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Molecular diagnostics of clinical strains of filamentous Basidiomycetes

Molekulare Diagnostik klinischer Stämme filamentöser Basidiomyzeten

G. S. de Hoog and A. H. G. Gerrits van den Ende

Key words. Basidiomycetes, ribosomal DNA, small subunit (18S) rDNA, variable domain 9, internal transcribed spacer, diagnostics, restriction fragment length polymorphism.

Schlüsselwörter. Basidiomyzeten, ribosomale DNA, small subunit (18S) rDNA, Variable Domäne 9, internal transcribed spacer, Diagnostik, RFLP.

Summary. Eleven clinical and veterinary strains of filamentous Basidiomycetes were compared with 15 reference strains representing the orders Aphyllophorales and Agaricales. The methods used were restriction analysis of small subunit (18S) (SSU) rDNA and internal transcribed spacers (ITS) 1 and 2 and variable domain 9 (V9)-ITS1 sequencing. Six strains were found to belong to the teleomorph genera *Schizophyllum* or *Coprinus*, whereas five could not be identified unequivocally. A rapid diagnostic overview is obtained with *Hae*III and *Hinf*I digestion of the ITS region.

Zusammenfassung. Elf klinische und veterinäre Stämme filamentöser Basidiomyzeten wurden untereinander und mit fünfzehn Referenzstämmen der Ordnungen Aphyllophorales und Agaricales verglichen. Angewandt wurden Restriktionsanalysen der SSU rDNA und der ITS 1 und 2 und Sequenzierung von Region V9-ITS1. Sechs Stämme wurden als Repräsentanten der teleomorphen Gattungen *Schizophyllum* oder *Coprinus* identifiziert; die übrigen fünf konnten nicht mit Sicherheit bestimmt werden. Eine rasche diagnostische Übersicht wurde durch *Hae*III und *Hinf*I Verdauung der ITS Region erreicht.

Centraalbureau voor Schimmelcultures CBS, Baarn, The Netherlands

Correspondence: Prof. Dr G.S. de Hoog, Centraalbureau voor Schimmelcultures, PO Box 273, NL-3740 AG Baarn, The Netherlands.

Abbreviations: SSU, small subunit (18S); ribosomal DNA (rDNA); V9, SSU variable domain 9; ITS, internal transcribed spacer; RFLP, restriction fragment length polymorphism.

Introduction

Of the fungi with clinical significance, only relatively few are of basidiomycetous affinity. Among these the heterobasidiomycetous yeasts are by far the most important: species of *Cryptococcus*, *Malassezia* and *Trichosporon* are among the classical pathogens, or opportunists, known since the previous century [1]. Homobasidiomycetes in culture generally produce a whitish, fluffy, filamentous mycelium rather than yeast cells, often with more or less differentiated arthroconidia. In the natural environment (wood, soil, compost, etc.) they are recognized by macroscopically visible fruit bodies. Clinical strains identified thus far belong to two orders of the Homobasidiomycetes, namely the Aphyllophorales and the Agaricales.

Until recently, only a few cases had been published in which a member of these orders was the aetiological agent [2]. Most of them belonged to the genera *Schizophyllum* (Aphyllophorales) or *Coprinus* (Agaricales). A significant increase in the number of cases has been noted during the past 5 years. Possibly, this is due to a growing awareness that such hyphal thalli may indeed represent agents of mycoses. Their mycelia may be rather undiagnostic as they are morphologically similar over larger taxonomic groups, and often lack key features such as clamp connections and conidia.

For the production of fruit bodies, and for the demonstration of septal ultrastructure, special methods are required [3, 4] that are not routinely available in clinical laboratories.

In the present article, the ribosomal gene of a number of recent clinical isolates that were suspected or proven to be of basidiomycetous affinity is compared with reference strains. On the basis of partial small subunit (18S) (SSU) and internal transcribed spacer (ITS) 1 sequencing and of restriction analysis of SSU and ITS1–2 amplicons, a diagnostic system applicable in the clinical laboratory is developed.

