG-1	−	−	++	+

Table 1 (*Continued*)

	Primer pair			
	ITS5+ITS4A	ITS1F+ITS4A	ITS5+ITS4	ITS1F+ITS4
R. solani AG-2	−	−	++	+
R. solani AG-3	−	−	++	++
R. solani AG-4	−	−	++	++
R. solani AG-5	−	−	++	++
R. solani AG-BI	−	−	++	++
Agaricus bisporus	−	−	+	++
Ustilago maydis	(+)	−	++	++
Plants				
Solanum tuberosum	−	−	−	−
Nicotiana tabacum	−	−	−	−

[a] The annealing temperatures used were 64°C for the primer pair ITS5+ITS4A, 62°C for ITS1F+ITS4A, and 53°C for ITS5+ITS4 and ITS1F+ITS4. The intensities of the bands are: ++, strong; +, moderate; (+), faint; −, no amplification.

for 15 min, the recovered pellet was washed with 80% cold ethanol, vacuum-dried, resuspended in 100 μl of water, and stored until further use at − 20°C. The final concentration of DNA was in the range of 1×10^{-2}–10×10^{-2} g.

Plant DNA was extracted according to Edwards et al. (1991). A disc of 1 cm^2 from leaf was macerated without buffer in a eppendorf at room temperature. The tube was filled with extraction buffer (200 mM Tris–HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS), vortexed for 5 s, and centrifuged at 13 000 rpm for 1 min. 300 μl of the supernatant was transferred to a fresh tube, mixed with an equal volume of isopropanol, and left at room temperature for 2 min. After centrifugation at 13 000 rpm for 5 min, the pellet was dried, resuspended in 100 μl of sterile double-distilled water and stored until further use at − 20°C.

Concentrations of DNA in each sample were estimated prior to PCR by comparing the intensity of DNA bands in 1% agarose gels with a series of DNA dilutions.

2.3. PCR conditions

The DNA was amplified by PCR using the complementary primers ITS5, ITS4 (White et al., 1990) and ITS1F (Gardes and Bruns, 1993) as previously described (White et al., 1990) with some modifications introduced by Boysen et al. (1996). The primer pairs ITS5 + ITS4A, ITS5 + ITS4, ITS1F + ITS4A and ITS1F + ITS4 were tested for amplification of DNA from the selected fungal species. Each 50 μl reaction contained 10 ng of genomic DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia, Sweden), 20 pmol of primer A (ITS5 or ITS1F), 0.4 pmol of primer B (ITS4A or ITS4), 0.0025% Tween 20, 10% dimethylsulphoxide (DMSO) and 1.25 U of Taq DNA polymerase (USB, Cleveland, Ohio). Each reaction was overlaid with one drop of mineral oil (Sigma). An automated thermal cycler (Perkin Elmer Cetus Corp. model 480, Norwalk, Connecticut, USA) was used for amplification reactions. An initial denaturation step at 94°C for 2.5 min was followed by 40 cycles of: 15 s denaturation at 94°C, 30 s annealing at 55°C or 64°C for the ITS5 + ITS4A pair, and 55°C or 62°C for the ITS1F + ITS4A pair, and 90 s elongation at 72°C. The annealing temperature for ITS5 + ITS4 and ITS1F + ITS4 pairs was 53°C. The amplification was terminated by an incubation for 10 min at 72°C. Control reactions with no DNA template were performed in every experiment to eliminate possible contamination of the reagents with fungal DNA. All PCR experiments were repeated at least twice, unless result was not clear enough, in that case they were repeated three times (as is the case for *Tricoderma harzianum* and *Verticillium dahliae*). PCR products were analyzed by electrophoresis on 1% agarose gels in TBE

buffer (45 mM Tris–borate, 1 mM EDTA, pH 8.0). After electrophoresis, the gels were stained with ethidium bromide and viewed under UV light (310 nm). *Bst* EII-cut λ DNA ladder was used as a molecular weight standard.

3. Results and discussion

Initially, primer pairs ITS5 + ITS4A and ITS1F + ITS4A were tested at an annealing temperature of 55°C because it is the optimal temperature for annealing of the fungal DNA with the known ITS primers (ITS1, ITS1F, ITS2, ITS3, ITS4, ITS4B, ITS5). At this annealing temperature, DNA of most of the samples was amplified. In experiments using primer pairs ITS5 + ITS4 and ITS1F + ITS4 at 53°C, which should amplify all fungal rDNA sequences, a band 650–700 bp long was observed in all reactions containing fungal DNA. Subsequently, it was established that the optimal annealing temperatures for selective amplification of the ascomycetous DNA were 64°C for the ITS5 + ITS4A primer pair and 62°C for the ITS1F + ITS4A primer pair. Under such conditions, amplification of the ITS region corresponded with the intended specificity of the ITS4A primer (Table 1). The obtained PCR products varied in length from 600 to 800 bp. Generally, all DNA samples from either anamorph or teleomorph ascomycetes were amplified efficiently with the ITS4A primer at 55°C as well as at 64°C or 62°C. Amplification of DNA from other fungi resulted in either no product or an extremely faint product at 64°C or 62°C, except for the samples of mitosporic fungi (*Helminthosporium victoriae*, *Verticillium dahliae*, *Aureobasidium pullulans*, and *Cladophialophora sp.*) in which the amplification was generally strong (Table 1).

