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Phylogenetic analysis of ten black yeast species using nuclear small subunit rRNA gene sequences

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Abstract

The nuclear small subunit rRNA genes of authentic strains of the black yeasts *Exophiala dermatitidis*, *Wangiella dermatitidis*, *Sarcinomyces phaeomuriformis*, *Capronia mansonii*, *Nadsoniella nigra* var. *hesuelica*, *Phaeoannellomyces elegans*, *Phaeococcomyces exophialae*, *Exophiala jeanselmei* var. *jeanselmei* and *E. castellanii* were amplified by PCR and directly sequenced. A putative secondary structure of the nuclear small subunit rRNA of *Exophiala dermatitidis* was predicted from the sequence data. Alignment with corresponding sequences from *Neurospora crassa* and *Aureobasidium pullulans* was performed and a phylogenetic tree was constructed using the neighbor-joining method. The obtained topology of the tree was confirmed by bootstrap analysis. Based upon this analysis all fungi studied formed a well-supported monophyletic group clustering as a sister group to one group of the Plectomycetes (Trichocomaceae and Onygenales). The analysis confirmed the close relationship postulated between *Exophiala dermatitidis*, *Wangiella dermatitidis* and *Sarcinomyces phaeomuriformis*. This monophyletic clade also contains the teleomorph species *Capronia mansonii* thus confirming the concept of a teleomorph connection of the genus *Exophiala* to a member of the Herpotrichiellaceae. However, *Exophiala castellanii* did not belong to this clade. Therefore, this species is not the anamorph of *Capronia mansonii* as it was postulated.

Introduction

Black yeasts are dematiaceous fungi that may occur as solitary cells during a part of their life cycle. Frequently, black yeasts are synanamorphs associated with dematiaceous hyphomycetes. The majority of black yeasts have teleomorphs in the ascomycetes, though in different orders (de Hoog & McGinnis 1987).

During the last decade there has been an increasing number of reports implicating black yeasts as causative agents of various kind of mycosis in humans and animals (Dixon & Polak-Wyss 1990; Kwon-Chung & Bennett 1992; Matsumoto et al. 1993). *Exophiala dermatitidis* (Kano) de Hoog is now regularly isolated from sputum of patients suffering from cystic fibrosis (Haase et al. 1991; Kusenbach et al. 1992). Systemic phaeohyphomycotic infections may also occur as brain lesions frequently with lethal outcome (Matsumoto et al. 1984, 1993; Hiruma et al. 1993). *Exophiala jeanselmei* is frequently involved in infections of nails, cutis, and subcutis (Schwinn et al. 1993). In addition to their role as human pathogens, black yeasts are involved in the degradation of marble and sand stone (Braams 1993; Wollenzien et al. 1994).

Conidiogenesis in black yeasts is either blastic, annellidic or phialidic. Upon changes in environmental conditions (e.g., during laboratory cultivation), black yeasts may respond by expressing different morphological forms, thereby changing their mode of conidiogenesis (Butterfield & Jong 1976; de Hoog 1993; de Hoog et al. 1994b). Since the form of conidiogenesis is the leading criterion for assigning black yeasts to certain anamorph genera (de Hoog 1977; Cole 1978), this variability of conidiogenesis has a negative impact

on successful species classification. Observing conidiogenesis is further complicated by the fact that the conditions and the media used for this analysis are not standardized. Dissimilar interpretations of phenomena have led to the assignment of a single isolate to different genera. For example, the strain CBS 207.35 was classified in the genus *Exophiala* Carmichael as *E. dermatitidis* based on the observed annellidic conidiogenesis. In the same year McGinnis (1977) established the genus *Wangiella* based on the same strain, but described the prevalent type of conidiogenesis as phialidic without collarettes. Problems of specific delimitation arose with strain ATCC 34100, classified by McGinnis (1977) as *Wangiella dermatitidis* McGinnis but by de Hoog (1977) as *Exophiala mansonii* (Castell.) de Hoog. It was questioned whether *E. castellanii* Iwatsu et al. (new name for *E. mansonii*) was a separate species or belonged to the *dermatitidis* cluster.

Physiological testing used for species delimitation and identification is tedious and time-consuming (de Hoog & Haase 1993). Furthermore, physiological data are incomplete or not yet available for most of the medically important black yeasts. Therefore, there is an urgent demand for a reliable and easy-to-perform procedure for the identification of black yeasts recovered from clinical specimens.

Recently, molecular techniques have become widely utilized in fungal taxonomy (Bruns et al. 1991; McGinnis et al. 1992). Few molecular studies have examined the dematiaceous fungi. Analysis of the genes coding for small subunit (SSU) ribosomal RNA indicated that the sequence of this gene can provide a reliable foundation upon which taxonomic problems may be resolved (Bruns et al. 1991; Kurtzman 1992; Wilmotte et al. 1993).

In the present study we have sequenced > 96% of the SSU-rRNA genes of *Exophiala dermatitidis*, of its putative synonyms and of some other *Exophiala* species [*E. jeanselmei* (Langer.) McGinnis & Padhye, *E. castellanii*] frequently recovered from clinical specimens. *Nadsoniella nigra* Issatchenko var. *hesuelica* Lyakh & Ruban was studied since this strain was supposed to be close to *E. jeanselmei* (de Hoog 1985). Since species of the genera *Phaeococcomyces* (de Hoog) de Hoog and *Phaeoannellomyces* synanamorphs in the genus *Exophiala* we have also included the type isolates (*Phaeococcomyces exophialae* (de Hoog) de Hoog and *Phaeoannellomyces elegans* (McGinnis & Schell) in our analysis. According to Schol-Schwarz (1968), and Müller et al. (1987) *Capronia mansonii* (Schol-Schwarz) E. Müller et al. should represent the holomorph of *E. castellanii* (formerly treated as *Exophiala mansonii*).

In the present paper we intend to address the questions outlined above by using molecular techniques. In addition, we aim to show the phylogenetic relationships of black yeasts to other main groups of Ascomyceta. The sequence data provided can potentially be used for the development of an identification system allowing quick and objective species assignment in the black yeasts.

Materials and methods

Strains and culture methods

Strains used listed in Table 1. Stock cultures were maintained on potato-dextrose agar (Merck AG, Darmstadt, FRG) slants at 8° C and as stock cultures on ceramic beads (Microbank™, Mast, Hamburg, FRG) at - 80° C. Inocula were prepared as exponential phase suspensions of yeast cells according to de Hoog et al. (1994b). Fungi were cultivated in a Sabouraud's broth (Difco, Detroit, USA) with constant shaking at 30° C for 5 days.

DNA extraction

DNA was prepared according to the procedure given by Goodwin & Lee (1993). Purified DNA was dissolved in TE buffer, aliquoted and stored at - 20° C.