Materials and methods

DNA extraction

The strains studied are listed in Table 1. Approximately 1 cm^2 of mycelium of 30-day-old cultures was transferred to a 2.0-ml Eppendorf tube containing 300 µl of CTAB (cetyltrimethylammoniumbromide) buffer and about 80 mg of a silica mixture (Silica Gel H, Merck 7736/Kieselgur Celite 545, Machery, 2:1, w/w). Cells were disrupted mechanically with a tight-fit sterile pestle for ≈ 1 min. Subsequently 200 µl of CTAB buffer was added, vortexed and incubated for 10 min at 65 °C. After the addition of 500 µl of chloroform, the solution was centrifuged for 5 min at 14 000 r.p.m., and the supernatant transferred to a new tube with 2 vols of cold 96% ethanol. DNA was allowed to precipitate for 30 min at −20 °C and then centrifuged again for 5 min at 14 000 r.p.m. Subsequently, the pellet was washed with cold 70% ethanol. After drying at room temperature, it was resuspended in 97.5 µl of TE buffer plus 2.5 µl of 20 U ml^{-1} RNAse and incubated for 5 min at 37 °C.

PCR amplification

Fragments of rDNA were amplified using the primer pairs (for RFLP) NS1–NS24 (SSU) plus ITS1–ITS4 [5] and (for sequencing) NS1–ITS4 in a reaction mixture containing 30 µl of distilled water, 5 µl of PCR buffer, 10 µl of N buffer, 1 µl of each primer (50 pmol), 1 µl of Ampliterm DNA polymerase and 2 µl of fungal DNA. Forty cycles were performed in a Bio-med 60 thermocycler: 94 °C for 60 s (delay); 94 °C for 60 s (denaturation); 58 °C for 60 s (annealing); 72 °C for 120 s (extension), with a final extension at 72 °C for 60 s.

Restriction analysis

The PCR products were digested with the following enzymes: *Hinf*I, *Hae*III, *Rsa*I and *Dde*I, according to the suppliers' instructions. Fragments were electrophoresed in 1.5% agarose gels, in TBE buffer (Tris–Borate–EDTA) at 3–6 V cm^{-1} for 3 h and stained with ethidium bromide. Biozym Low Ladder was used as the marker. The gels were analysed and photographed using the Image Master System (Pharmacia). The lengths of the resulting bands were measured using VDS software version 2.0 and the data were transferred to a Quattro Pro 5.0 matrix. Molecular weights were compared with those present in a database available at CBS.

Sequencing

The remaining primers and dNTPs were removed with Microspin S-300 HR (Pharmacia). The sequencing primers used were V9G (5′-TTACGTCCCTGCCCTTTGTA-3′) and 5.8G (5′-AATGTGCGTTCAAAGATTCG-3′), spanning the V9 variable domain of the SSU rDNA until the central region of the 5.8S rDNA. Sequencing was performed using a primer terminating protocol in a Perkin-Elmer 9600 automatic sequencer, using 25 PCR cycles as follows: 96 °C for 10 s (denaturation), 50 °C for 5 s (annealing), 60 °C for 4 min (extension).

Sequence alignment and tree construction

The sequences obtained with the two primers were aligned and adjusted using the Align and DCSE package [6]. Trees were calculated using the neighbour joining method of the Treecon program [7].

Results

The SSU amplicon of the majority of the strains had a length of about 1800 bp, as expected (Table 1). In two strains of *Schizophyllum commune* the amplicons were about 2100 bp in length, indicating the presence of an intron-like element. Despite the insertion in the 18S ribosomal gene, the three strains of this species showed identical patterns when ITS amplicons were digested with *Hae*III or *Hinf*I (Table 1). This indicates that these strains are closely related and that the 18S gene outside the insertion is likely to be identical or nearly identical.