To determine if concentration and quality of plant DNA were suitable for rDNA amplification, experiments using the universal primers NS3 and NS4 were performed. These primers amplify a region of approximately 600 bp in the 18 S rDNA (White et al., 1990). This region was amplified efficiently in the plant samples (data not shown).

Dilution experiments were performed at 55°C to determine the sensitivity of the amplification

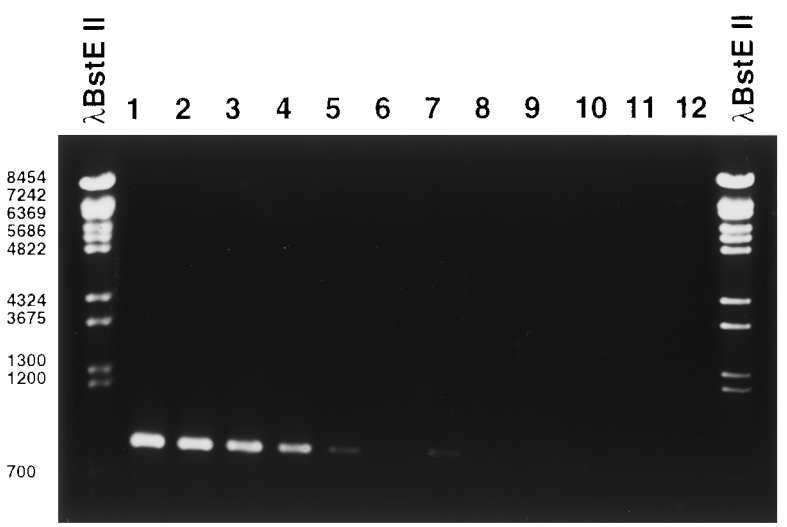

Fig. 3. PCR amplification using decreasing concentrations of DNA as template. DNA from the ascomycete *C. parasitica* and the basidiomycete *R. solani* AG-2 were used with the ITS5 + ITS4A primer pair annealed at 55°C. Lanes 1–5 are amplification products of the *C. parasitica* DNA, with the following amounts of template: 1, 2.5×10^{-2} g; 2, 2.5×10^{-5} g; 3, 2.5×10^{-8} g; 4, 2.5×10^{-12} g, and 5, 2.5×10^{-14} g. Lanes 7–11 are *R. solani* DNA amplification products with the following amounts of the template: 7, 2.5×10^{-2} g; 8, 2.5×10^{-5} g; 9, 2.5×10^{-8} g; 10, 2.5×10^{-12} g; and 11, 2.5×10^{-14} g. Lanes 6 and 12 are controls without DNA template.

reactions towards ascomycetous and not ascomycetous DNA. It was also observed the lowest amount of template DNA that would result in a PCR product with the ITS4A primer annealed at 55°C. DNA amounts tested ranged from 2.5×10^{-2} to 2.5×10^{-14} g (Fig. 3). With the ITS5 + ITS4A primer pair, 2.5×10^{-2} g of DNA from the basidiomycete *R. solani* yielded a product detectable by ethidium bromide staining of the gel (Fig. 3, lane 7), and any lower DNA concentration did not result in a detectable band. When DNA from the ascomycete *Cryphonectria parasitica* was used as template the sensitivity level increased to 2.5×10^{-14} g (Fig. 3, lanes 1–5). With the ITS1F + ITS4A primer pair the results were similar to those obtained with the ITS5 + ITS4A primer pair.

As mentioned above, for those samples belonging to mitosporic fungi the amplification was generally strong (Table 1). Mitosporic fungi constitutes an artificial assemblage of genera and species that comprises approximately all the known fungi which have not been correlated with any meiotic state. Morphological systematics have been traditionally removing some well-known anamorph fungi from the mitosporic fungi and named together with the sexual state (teleomorph) when sexual reproduction has been demonstrated for those species. Some of those mitosporic fungi have been correlated with teleomorphs in ascomycetes or basidiomycetes (more rarely zygomycetes), being named as the anamorphs of these groups. Within the group of true fungi, the majority of anamorph/teleomorph connections established up to date are known to belong to the ascomycetes.

As recommended in the last edition of Ainsworth and Bisby's Dictionary of the Fungi (Hawksworth et al., 1995), we have used the term mitosporic fungi for those imperfect fungi which presumptively lack a perfect state (Table 1). Since no teleomorph state has been correlated up to date with genus such as *Verticillium*, *Helminthosporium*, *Aureobasidium* and *Cladophialophora*, the strong amplification signal detected with the use of this new primer could provide some accurate clues on the type of teleomorph for these genera.

In summary, we describe the design of an oligonucleotide primer ITS4A having enhanced specificity for DNA from ascomycetes. This ITS4A primer reported provides a simple method for identifying ascomycetes. The method offers advantages over classical methods of identification: the entire assay is fast and reliable, and no taxonomy-specialized personal is demanded. In combination with other fungal primers ITS5 or ITS1F, this primer preferentially amplified the ITS region of fungi belonging to this taxonomical class and some others mitosporic fungi with presumptively ascomycetous teleomorph. In this sense, it can be also used to determine whether a mitosporic fungus is more likely to belong to the ascomycetes than the basidiomycetes or zygomycetes, as well as to examine the ascomycete component in many natural substrates (i.e. complex environmental samples such as mycorhizospheres, soil samples, composts, etc.).

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