PCR amplification

The SSU-rRNA gene was amplified using the NS1/NS8 primers of White et al. (1990). The PCR reaction was performed with 1 μg DNA and 10 μl of 10 × PCR buffer (500 mM KCl, 100 mM Tris HCl, pH 8.3, 15 mM $MgCl_2$, 0.01% gelatin), 62.5 μM (final concentration) of deoxyribonucleotide triphosphates [using the desoxynucleoside-triphosphate-set® (Boehringer, Mannheim, FRG)], 5 pmol each of NS1 and NS8 primers (for some isolates NS8 was replaced by ITS2; White et al. 1990), and 2.5 units of Taq polymerase (Boehringer). Distilled deionized water was added to a final volume of 100 μl. Thermal cycling parameters were set at 94° C for 1 min (denaturation), 55° C for 1 min (annealing), and 72° C for 1.5 min (extension) for 27 cycles. An extension step at 72° C for 7 min was performed after the final cycle. Amplifi-

Table 1. Fungal strains used for sequencing of SSU-rRNA.

Species	Strain-number	EMBL-accession numbers	Taxon-Abbreviation
Exophiala dermatitidis (Kano) de Hoog	CBS 207.35^T	X 79312–79314	E.der
Wangiella dermatitidis (Kano) McGinnis	ATCC 34100	X 71315–79317	W.der
Wangiella dermatitidis (Kano) McGinnis (= *Phaeotheca* synanamorph of *W. dermatitidis*)	KU A-0052	X 80702	P.der
Sarcinomyces phaeomuriformis Matsumoto et al.	CBS 131.88^T	X 80710	S.pha
Exophiala jeanselmei (Langeron) McGinnis & Padhye var. *jeanselmei* (Benedek & Specht) de Hoog	ATCC 34123^T	X 80705	E.jea
Nadsoniella nigra Issatschenko var. *hesuelica* Lyakh & Ruban	CBS 546.82	X 80707	N.nig
Phaeoannellomyces elegans McGinnis & Schell	UTMB 1286^T	X 80708	P.ele
Phaeococcomyces exophialae (de Hoog) de Hoog	CBS 668.76^T	X 80709	P.exo
Exophiala castellanii (Iwatsu et al.) (= *Exophiala jeanselmei* (Langeron) McGinnis & Padhye var. *castellanii*) Iwatsu & Udagawa	CBS 158.58^{NT}	X 78480	E.man
Capronia mansonii (Schol-Schwarz) E. Müller	CBS 101.67^T	X 79318	C.man

cation products were purified using the 'Wizard DNA clean up' kit® (Promega, Madison, USA).

Direct sequencing

Direct sequencing was done with 10 μl of purified amplification products and 10 pmol primer (NS1–NS8) using the PRISM™ Ready Reaction DyeDesoxye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, FRG) according to the manufacturer's instructions. Sequencing assays were analyzed on an automated DNA Sequencer 373 A (Applied Biosystems). In order to achieve optimal overlap of the partial sequences, two additional primers were designed: LS-292F (5'-ATTCAAATTTCTGCCCTATCAAC-3') and LS-825F (5'-GTTCTATTTTGTTGGTTTCT-3'). All primers used were synthesized with a DNA Synthesizer Oligo 1000 (Beckman, Munich, FRG). Compilation and analysis of sequence data were done with the aid of the PC Gene® program (IntelliGenetics, Mountain View, USA).

Reconstruction of secondary structure

The mature SSU-rRNA of *Exophiala dermatitidis*, CBS 207.35 was folded into the 'universal' secondary structure adopted for eukaryotic SSU-rRNA (Neefs et al. 1993; Van de Peer et al. 1994). Drawing of this structure was performed by using the program 'CARD' (Winnepenninckx et al. 1995).

Sequence alignment and phylogenetic tree-construction

The sequences determined in this study were aligned with all other complete (meaning > 90% sequence information of this gene) ascomycete SSU-rRNA sequences (Neefs et al. 1993) so far published (October 1994) on the basis of similarity in primary and secondary structures, by means of a specially developed sequence editor (de Rijk & de Wachter 1993). The phylogenetic tree was constructed using the neighbor-joining method (Saitou & Nei 1987), based on a dissimilarity matrix corrected for multiple mutations per site (Jukes & Cantor 1969). The complete alignment of all sequences was taken into account for distance calculation. The basidiomycete *Filobasidiella neoformans* Kwong-Chung was chosen as an outgroup organism. Confidence values for individual branches were determined by bootstrap analysis in which 500 bootstrap trees were generated from resampled characters (Felsenstein 1985). Distance calculations, tree construction, and bootstrap analysis were performed with

the software package TREECON (Van de Peer & de Wachter 1993, 1994).

Results

PCR-based DNA amplification performed with the primers NS1/NS8 yielded products of different lengths. Amplicons obtained from *Exophiala* (*Wangiella*) *dermatitidis* and its *Phaeotheca*-like synanamorph, and *Sarcinomyces phaeomuriformis* showed a lenght of approximately 2.7 kb, whereas those from *Nadsoniella nigra* var. *hesuelica* and *Phaeoannellomyces elegans* showed a length of 2.2 kb and 2.45 kb, respectively. Amplification products of all other fungi tested, including *Neurospora crassa* Shear & Dodge, exhibited the expected length of 1.8 kb.

Subsequent sequencing of the amplification products revealed the presence of two different putative introns in *E. dermatitidis*, the *Phaeotheca*-like synanamorph, in ATCC 34100 and in *S. phaeomuriformis*. These introns were situated downstream of nucleotide positions 561 and 1164, respectively, according to the SSU-rRNA sequence-numbering of *N. crassa* (EMBL accession number X04971). The SSU-rRNA gene of *N. nigra* var. *hesuelica* harbored a different putative intron downstream of nucleotide position 1210 whereas the corresponding sequence from *P. elegans* carried yet another intron downstream of nucleotide position 1764. Details about these introns will be presented elsewhere.

Alignment of the obtained sequences is shown in Fig. 1. The SSU-rRNA gene sequence from *N. crassa* was chosen as the leading sequence. For comparison the SSU-rRNA sequence of a saprobic black yeast, *Aureobasidium pullulans* (de Barry) Arnaud, was added (EMBL accession number M55639). In Fig. 1 the locations of the primers used are marked by bold letters. Sequence differences clustered primarily between the nucleotide positions 65–82, 122–187, 322–386, 644–710, 752–770, 1224–1267, 1355–1378, and 1720–1747 (Fig. 1). As compared to *N. crassa*, most of these sequence differences occur in all species studied, including *A. pullulans*.

The predicted secondary structure of the gene products of CBS 207.35, *E. dermatitidis* is given in Fig. 3. Secondary structures of helices 19, 20, and 21 are different from that of *Candida krusei* (Castell.) Berkhout (Hendriks et al. 1981), *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Gutell 1993) and *Ustilago maydis* (de Candolle) Corda (de Wachter et al. 1992), but nearly identical to that of *Endomyces fibuliger* Lindner (Wilmotte et al. 1993) and *Acanthamoeba griffini* Sawyer (Gast et al. 1994).

A phylogenetic tree calculated by utilizing each (nearly) complete ascomycetal SSU-rRNA gene published thus far is given in Fig. 3. In this tree the ten species studied cluster as a monophyletic sister-group to the Plectomycetes (Eurotiales and Onygenales).

