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rDNA-ITS SEQUENCE OF *ARMILLARIA* SPECIES AND A SPECIFIC PRIMER FOR *A. MELLEA*

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ABSTRACT

The internal transcribed spacer (ITS) of the nuclear ribosomal DNA of *Armillaria borealis*, *A. cepistipes*, *A. gallica*, *A. mellea* and *A. ostoyae* was amplified by the polymerase chain reaction (PCR) and sequenced. There was a considerable sequence similarity among *A. borealis*, *A. cepistipes*, *A. gallica* and *A. ostoyae* and intraspecific variation among isolates. The *A. mellea* isolates were rather uniform and differed considerably from the other fungi. A primer, designed on the basis of the sequence difference, specifically identified isolates of *A. mellea* by the technique of 'taxon-specific priming PCR'.

Keywords: rDNA, ITS, 'taxon-specific priming PCR', *Armillaria* spp.

INTRODUCTION

In Europe *Armillaria* (Fr.: Fr.) Staude comprises five annulate species of significance for forest trees: *A. borealis* (Marxmüller & Korhonen), *A. cepistipes* (Velenovsky), *A. gallica* (Marxmüller & Romagnesi), *A. mellea* (Vahl: Fr.) Kummer, and *A. ostoyae* (Romagnesi) Herink. Their different biology, pathogenicity, host preference and geographical distribution have already been described (GUILLAUMIN *et* al. 1993; Shaw and Kile 1991).

Among the molecular methods used for characterization and identification of *Armillaria* species, some techniques are based on the sequence of the internal transcribed spacer (ITS) of rDNA. The PCR amplification of rDNA-ITS sequences using taxon-specific primers, derived from the sequence data and checked for cross reaction with related fungi, has been shown to be a powerful molecular tool for fungal diagnosis. The method has been named 'taxon-specific priming PCR' (TSPP). With regard to wood decay basidiomycetes, MORETH and SCHMIDT (2000) and SCHMIDT and MORETH (2000) obtained primers within the ITS II region, which specifically identified the indoor wood decay fungi *Serpula lacrymans* (Wulfen: Fr.) Schroeter apud Cohn, *S. himantoides* (Fr.: Fr.) P. Karsten, *Antrodia vaillantii* (DC.: Fr.) Ryv., *Tyromyces placenta* (Fr.) Ryv. and *Gleophyllum sepiarium* (Wulfen: Fr.) P. Karsten. Among pathogenic basidiomycetes, *Heterobasidion annosum* (Fr.) Bref. and *A. ostoyae* have been investigated by TSPP (GARBOLETTO *et al.* 1996; SCHULZE *et al.* 1997). During our experiments, CHILLALI *et al.* (1998a) published partial ITS sequences of *Armillaria* species, but did not attempt to use the information for identification.

This paper deals with the complete ITS sequences of 15 isolates of five *Armillaria* species and a specific primer for *A. mellea*, which may be used for identification.

MATERIAL AND METHODS

Fungi and culture conditions

The isolates from *Armillaria borealis*, *A. cepistipes*, *A. gallica*, *A. mellea* and *A. ostoyae* (Table 1) were identified by the mating technique of KORHONEN (1978) and grown on 2% (w/v) malt extract / 1.5% agar (Oxoid, Unipath, Basingstoke, UK) plates (pH 5.5) at 20°C for about 2 weeks to obtain young aerial mycelium.

Sample preparation and PCR

About 3 mg fresh weight of aerial mycelium was sampled from agar plate cultures into 1.5 ml Eppendorf tubes containing 0.2 ml autoclaved tap water. Rhizomorphic tissue was avoided because extraction of its DNA was difficult. The hyphae were broken by an

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Table 1. ***Armillaria*** **species and isolates with coding, origin and EMBL accession number.**