Restriction patterns of 1800-bp SSU rDNA were identical or very similar in all strains analysed when *Rsa*I or *Dde*I was used (Table 1). With *Hinf*I four patterns were generated, and seven were

rains examined with their SSU and ITS restriction patterns

	Number	Source	NS1–NS24	*Hae*III	*Hinf*I	*Rsa*I	*Dde*I	ITS1–ITS4	*Hae*III	*Hinf*I	*Rsa*I	*Dde*I
commune	CBS 405.96	Lung [3]	2100	A	A	A	A	600	A	A	A	A
commune	CBS 333.85	Liquor	2100	A	B	B	B	600	A	A	B	B
commune	CBS 340.81		1800	B	C	C	C	600	A	A	B	A
us	FMR 5149	Air, landfill	1800	C	D	C	C	650	B	B	C	C
us	FMR 5150	Leaves, landfill	1800	C	D	C	C	650	B	B	C	C
a aspergillata	CBS 519.91(T)	Skin	ND					650	B	B	C	C
a aspergillata	FMR 5148	Air, landfill	1800	C	D	C	C	650	B	B	C	C
a aspergillata	FMR 5151	Air, landfill	1800	C	D	C	C	650	B	B	C	C
a aspergillata	FMR 5153	Soil, landfill	1800	C	D	C	C	650	B	B	C	C
a verticillata	CBS 509.94	Nervous tissue	1800	C	D	C	C	650	B	B	C	C
a aspergillata	CBS 106.97	Lung	1800	C	D	C	C	650	B	B	C	C
a aspergillata	CBS 833.96	Lung [12]	1800	C	D	C	C	650	B	B	C	C
es	CBS 258.96	Sputum [13]	1800	D	D	C	C	650	C	C	C	D
a candelabrata	CBS 517.9(T)	Dung	1800	D	D	C	C	650	H	C	C	E
a verticillata	FMR 3936(T)	Spinal catheter	1800	E	D	C	C	650	H	D	C	B
a verticillata	FMR 5154	Soil, landfill	1800	E	D	C	C	650	H	D	C	B
a verticillata	FMR 5155	Air, landfill	1800	E	D	C	C	650	H	D	C	B
a verticillata	FMR 5156	Air, landfill	1800	E	D	C	C	650	H	D	C	B
mycete	CBS 237.96	Eye [14]	1800	F	E	C	C	630	E	H	E	B
e	CBS 800.95	Dolphin	1800	C	E	D	C	600	F	F	D	A?
hrysosporium	CBS 129.27(T)	Soil	1800	G	E	C	C	600	F	F	B	B
hrysosporium	CBS 255.65	Soil	1800	G	E	C	C	600	F	F	B	B
hrysosporium	CBS 363.65	Soil	1800	G	E	C	C	600	F	F	B	B
dimorphosporum	CBS 419.70(T)	Potato	1800	H	E	D	C	650	G	G	C	C
lusta	CBS 230.93	Eucalyptus	1800	F	?	C?	?	600	D	E?	B	A?
a species	CBS 257.96	Liquor	1800	F	D?	C?	C?	600	D	E	D	A?

lbureau voor Schimmelcultures, Baarn; FMR, Facultad de Medicina, Reus; T, type strain.

generated with *Hae*III. ITS restriction patterns were particularly variable with *Hae*III and *Hinf*I, both generating seven different patterns.

In main traits, RFLP of 18S and ITS allows the distinction of five groups, each containing more than one strain (Table 1). Group 1 contains the three strains identified as *S. commune*. Among these are two clinical strains and one environmental strain (Table 1). Some heterogeneity is found in ITS restriction patterns. Group 2, including four clinical strains, contains *Coprinus cinereus* and its anamorph *Hormographiella aspergillata* plus one strain identified as *Hormographiella verticillata*, which apparently is a misidentification. The core of *H. verticillata* is found in group 3, which contains a single clinical strain but as yet no teleomorph. Group 4 contains three strains of *Phanerochaete chrysosporium* but no clinical strain. Digestion patterns of group 5, containing *Bjerkandera adusta* and a clinical strain, were interpreted with difficulty as the length differences between fragments were minute.