Discussion

Analysis of sequence data

Comparison of ten (nearly) entire SSU-rRNA sequences to the corresponding sequence from *Neurspora crassa* revealed a remarkable variability in segments corresponding to stems of helices 12 and 13 (Figs 1, 2). These regions are supposed to be highly conserved (Neef et al. 1993). This false assumption may have arisen through the common practice of comparing only partial sequences of selected variable regions (for example, Spatafora & Blackwell 1993), supposing that construction of a phylogenetic tree based on entire SSU-rRNA sequences or on partial variable regions of the genes would lead to identical results (McCarrol et al. 1983). Our data indicate that it is advisable to sequence the entire SSU-rRNA gene when primers are designed for recognition of taxa above the level of species. Furthermore, there is a need to confirm the assumed conservation of target sites for 'universal' primers (White et al. 1990). This sequence information is obtainable only by using additional primers. By employing the primer LS-292F we revealed sequence diversity located at the NS3 primer site (Fig. 1).

Sequencing of the SSU-rRNA gene amplified by PCR instead of rRNA-derived cDNA templates also revealed the occurrence of introns in the nuclear SSU-rRNA genes of fungi (de Priest 1993; de Wachter et al. 1992). These insertions could severely affect hybridization assays with genomic DNA (e.g., 'ribotyping' experiments) or PCR-based species assignment using restriction fragment patterns of amplification products (for example, Shen & Lanchance 1993; Bernier et al. 1994). Thus, for the development of molecular detection or diagnostics of black yeasts using SSU-rRNA genes, sequencing of the entire gene is recommended.

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                                  ⇨ NS 1 ⇦
N.cra  TACCTGGTTG ATTCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTTTAAGC A-ATTAAACC GCGAAACTGC GAATGGCTCA - 100
A.pul  A.......... ..C..... .......... .......... .......... .......... ....A..... ...C..T..G .T..... .......... ..........
E.der  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNN.A... ..C..... .......... ....A..... .CT...T... .......... ......T...
W.der  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN N......... .......... ....A..... .CT...T... .......... ..........
P.der  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNN... ..T..... .......... ....A..... .CT...T... .......... ..........
S.pha  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNN... ..T..... .......... ....A..... .CT...T... .......... ..........
C.man  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN N.C..... .......... ....A..... .CT...T... .......... ......T...
N.nig  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN .......... ....A..... ....C.T..T .T..... .......... ..........
P.ele  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN N.C..... .......... ....A..... ....C.T..T .T..... .......... ..........
P.exo  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNN...... ....A..... ....C.T..T .T.......T ..........
E.jea  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN N.C...C... .......... ....A..... ....C.T..T .T..... .......... ..........
E.man  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNN... .......... ....A..... ....C.T..T .T..... .......... ..........

N.cra  TTAAATCAGT TATAGTTTAT TTGATAGTAC CTTACTACAT GGATAACCGT GGTAATTCTA GAGCTAATAC ATGCTAAAAA CCCCGACTTC GGAAGGGGTG - 200
A.pul  .......... ...C...... .......... ........T. .......... .......... .......... .......... ....A..... ..........
E.der  .......... ...C...... .......... ...--...C. .........G .......... .......... .........C .......... ..........
W.der  .......... ...C...... .......... ...--...C. .......... .......... .......... .......... .......... ..........
P.der  .......... ...C...... .......... ...--...C. .......... .......... .......... .......... .......... ..........
S.pha  .......... ...C...... .......... ...--...C. .......... .......... .......... .......... .......... ..........
C.man  .......... ...C...... .......... ...--...C. .......... .......... .......... .......... .......... ..........
N.nig  .......... ...C...... .......... ...--...C. .......... .......... .......... ....G..... .......... ..G.......
P.ele  .......... ...C...... .......... ...--...C. .......... .......... ...T...... ....G..... .......... ..G.......
P.exo  .......... ...C.C.... .......... ...--...C. .......... .......... .......... ......C... .......... ..G.......
E.jea  .......... ...C...... .......... ...--...C. .........G .......... .......... .......C.C .......... ..G.......
E.man  .......... ...C...... .......... ...--...C. .......... .......... .......... .......... ..T..... ..........

N.cra  TATTTATTAG ATTAAAAACC AATGCCCTTC GGGGCTAACT GGTGATTCAT AATAACTTCT CGAATCGCAT GGCCTTGCGC TGGCGATGGT TCATTCAAAT - 300
A.pul  .......... ..A..... .......... ......CCT. .......... .......AAA .......... .......... C......... ..........
E.der  .......... ..A..... .......... ......CCT. .......... ........AA .......... .......... C......... ..........
W.der  .......... ..A..... .......... ......CCT. .......... ........AA .......... .......... C......... ..........
P.der  .TAG...... ..A..... ....A..... ......CCT. .......... ........AA ...T...... .......... C......... ..........
S.pha  .TAG...... ..A..... ...CA..... ......CCT. .......... ........AA ...T...... .......... C......... ..........
C.man  .......... ..A..... .......... ......CCT. .......... ........AA .......... .......... C......... ..........
N.nig  .T........ ..A..... .......... ....T.CCT. .......... ........AA .......... .......T.. C......... ..........
P.ele  .T........ ..A..... .......... ......CCT. .......... ........AA .......... .......T.. C......... ..........
P.exo  .......... ..A..... .C........ ......CCT. .......... .......CAA ...C...... .......... C......... ..........
E.jea  .T........ ..A..... .......... ....T.CCT. .......... ........AA .......... .......... C......... ..........
E.man  .......... ..A..... .......... ......CTT. .......... ........AA .......... .......... C......... ..........
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Fig. 1. Alignment of the 10 obtained SSU-rRNA sequences with *Neurospora crassa* (N.cra) SSU-rRNA as the leading sequence. The corresponding sequence of *Aureobasidium pullulans* (A.pul) was added because of its status as a familiar representative of the black yeasts. Identical nucleotides are indicated by dots. Abbrevations of the fungal strains are explained in Table 1. Location of primers used for sequencing are marked by bold face and are underlined.

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                ⇨  LS-292F  ⇨
N.cra  -TTCTGCCCT ATCAACTTTC GACGGCTGGG TCTTGGCCAG CCATGGTGAC AACGGGTAAC GGAGGGTTAG GGCTCGACCC CGGAGAAGGA GCCTGAGAAA - 400
A.pul  ......... ......... ..T..TA..A .AG.....TA .......AT. ......... ..G.AA.... ..T..T.TT. ......G... .........
E.der  ......... ......... ..TT.TAA.A .AGA...TTA .A.....CTT ......... ..G.AA.... ..T..T.TT. ......G... .........
W.der  ......... ......... ..TT.TAA.A .AGA...TTA .A.....CTT ......... ..G.AA.... ..T..T.TT. ......G... .........
P.der  CA....... ......... ..TT.TAA.A .AGA...TTA .A.....CTT ......... ..G.AA.... ..T..T.TT. ......G... .........
S.pha  CA....... ......... ..TT.TAA.A .AGA...TTA .A.....CTT ......... ..G.AA.... ..T..T.TT. ......G... .........
C.man  ......... ......... ..TT.TAA.A .AGA...TTA .A.....CTT ......... ..G.AA.... ..T..T.TT. ......G... .........
N.nig  ......... ......... ..TT.TA..A .AGA....TA .A.....CTT ......... ..G.AA.... ..T..T.TT. ......G... .........
P.ele  ......... ......... ..TT.TA..A .AGA....TA .A.....CTT ......... ..G.AA.... ..T..T.TT. ......G... .........
P.exo  ......... ....C..... ..TT.TAA.A .AGA...TTA .A....GAG. ......... ..G.AA.A.. ..T..T.TT. ......G... .........
E.jea  ......... ....C..... ..TT.TAA.A .AGA...TTA .A....GAG. ......... ..G.AA.A.. ..T..T.TT. ......G... .........
E.man  ......... ......... ..T..TAA.A .AG....TTA .......CT. ......... ..G.AA.... ..T..T.TT. ......G... .........