Species	Isolate	Coding		n / $2n$	Host	Origin	Collector	Year of isolation
A. borealis	*92142/2	B1	AJ250052	n	*Alnus incana*	Finland	K. Korhonen	1992
	*9321	B2		n	*A. incana*	Finland	K. Korhonen	1993
	93035	B3		$2n$	*Quercus sp.*	Finland	P. Łakomy	1993
	*51/21/4/97	B4		$2n$	*Q. robur*	Poland	K. Przybył	1997
A. cepistipes	*93299/2	C1	AJ250053	n	*Quercus sp.*	Poland	A. Żołciak	1993
	*93623/1	C2		n	*Quercus sp.*	Poland	A. Żołciak	1993
	93688	C3		$2n$	*Picea sp.*	Greece	K. Korhonen	1993
	*92159	C4		$2n$	*Picea abies*	Finland	K. Korhonen	1992
A. gallica	*88104/3	G1	AJ250054	n	*Quercus sp.*	Italy	R. Grillo	1988
	*91275/3	G2		n	–	Switzerland	K. Korhonen O. Holdenrieder	1991
	*98332	G3		$2n$	*Quercus sp.*	Poland	P. Łakomy	1998
	55/21/4/97	G4		$2n$	*Q. robur*	Poland	K. Przybył	1997
A. mellea	*90260/1	M1	AJ250051	n	–	Slovenia	A. Munda	1990
	*90254/7	M2		n	–	Italy	R. Grillo	1990
	*94056	M3		$2n$	*Q. petrea*	Hungary	M. Szanto	1989
	94075	M4		$2n$	*P. sylvestris*	Hungary	M. Szanto	1990
A. ostoyae	*93415/1	O1	AJ250055	n	*Quercus sp.*	Poland	A. Żołciak	1993
	*93569/1	O2		n	*Carpinus sp.*	Poland	A. Żołciak	1993
	*97030	O3		$2n$	*P. sylvestris*	Poland	P. Łakomy	1997
	97036	O4		$2n$	*P. sylvestris*	Poland	P. Łakomy	1997

autoclaved plastic pistil (Bel-Art Products, Pequannock, NJ, USA), which fitted into the tube and was rotated by a mechanical device at 2,000 rpm for 3 min. The resulting suspension was boiled for 3 min to inactivate enzymes, and diluted 1:5, 1:25 and 1:125 with tap water for a suitable DNA template concentration for PCR. No special chemical extraction and purification were performed.

For amplification of the ITS region, 50 µl PCR mixtures were prepared from the DyNAzyme I DNA polymerase kit (Finnzymes Oy, Espoo, Finland) and contained a final concentration of 10 mM Tric-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 µM of each dNTP, 400 nM of the primers ITS 1 and ITS 4 each (Biometra, Göttingen, Germany) of WHITE *et al.* (1990), 0.6 unit of polymerase and 2 µl DNA solution from the dilutions.

Amplifications were performed in a PTC-100 thermocycler (MJ Research, Watertown, Ma, USA) with a heated lid for 35 cycles of 94 °C denaturing, 55 °C annealing and 72 °C extension, each for 1 min. Initial denaturing at 94 °C was extended to 2–4 min to improve DNA extraction. The final extension was at 72 °C for 7 min.

Sequencing of the rDNA-ITS region

Sequencing of the PCR products was carried out by MWG-Biotech (Ebersberg, Germany) with a Li-COR 4200 sequencer. Three isolates of five species (Table 1) were sequenced in both directions.

Taxon-specific priming PCR

Using base sequence differences in the ITS II region between the fungi, 20mer primers were designed and commercially synthesized (Biometra). Each primer (reverse) was combined with the universal primer ITS 1 (forward). The PCR protocol was as above.

Electrophoresis

The PCR products were analysed by horizontal electrophoresis ($4 V \cdot cm^{-1}$) in 1.5% (w/v) DNA agarose (Biozym, Hess. Oldendorf, Germany) gels in 0.5× TBE buffer for 2 hours using a GNA-200 apparatus (Pharmacia Biotech, Freiburg, Germany). A DNA marker (50–2,500 bp; FMC BioProducts, Rockland, Me, USA) was used for size estimation. Gels were stained with GelStar (FMC BioProducts) by shaking for

30 min. DNA was visualised by 312 nm UV transillumination and photographed with a Leicaflex SL, a close-up lens, a Wratten No. 9 filter, and an AGFA APX 100 film.

With the exception of sequencing, experimental runs were repeated. The use of impurified DNA implied variation in band intensity among replicate experiments with regard to the most reliable DNA dilution for PCR. Thus different DNA dilutions were used for PCR. Non-amplifying isolates and 'false positives' were repeated.