Five strains could not be unambiguously attributed to any of the groups above. Among these was *Disporotrichum dimorphosporum*, which could not be assigned to any of the remaining reference teleomorphs. None of the clinical strains appeared similar to this species. The type strain of *Hormographiella candelabrata* was found to resemble group 3 but showed different patterns with *Hinf*I and *Dde*I. Three clinical strains could not be identified with certainty. CBS 258.96 from sputum was found to be intermediate between *H. aspergillata* and *H. verticillata*. CBS 237.96 had three 18S RFLP patterns in common with *P. chrysosporium* but had clearly different ITS restriction profiles. A strain from dolphin could not be identified either.

A distance tree of the moderately variable V9 domain of SSU rDNA plus the partial 5.8S rDNA gene is presented in Fig. 2, and a tree of the highly variable ITS1 spacer is presented in Fig. 3, both based on the sequence alignment presented in Fig. 1. The V9 domain as well as the SSU terminus and the 5.8S gene could be aligned with confidence. A tree using 199 SSU and 108 5.8S positions of all strains analysed is presented in Fig. 2. The RFLP groups 2 and 3 cannot be clearly separated from each other. The two sequenced *S. commune* strains (group 1) are found together but at a rather large distance. The three strains of *P. chrysosporium* (group 4) are found to be very similar in the 18S distance tree (Fig. 2) and show very little variation in ITS1 sequences (Fig. 1). *D. dimorphosporum* and *B. adusta* are found at rather isolated positions (Fig. 2). The clinical strains that showed ambiguous results with RFLP could not be identified with any of the reference strains used.

A finer resolution of the *Coprinus–Hormographiella* complex is achieved when the positions 189–455, spanning the ITS1 spacer, are taken into account (Fig. 3); the remaining species could not be aligned with sufficient confidence. There is a marked distinction between *C. cinereus–Hormographiella aspergillata* (RFLP group 2), clearly representing a single taxon, and the remaining strains (RFLP group 3). The latter group is significantly more heterogeneous.

Discussion

In recent years it has become apparent that higher, filamentous Basidiomycetes occasionally do occur as causative agents of human and animal mycoses. Teleomorph connections of clinical fungi have been proven by a demonstration of fruit bodies [4] and septal ultrastructure [3, 8]. Aetiological agents identified thus far belong to different orders of the class Basidiomycetes: Aphyllophorales (*Phanerochaete*, *Schizophyllum*) and Agaricales (*Coprinus*). These taxonomic groups are broadly recognized in the grouping made on the basis of RFLP patterns: group 1 corresponds to the *Schizophyllum* relationship (Aphyllophorales), groups 2 and 3 to the Agaricales, and groups 4 and 5 to the Aphyllophorales. *B. adusta* and the clinical strain CBS 257.96 were found at a larger distance from all groups, including the aphyllophoralean species *P. chrysosporium*. With the remaining species, despite the fact that the taxonomic distances between groups are currently recognized as being at the ordinal level, SSU RFLP patterns generated using *Rsa*I and *Dde*I were found identical or similar in all groups except *B. adusta*. Patterns generated with *Hinf*I were found to differ according to the taxonomic borderlines of the orders and this enzyme is therefore a useful indicator of main relationships.

Of the 11 clinical strains analysed, six were attributed to known species: *S. commune, C. cinereus* or *H. verticillata*. Strains CBS 258.96 and 517.91 (type strain of *H. candelabrata*) were similar to *C. cinereus* and thus might represent another *Coprinus* species. Similarly, CBS 257.96 might represent another *Bjerkandera* species. The strains CBS 237.96 and 800.95 seem too remote to be associated with any of the reference taxa. None of the clinical strains came close to *D. dimorphosporum*. The number of Basidiomycetes involved in human or animal mycoses is apparently larger than the ones mentioned in the literature [9], but, on the other hand, they are not randomly distributed over the orders of Basidiomycetes. Some relatively limited groups seem to possess more virulence