N.cra  CGGCTACTAC ATCCAAGGAA GGCAGCAGGC GCGCAAATTA CCCAATCCCG ACACGGGG-A GGTAGTGACA ATAAATACTG ATACAGGGCT CTTTTGGGTC - 500
A.pul  .......C.. ......... ......... ......... ......... ......... ......... ......... ......... .........
E.der  .......C.. ......... ......... ......... ......... ......... ......... ......... ....G..... ....C.....
W.der  .......C.. ......... ......... ......... ......... ......... ......... ......... ....G..... ....C.....
P.der  .......C.. ......... ......... ......... ......... ......... ......... ......... ....G..... ....C.....
S.pha  .......C.. ......... ......... ......... ......... ......... ......... ......... ....G..... ....C.....
C.man  .......C.. ......... ......... ......... ......... ......... ......... ......... ....G..... ....C.....
N.nig  .......C.. ......... ......... ......... ......... .T....... ......... ......... ....G..... ....C.....
P.ele  .......C.. ......... ......... ......... ......... .T....... ......... ......... ....G..... ....C.....
P.exo  .......C.. ......... ......... ......... ......... .T....... ......... ......G... ....G..... ....C.....
E.jea  T..G...C.. ......... ......... ......... ....G..... .T......C. ......... ......... ....G.C... ...CC.....
E.man  .......C.. ......... ......... ......... ......G.T. ....A.C... ......... ......... ....G..... ....C.....

                                                          ⇨  NS 3  ⇨
N.cra  TTGTAATTG GAATGAGTACA ATTTAAATCC CTTAACGAGG AACAATTGGA GGGCAAGTCT GGTG-CCAGC AGCCGCGGTA ATTCCAGCTC CAATAGCGTA - 600
A.pul  ......... ........... ......... ......... ......... ......... ......... ......... ......... .........
E.der  .C....... ........... ..C...... ......... ......... ......... ....G..... .C....... ......... .........
W.der  .C....... ........... ..C...-... ......... ......... ......... ....G..... .T....... ......... ........C.
P.der  .C....... ........... ..C...... ......... ......... ......... ....G..... .C....... ......... .........
S.pha  .C....... ........... ..C...... ......... ......... ......... ....G..... .C....... ......... .........
C.man  .C....... ........... ..C...... ......... ......... ......... ..A...... ......... ......... .........
N.nig  .C....... ........... ..C...... ......... ......... ......... ......... ......... ......... .........
P.ele  .C....... ........... ..C...... ......... ......... ......... ......... ......... ......... .........
P.exo  .C....... ........... ..C...... ......... ......... ......... ......... ......... ......... .........
E.jea  .C.....A. ........... ..C...... .G......A. ........A. ...T..C... ..C...... ..A...... ......... .........
E.man  .C....... ...........C....... ......... ......... ......... ......... ......... ......... ......T...
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Fig. 1. Continued

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N.cra  TATTAAAGTT GTTGAGGTTA AAAAGCTCGT AGTTGAACCT TGGGCTCGGC CG-TCGGTCC GCCTCACCGC GTGCACTGAC TGGGTCGGGC CTTT-TTTCC - 700
A.pul  .......... ....CA.... .......... .......... .....CT... T.GC...... .......... ...T....GT CC..C..... .....CC.T.
E.der  .......... ....CC.... .......... .......... ...A.CT... T.A..T.... T...A.T..A .C....G..T .C.......T .....CC.T.
W.der  .......... ....CC.... .......... .......... ...A.CT... T.A..T.... T...A.T..A .C....G..T .C.......T .....CC.T.
P.der  .......... ....CC.... .......... .......... ...A.CT... T.A..T.... T...A.T..A .C....G..T .C.......T .....CC.T.
S.pha  .......... ....CC.... .......... .......... ...A.CT... T.A..T.... T...A.T..A .C....G..T .C.......T .....CC.T.
C.man  .......... ....CCS... .......... .......... ...A.CT... T.A..T.... T...A.T..A .C....G..T .C.......T ....TCC.T.
N.nig  .......... ....CC.... .......... .......... ...A.CT... T.A..T.... T...A.T..A .C....G..T .C.......T .....CC.T.
P.ele  .......... ....CC.... .......... .......... ...A.CT... T.A..T.... T...A.T..A .C....G..T .C.......T .....CC.T.
P.exo  .......... ....GC.... .......... .......... ...C.CT... T.A..T.... T...A.T..A .C....G..T .C.......G .....CC.T.
E.jea  .......... ....CC.... .......... .......... ...C.CT... T.A..T.... T...A.T..A .C....G..T .C.......G .....CC.T.
E.man  .......... ....CC.... .......... .......... ...A.CT... T.A..T.... T.....T..A .C....G..T .C.......T .....CC.T.

N.cra  TGGAGAACCG CATGCCCTTC ACTGGGTGTG TCGGGGAACC AGGACTTTTA CCGTGAACAA ATCAGATCGC TCAAAGAAGG CCTATGCTCG AATGTACTAG - 800
A.pul  ...G..G... .......... ......C... .......... .......... .TT....A.. ..T...GT.T ......C... ...T...... ...AC.T...
E.der  ...G.....C .......... .......... G......... .......... ..T....A.. ..T...GT.T ......C... ...T...... ...AC.T...
W.der  ...G.....C .......... .......... G......... .......... ..T....A.. ..T...GT.T ......C... ...T...... ...AC.T...
P.der  ...G.....C .......... .......... G......... .......... ..T....A.. ..T...GT.T ......C... ...T...... ...AC.T...
S.pha  ...G.....C .......... .......... G......... .......... ..T....A.. ..T...GT.T ......C... ...T...... ...AC.T...
C.man  ..AG.....C .......... .......... GT........ .......... ..T....A.. ..T...GT.W C......... ...TC.A... ...AC.T...
N.nig  ...G..G..C .......... .......... GT........ .......... ..T....A.. ..T...GT.T ......C... ...T...... ...AC.T...
P.ele  ...G..G..C .......... .......... GT........ .......... ..T....A.. ..T...GT.T ......C... ...T...... ...AC.T...
P.exo  ...G..G..C .......... .......... G......... .......... ..T....A.. ..T...GT.T ......C... ...T...... ...AC.T...
E.jea  ...G..G..C .......... .......... G......... .......... ..T....A.. ..T...GT.T ......C... ...T...... ...AC.T...
E.man  ...G..G..T .......... .......... .G........ .......... ..T....A.. ..T...GT.T ......C... ...T..T... ...AC.T...