RESULTS AND DISCUSSION

ITS region and base sequence

As formerly described for *Armillaria* species (HARRINGTON & WINGFIELD 1995), *Heterobasidion annosum* (PAPPINEN *et al.* 1996), *Serpula lacrymans* (SCHMIDT & MORETH 1999, 2000) and other house rot fungi (MORETH & SCHMIDT 2000), the PCR was carried out with no special preceding DNA extraction or purification. In the present study, young aerial mycelium just grown near the inoculum on the agar plate was sampled. Initial tests had revealed that it is difficult to extract DNA from old mycelium and especially from rhizomorphic tissue. The hyphae were broken by a rotating pistil and after dilution transferred directly to the PCR mixture. The direct transfer of hyphae from *Armillaria* species into the PCR mix, as described by HARRINGTON and WINGFIELD (1995), did not work with our fungi.

The combination of ITS 1 and ITS 4, universal primers described by WHITE et al. (1990), successfully amplified the rDNA-ITS region of all isolates listed in Table 1. The size of the amplified ITS product was estimated after gel electrophoresis to be about 880 bp for the five *Armillaria* species.

The complete sequences of internal transcribed spacer regions of all three isolates of the five species were rather uniform and therefore aligned by eye. In some sequences, the adjacent bases, up to eight bases, were taken from other fungi (MORETH & SCHMIDT 2000), because this information fitted for the nested PCR used by the sequencing service. Some gaps and ambiguities were filled from the information in the complementary DNA strand.

The sizes of the complete ITS products without gaps are 871 to 872 bp for *A. borealis*, *A. cepistipes*, *A. gallica*, *A. ostoyae*, and 882 to 883 bp for *A. mellea*. By contrast, the product sizes obtained with the ITS 1/ITS 4 combination for the various house rot basidiomycetes were 639 to 727 bp (MORETH & SCHMIDT 2000).

The rDNA portion encoding the 5.8S rRNA gene shares homology with the data for 12 other basidiomycetes (ZHANG *et al.* 1997). It contains 157 base pairs and reaches from 282 to 438 bp. The 5.8S rDNA sequence from our five *Armillaria* species was highly conserved and did not show any base difference among all isolates. This sequence identity shows the close relationship of the five *Armillaria* species. It also attests to the quality of the sequencing obtained. Identical 5.8S rDNA sequences between two species of the same genus have also been found for *S. lacrymans* and *S. himantioides* (Schmidt and Moreth 2000). Compared with the 5.8S rDNA sequence of the seven house rot fungi (MORETH & SCHMIDT 2000), the *Armillaria* species revealed base differences at 7–13 positions.

CHILLALI *et al.* (1998a) published for the above species partial ITS sequences of 744 bp. These sequences are shorter and show slight differences compared to our data.

The ITS sequences of our 15 isolates of the five *Armillaria* species were rather similar. *A. mellea* is the only species that is distinctly different. The sequence of each one isolate per species is deposited in EMBL (Table 1). A phylogenetic tree (Figure 1) indicates that the *A. mellea* isolates form a separate branch of the dendrogram. The remaining four species tend to form a distinct phylogenetic group. This is basically consistent with other dendrograms based on the ITS region (CHILLALI *et al.* 1998a, b).

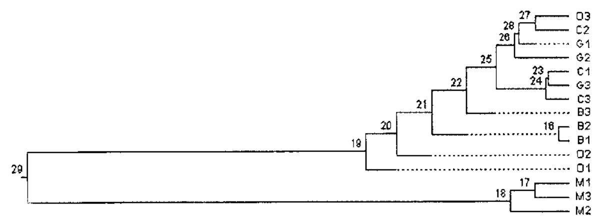

Figure 1. Phylogenetic tree. Based on cluster analysis of the nucleotide sequences of the ITS region in 15 *Armillaria* isolates. Isolates coding according to Table 1. The length of horizontal pair of branches represents the distance.

Primer and taxon-specific priming PCR

In spite of the similarity of the internal transcribed spacer region of the five *Armillaria* species, sequence differences in the ITS II were used to design 20mer oligonucleotides to attempt fungal differentiation by taxon-specific priming PCR. For *A. gallica*, two primers were selected.

The suitability of taxon-specific primers is based on intraspecific identity in the target region of rDNA. For all species, three isolates were sequenced. An additional precaution was to sequence the ITS in both directions. So far, intraspecific variation within the ITS base

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