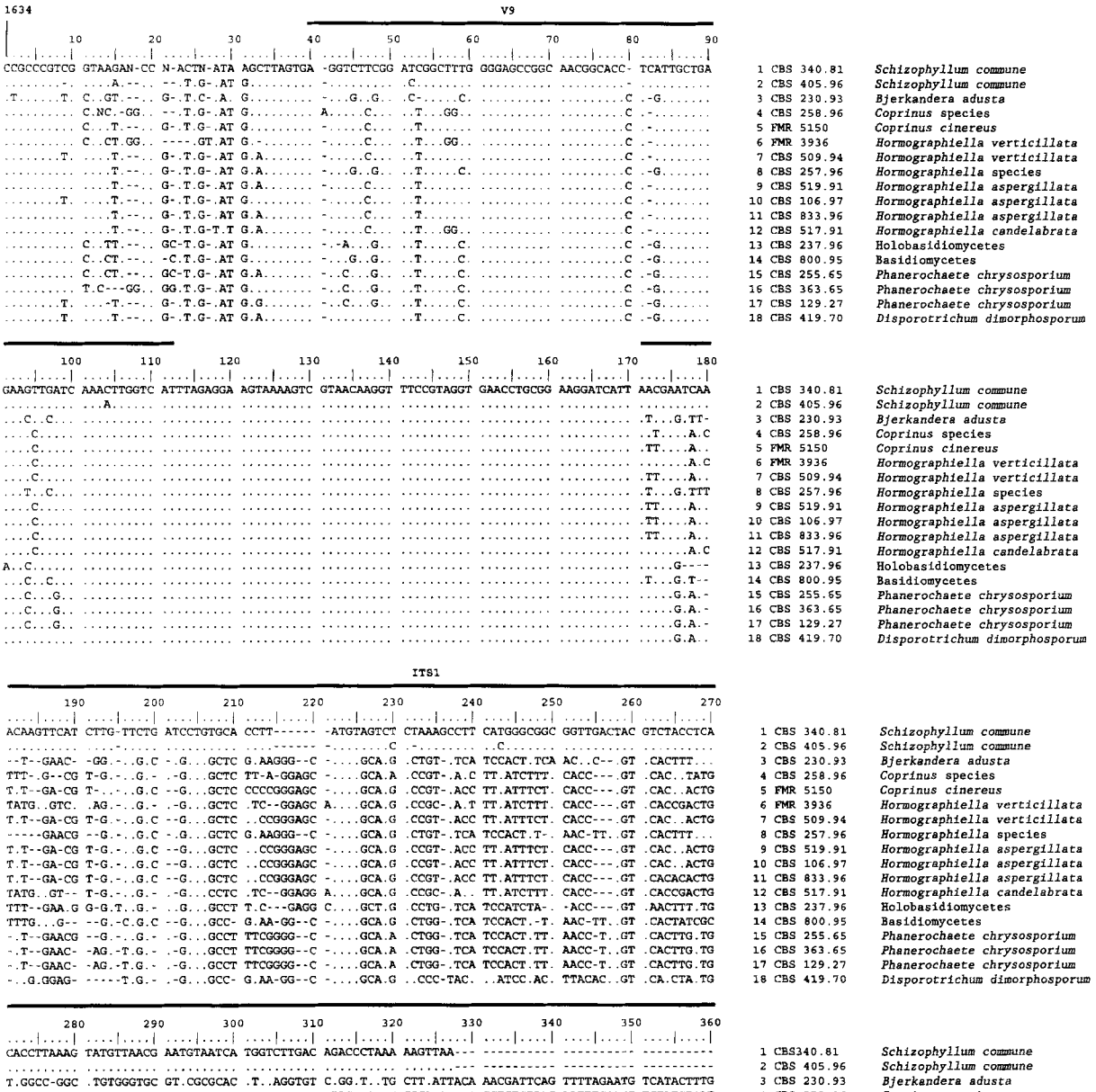

Figure 1. Alignment of partial SSU rDNA and ITS1 sequences starting at position 1634 of the 5′ end with reference to *S. cerevisiae. Schizophyllum commune*, CBS 340.81 is used as leading strand. Dots indicate nucleotides identical to this reference; dashes represent deletions necessary for alignment. The upper lines mark the positions of the variable domain V9 of SSU rDNA, the ITS1 spacer and the 5.8S rDNA gene.

factors than the remaining thousands of culturable species of filamentous Basidiomycetes.