                                          ⇨  LS-825F  ⇨
N.cra  CATGGAATAA TAGAATAGGA CGTGTGGTTC TATTTTGTTG GTTTCTAGGA CCGCCGTAAT GATTAATAGG GACAGTCGGG GGCATCAGTA TTCAATTGTC - 900
A.pul  .......... .......... ....C..... .......... .......... .......... .......... ..T....... .......... Y.........
E.der  .......... .......... .A.-C..... .......... .......... .......... ....T..... .......... ..T....... ...G...... ..........
W.der  .......... .......... .A.-C..... .......... .......... .......... ....T..... .......... ..T....... ...G...... ..........
P.der  .......... .......... .A.-C..... .......... .......... .......... ....T..... .......... ..T....... ...G...... ..........
S.pha  .......... .......... .A.-C..... .......... .......... .......... ....T..... .......... ..T....... ...G...... ..........
C.man  A......... .......... .A.-C..... ...A...... .......... .......... ....T..... .......... ..T....... ...G...... ..........
N.nig  .......... .......... .A.-C..... .......... .......... .......... ....T..... .......... .......... ...G...... ..........
P.ele  .......... .......... .A.-C..... .......... .......... .......... ....T..... .......... .......... ...G...... ..........
P.exo  .......... .......... .A.-C..... ........G. .......... .......... .-..T..... .......... .......... ...G...... ..........
E.jea  .......... .......... .A.-C..... .......... .......... .......... ....T..... .......... .......... ...G...... ..........
E.man  .......... .......... .A.-C..... .......... .......... .......... ....T..... .......... ..T....... ...G...... ..........
```

Fig. 1. Continued

```
N.cra  AGAGGTGAAA TTCTTGGATT TATTGAAGAC TAACTACTGC GAAAGCATTT GCCAAGGATG TTTTCATTAA TCAG-GAACG AAAGTTAGGG GATCGAAGAC - 1000
A.pul  .......... .......... .......... .......... .......... .......... .......... ....T..... .......... ..........
E.der  .......... .......... .......... .......... .........C .......... .......... ....T..... .......... ..........
W.der  .......... .......... .......... .......... .........C .......... .......... ....T..... .......... ..........
P.der  .......... .......... .......... .......... .........C .......... .......... ....T..... .......... ..........
S.pha  .......... .......... .......... .......... .........C .......... .......... ....T..... .......... ..........
C.man  .......... .......... .......... .......... .........C .......... .......... ....T..... .......... ..........
N.nig  .......... .......... .......... ......T... .........C .......... .......... ....T..... .......... ..........
P.ele  .......... .......... .......... ......T... .........C .......... .......... ....T..... .......... ..........
P.exo  .......... .......... .......... .......... .........C .......... .......... ....T..... .......... ..........
E.jea  .......... .......... .......... .......... .........C .......... .......... ....T..... .......... ..........
E.man  .......... .......... .......... .......... .........C .......... .......... ....T..... .......... ..........

N.cra  GATCAGATAC CGTCGTAGTC TTAACCATAA ACTATGCCGA TTAGGGATCG GACGGT-GTT ATTTTTTGAC CCGTTCGGCA CCTTACGATA AATCAAAATG - 1100
A.pul  .......... .......... .......... .......... C.......... .G..A..... .CA..... T..C..... ........G. .......G.C
E.der  .......... .......... .......... .......... C.......... ......G... T....A..C. .......... ........G. .......G..
W.der  .......... .......... .......... .......... C.......... ......G... T....A..C. .......... ........G. .......G..
P.der  .......... .......... .......... .......... C.......... ......G... T....A..C. ........A. ........G. .......G..
S.pha  .......... .......... .......... .......... C.......... ......G... T....A..C. ........A. ........G. .......G..
C.man  .......... .......... .......... .......... C.......... ......G... T....A.... .......... ........G. .......G..
N.nig  .......... .......... .......... .......... C.......... ......G... T....A..C. .......... ........G. .......G.T
P.exo  .......... .......... .......... ......A... C.......... ......G... T....A..C. ........A. ........G. .......G.T
P.ele  .......... .......... .......... .......... C.......... ......G... T....A..C. .......... ........G. .......G.T
E.jea  .......... .......... .......... .......... C.......... ......G... T....A..C. .......... ........G. .......G.T
E.man  .......... .......... .......... .......... C.......... ......G... T....A..C. ........A. ........G. .......-.T

                                                                ⇨  NS 5  ⇨
N.cra  TTTGGGCTCC TGGGGGAGTA TGGTCGCAAG GCTGAAACTT AAAGAAATTG ACGGAAGGGC ACCACCAGGG GTGGAGCCTG CGGCTTAATT TGACTCAACA - 1200
A.pul  ......T..T G......... .......... .......... .......... .......... .........C .......... .......... ..........
E.der  .........G G......... .......... .......... .......... .......... .........C .......... .......... ..........
W.der  .........G G......... .......... .......... .......... .......... .........C .........C .......... ..........
P.der  .........G G......... .T........ .......... .......... .......... .........C .......... .......... ..........
S.pha  .........G G......... .K........ .......... .......... .......... .........C .......... .......... ..........
C.man  .........G G......... .......... .......... .......... .......... .......A.. .......... .......... ..........
N.nig  .........G G......... .......... .......... .......... .......... .........C .......... .......... ..........
P.exo  .A.......G G......... .......... .......... .......... .......... .........C .......... .......... ..........
P.ele  .........G G......... .TT....... .......... .......... .......... .......... T......... ...G..T... ..G.....A.
E.jea  .........G G......... .......... .......... .......... .......... .........C .......... .......... ..........
E.man  .........G G......... .......... .......... ..N....... ....N..... .........C ..C....... ........A. ..........
```

Fig. 1. Continued

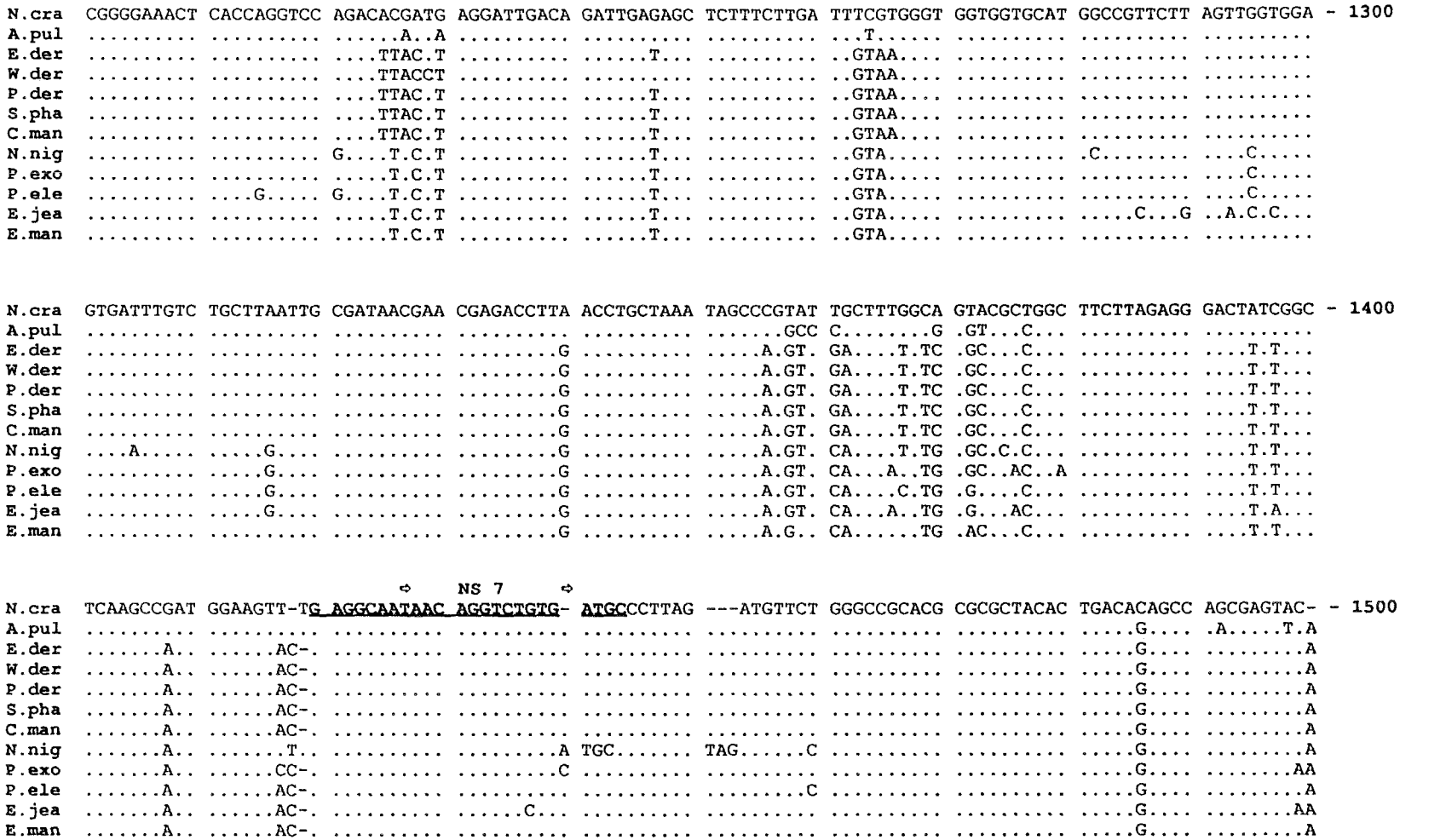

Fig. 1. Continued

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N.cra  TC-CCTTGGC CGGAAGGTCC GGGTAATCTT GTTAAACTGT GTCGTGCTGG GGATAGAGCA TTGCAATTAT TGCTCTTCAA CGAGGAATCC CTAGTAAGCG - 1600
A.pul  .TT.....C. .......GTT .......... ........C. .......... .......... .......... .......... ........G. ..........
E.der  ..A..... ..A......T .......... ........C. .......... .......... .......... .......... ........G. ..........
W.der  ..A..... ..A......T .......... ........C. .......... .......... .......... .......... ........G. ..........
P.der  ..A..... ..A......T .......... ........C. .......... .......... .......... .......... ........G. ..........
S.pha  ..A..... ..A......T .......... ........C. .......... .......... .......... .......... ........G. ..........
C.man  ..A..... ..A......T .......... ........C. .......... .......... .......... .......... ........G. ..........
N.nig  ..A..... ..A......T .......... ........C. .......... .......... .......... .......... ........G. ..........
P.exo  ..A..... ..A......T .......... ........C. .......... .......... .......... .......... ........G. ..........
E.ele  ..A..... ..A......T .......... ........C. .......... .......... .......... .......... ........G. ..........
E.jea  ..A..... ..A......T .......... ........C. .......... .......... .......... .......... ........G. ..........
E.man  ..A..... ..A......T .......... ........C. .......... .......... .......... .......... ........G. ..........

N.cra  CAAGTCATCA GCTTGCGTTG ATTACGTCCC TGCCCTTTGT ACACACCGCC CGTCGCTACT ACCGATTGAA TGGCTCAGTG AGG-CTTCCG GACTGGCCCA - 1700
A.pul  T.C..... ..G..... .......... .......... .......... .......... .......... .....G.... ...C....GG -.........
E.der  .......... .......... .......... .......... .......... .......... .......... .......... ...C...-G. .......T..
W.der  .......... .......... .......... .......... .......... .......... .......... .......... ...C...-G. .......T..
P.der  .......... .......... .......... .......... .......... .......... .......... .......... ...C...-G. .......T..
S.pha  .......... .......... .......... .......... .......... .......... .......... .......... ...C...-G. .......T..
C.man  .......... .......... .......... .......... .......... .......... .......... .......... ...C...-G. .......T..
N.nig  .......... .......... .......... .......... .......... .......... .......... .......... ...C...-G. .......T..
P.exo  .......... .......... .......... .......... .......... .......... ......C... .....A.... ...A..-.G. .......T..
P.ele  .......... .......... .......... .......... .......... .......... .......... .......... ...C...-G. .......T..
E.jea  .......... .......... .......... .......... .......... .......... .......... .......... ...C..-.G. .......T..
E.man  .......... .......... .......... .......... .......... .......... .......... .......... ...C...TG. .......T..

                                                                                                    ⇦   NS 8   ⇦
N.cra  GGGAGGTCGG CAACGACCAC CCAGGGCCGG AAAGCTATCC AAACTCGG-T CATTTAGAGG AAGTAAAAGT CGTAA-CAAG GTCT-CCGTT GGTGAACC-A - 1800
A.pul  .......... .......... .......... ....T.GGT.........C.. .......... .......... ........... ..T......A .........T
E.der  .A........ .......... T...A..... ...CT.GGT. .....T.... .......... .......... .......... .........A .........CT
W.der  .A........ .......... T...A..... ...CT.GGT. .....T.... .......... .......... .......... .........A ..........
P.der  .A........ .......... T...A..... ...CT.GGT. .....T.... .......... .......... .......... .........A .........T
S.pha  .A........ .......... T...A..... ...CT.GGT. .....T.... .......... .......... .......... .........A NNNNNNNN?N
C.man  .A........ .......... T...A..... ...CT.GGT. .....T.... .......... .......... .......... T......NNN NNNNNNNN?N
N.nig  .A........ .......... T...A..... ...CT.GGT. .....T.... .......... .......... .......... ..T......A .........T
P.exo  .A........ .......... T...A..... ...CT.G.A. .....T.... .......... .......... .......... ....T...GA .........T
P.ele  .A........ .......... T...A..... ...CT.GGT. .....T.... .......... .......... .......... ..T......A .........T
E.jea  .A........ .......... T...A..... ...CT.GGT. .....T.... .......... .......... .....A.... .........A .........T
E.man  .A........ .......... T...A..... ...CT.GGT. .T......C. ..CA.N.... .......... ...CNA..C. .......NNN  NNNNNNN?N
```