RFLP of the complete, PCR-amplified SSU ribosomal gene provides a fairly confident insight into the relationships of the fungi under study. The three groups, namely *Schizophyllum* (Aphyllophorales), Agaricales and other Aphyllophorales are distinguishable with *Hinf*I digestion, only *Bjerkandera* being found as a fourth group (Table 1). *Disporotrichum* is an as yet unclassified anamorph [10]. *Hae*III generates more patterns, leading to differences within the orders. Earlier RFLP studies [11] have shown that also in hyphomycetes of ascomycetous affinity *Hae*III has restriction sites in

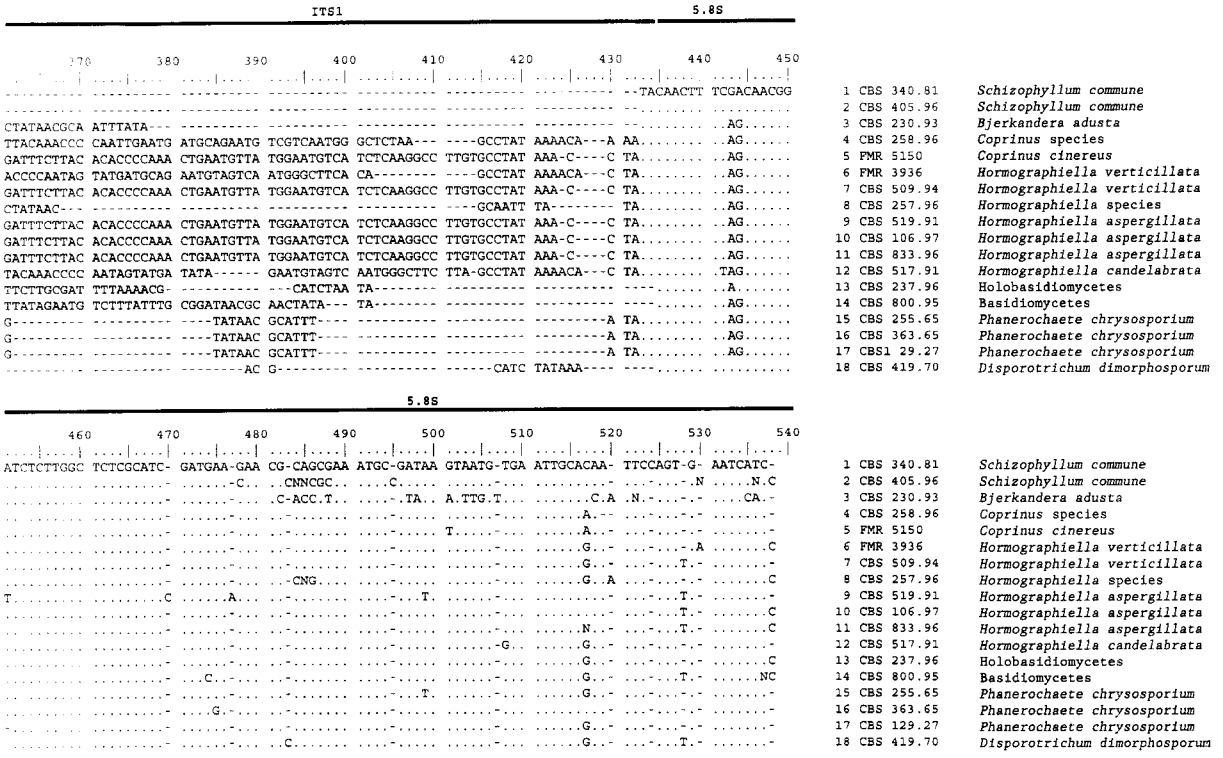

Figure 1. (*continued*)

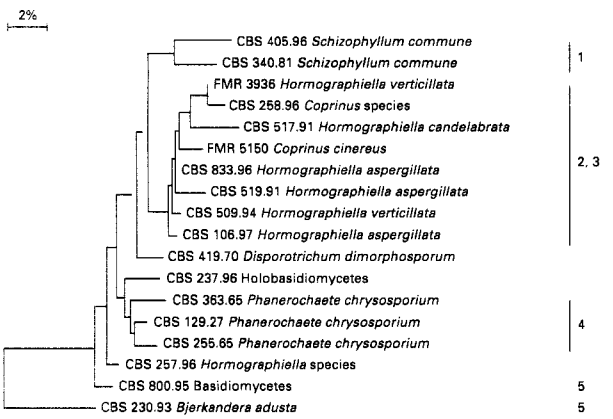

Figure 2. Distance tree of studied filamentous Basidiomycetes generated with the neighbour-joining algorithm using the Treecon software package, based on unambigously aligned positions of SSU rDNA. *Bjerkandera adusta* was used as outgroup.

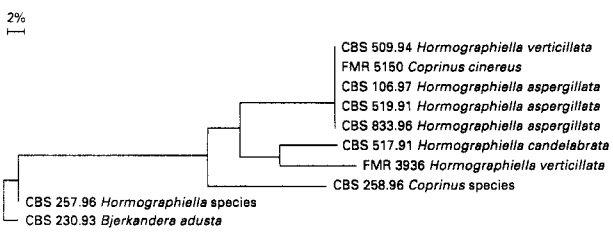

Figure 3. Distance tree of the *Hormographiella–Coprinus* complex generated with the neighbour-joining algorithm using the Treecon software package, based on unambiguously aligned positions of ITS1. *Bjerkandera adusta* was used as outgroup.

relatively variable 18S rDNA domains, whereas *Hinf*I and *Sau*3A1 (with restriction sites identical to those of *Dde*I) cut in conserved areas and thus the resulting patterns are expected to be identical when the fungi are phylogenetically not too remote. *Dde*I digestion patterns are nearly invariant, whereas with *Hinf*I and *Sau*3A1 three and four groups, respectively, could be distinguished.

Basic relationships of the strains studied are evaluated on the basis of a sequence comparison of the terminal portion of 18S rDNA that includes the V9 variable domain. This could be sequenced in a single run together with ITS1 when the primer V9G was used. The number of informative sites, however, is rather low, and the groups distinguished in the distance tree (Fig. 2) are mostly statistically insignificant having low bootstrap values (not shown). The RFLP groups 2 and 3 could not be distinguished as strain FMR 5150 proved to be a sister group to the remaining species. Figure 3 shows that RFLP group 2 probably comprises a single species, whereas group 3 is more heterogeneous. The resolution power of morphology is insufficient in the Homobasidiomycetes as strain CBS 509.94 was incorrectly identified as *H. verticillata,* and the remote, *Bjerkandera*-like strain CBS 257.96 had been supposed to be a *Hormographiella* on the basis of the presence of arthroconidial elements.

The hypervariable ITS1 spacer was sequenced

in strains representing all groups found with restriction analysis. Most sequences could hardly be aligned. In contrast, the type of *H. aspergillata*, from a clinical source, and *C. cinereus* proved to be nearly identical (Fig. 1), confirming the conclusion presented by Gené *et al.* [4] on the basis of nDNA restriction analysis. *H. verticillata*, represented by clinical isolate FMR 3936 and three environmental isolates (Table 1), had similar 18S RFLP patterns but was otherwise clearly different from *H. aspergillata* with ITS1–2 RFLP (Table 1) and ITS1 sequencing (Fig. 3); probably another *Coprinus* species is concerned.

The applied primers V9G and 5.8G worked well in all fungal orders studied, despite the fact that in some strains a base substitution was found near the 5′ ends of these primers. To increase amplification confidence in filamentous Basidiomycetes it is therefore recommended that one base is replaced and three bases omitted at the 5′ end of these primers and that they are replaced by the primers V9D (5′-TTAA-GTCCCTGCCCTTTGTA-3′) and 5.8D (5′-GTGCGTTCAAAGATTCG-3.

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