Fig. 1. Continued

```
N.cra  GCGGAGGGAT CATTA - 1815
A.pul  .A..A.... ..AGC
E.der  ..........  .....
W.der  ..........  .....
P.der  ..........  .....
S.pha  NNNNNNNNNN NNNNN
C.man  NNNNNNNNNN NNNNN
N.nig  .....A....  .....
P.exo  ..........  .....
P.ele  ......A...  .....
E.jea  ......A...  .....
E.man  NNNNNNNNNN NNNNN
```

Fig. 1. Continued

Phylogenetic analysis

Phylogenetic tree construction with eukaryotic SSU-rRNA gene sequences using the neighbor-joining method has been proven to be a reliable predictor of taxonomic relationships (Bruns et al. 1991; Hendriks et al. 1991). The ten strains of black yeasts studied formed a monophyletic branch (Fig. 3), thus strongly supporting the close interrelationship of the organisms concerned. They are a sister-group to the Plectomycetes (Eurotiales and Onygenales). In contrast, the black yeast *Aureobasidium pullulans* appears unrelated. The exclusion of *A. pullulans* from the combined group of black yeasts and Plectomycetes indicates that Loculoascomycetes do not form a natural, monophyletic group. The precise phylogenetic arrangement relative to the Pyrenomycetes (Ophiostomatales and Sphaeriales) remains unclear since these branches are not well supported. The black yeasts studied are strongly separated from the white yeasts and the basidiomycetous outgroup *F. neoformans*, so that formation of yeast cells must be a secondarily derived character. Using an ascomycetous organism as outgroup organism did not alter the topology of the tree obtained.

Taxonomy

The close interrelationship of *Sarcinomyces phaeomuriformis* and *Exophiala* (*Wangiella*) *dermatitidis* and its purported *Phaeotheca*-like anamorph (Matsumoto et al. 1990) was well supported by the phylogenetic analysis. The type strain of *Exophiala* (*Wangiella*) *dermatitidis* and strain ATCC 34100 exhibit 9 nucleotides difference (= 0.5%; Fig. 1). The branch between these two species is statistically not significant, indicating that these two strains belong to the same species. The four strains mentioned above are indistinguishable from each other by exoantigen test (Matsumoto et al. 1986). All have two different introns (but with identical sequences) inserted at the same position in the SSU-rRNA gene.

The ascomycetous teleomorph *Capronia mansonii* was found in the same monophyletic group as *E. dermatitidis*. This strongly supports the concept that *Exophiala* anamorphs have teleomorph connections in family Herpotrichiellaceae Munk (Schol-Schwarz 1968; Samuels & Müller 1978; Müller et al. 1987; de Hoog et al. 1994a). Further members of the Herpotrichiellaceae, as well as of supposed related anamorphic genera (e.g., *Fonsecaea* Negroni, *Ramichloridium* Stahel ex de Hoog, *Rhinocladiella*

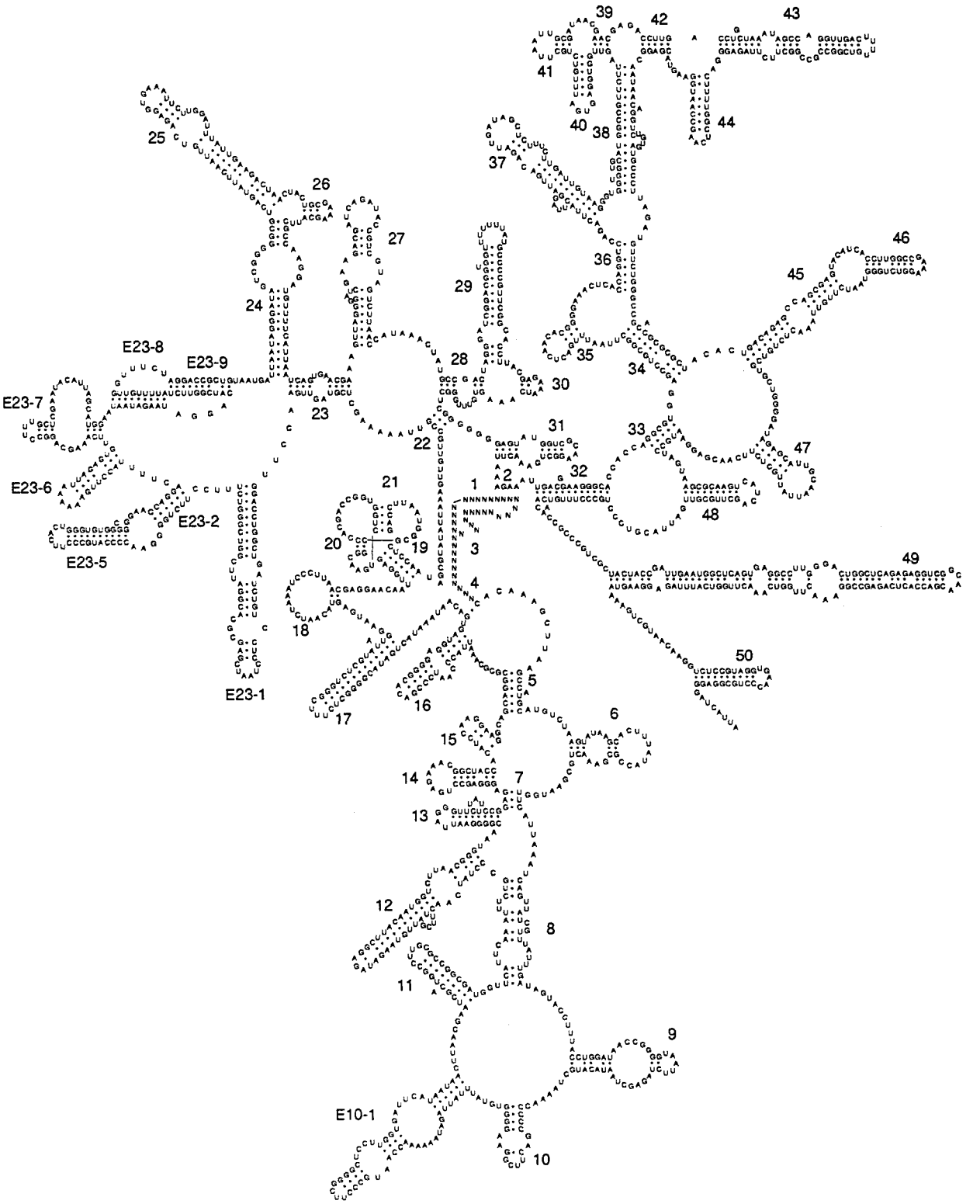

Fig. 2. Predicted secondary structure model of the SSU-rRNA of *Exophiala dermatitidis* CBS 207.35^{T}. Helix numbering according Neefs et al. (1993).

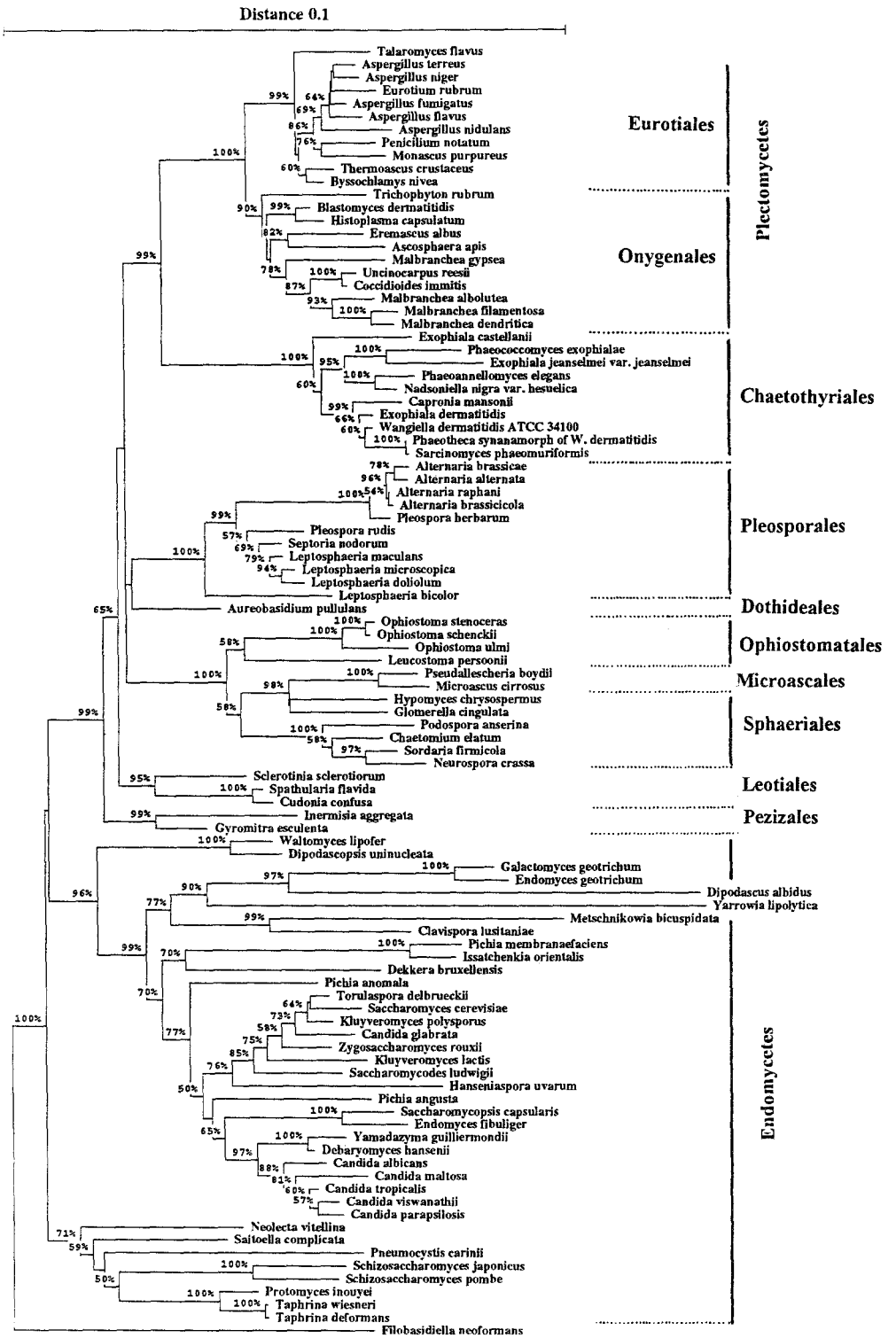

Fig. 3. Rooted phylogenetic tree of ascomycetes (with more than 90% sequence information of the SSU-rRNA gene) including the ten new SSU-rRNA sequences determined in this study. The basidiomycete *Filobasidiella neoformans* was used as outgroup organism. The distance between two organisms or groups of organisms, measured in substitutions per nucleotides, is obtained by summing the lengths of the connecting branches along the horizontal axis, using the scale on the top. Bootstrap analysis percentage (out of 500 samples) higher than 50% are indicated alongside the branch considered.

Nannf.) need to be studied for a further elucidation of teleomorph connections of dematiaceous human pathogens.

The strain CBS 158.58 was designated by de Hoog (1977) as neotype of *E. mansonii* because the original material described by Castellani (1905) was lost. McGinnis (1977) pointed out that this designation was in conflict with the International Code of Botanical Nomenclature, since Castellani's original description (1905) of the patient's infection was in disagreement with the clinical picture caused by CBS 158.58. Therefore, Iwatsu et al. (1984) proposed the name *Exophiala castellanii* for this strain. In 1990 the new combination *Exophiala jeanselmei* var. *castellanii* (Iwatsu et al.) Iwatsu & Udagawa was proposed for this strain since it would differ only in minor morphological characters from the other varieties of *E. jeanselmei*. Our phylogenetic analysis strongly supports a separation of *E. jeanselmei* and *E. castellanii*. The taxon is also clearly different from *E. dermatitidis* (Fig. 3). Therefore, we recommend retention of *E. castellanii* as a separate species, as proposed by de Hoog (1977), Iwatsu et al. (1984) and Matsumoto et al. (1993). *Capronia mansonii* (formerly treated as *Dictyotrichiella mansonii* Schol-Schwarz) was described as the teleomorph of *E. mansonii* (Schol-Schwarz 1968; Müller et al. 1987). This finding is in conflict with our phylogenetic analysis (Fig. 3).

De Hoog (1985) suggested that *N. nigra* var. *hesuelica* was an *Exophiala* species close to *E. jeanselmei*. Our analysis favours the maintenance of this strain as a separate species. *Phaeococcomyces exophialae* and *Phaeoannellomyces elegans* were described as synanamorphs of *Exophiala* which could not be assigned to any particular species (de Hoog 1977; McGinnis et al. 1985). The coherence of these taxa with species of *Exophiala* is in good agreement with the results of our phylogenetic analysis.

In conclusion, direct sequencing of nuclear SSU-rRNA genes of black yeasts and a subsequent phylogenetic analysis with the obtained data appears to be suitable for resolving taxonomic problems in this pleomorphic group of fungi. For the development of a reliable diagnostic system, more species of the genus *Exophiala* and of related genera should be examined. Particularly intraspecific variability of the SSU-rRNA gene needs to be determined. Yet our results already indicate that the segment of dematiaceous SSU-rRNA genes corresponding to the stem of helix 43 (Fig. 2) of the secondary structure model (corresponding nucleotide position 1340–1389 in Fig. 1) is a promising region to be used for designing species-specific oligonucleotides.

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