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Coniosporium perforans and *C. apollinis*, two new rock-inhabiting fungi isolated from marble in the Sanctuary of Delos (Cyclades, Greece)

Katja Sterflinger[1], Raymond De Baere[2], G.S. de Hoog[3], Rupert De Wachter[2], Wolfgang E. Krumbein[1] & G. Haase[4]

[1]*Geomikrobiologie, ICBM, Carl von Ossietzky Universität Oldenburg, P.O. Box 2503, D-26111 Oldenburg, Germany;* [2]*Universitaire Instelling Antwerpen, Department of Biochemistry, Universiteitsplein 1, B-2610 Antwerpen, Belgium;* [3]*Centraalbureau voor Schimmelcultures, P.O. Box 273, NL-3740 AG Baarn, The Netherlands;* [4]*Institut für Medizinische Mikrobiologie, University Hospital RWTH, Pauwelsstr. 30, D-52074 Aachen, Germany*

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Abstract

Coniosporium perforans and *C. apollinis*, orginating from marble in the Mediterranean basin, are described as new species of rock inhabiting microcolonial fungi. The morphologically similar species *Monodictys castaneae* (Wallr.) S. Hughes, *Phaeosclera dematioides* Sigler et al., and a *Coniosporium*-like strain are compared using 18S rDNA phylogeny and Restriction Length Fragment Polymorphism analysis of Internal Transcribed Spacer regions. *Sarcinomyces crustaceus* Lindner is additionally compared on the basis of 18S rDNA sequencing data. Phylogenetic analysis suggests that *Phaeosclera dematioides* is related to the ascomycetous order Dothideales and *Monodictys castaneae* to the Pleosporales, whereas the three *Coniosporium* species studied are a sister group to the Herpotrichiellaceae (Chaetothyriales). A similar affinity was suggested previously for the recently described meristematic rock-fungus *Sarcinomyces petricola* Wollenzien & de Hoog. *Sarcinomyces crustaceus* appears unrelated to this group, and hence the present new taxa cannot be described in this genus.

Introduction

Bare calcareous and quartzitic rock surfaces in arid and semi-arid environments provide very special living conditions for microorganisms. The term 'extreme environment' is frequently used for such biotopes. Fungi which are prevalent in such environments show a high versatility and adaptability to a wide range of physical and chemical environmental parameters. Organisms occurring in such extreme habitats show a remarkable morphological similarity on the natural substrate (habitat specific ecotypes; Sterflinger & Krumbein, 1995; Stackebrand et al., 1993), but may be phylogenetically unrelated. The biodiversity of such habitats is considerably higher than originally supposed (Urzì et al., 1995; Eppard et al., 1996). Rock as a substrate represents one of the largest and most poorly studied biotopes on the globe.

Under conditions of low humidity, high temperatures and rapid environmental changes, fungi, cyanobacteria and actinomycetes frequently form black, nodose colonies (Krumbein, & Jens 1981; Henssen, 1987). These colonies show extremely little expansion growth, and the respective fungi have therefore been termed microcolonial fungi (MCF; Staley et al., 1982). In culture they often grow meristematically and may be classified in genera like *Sarcinomyces* Lindner (Wollenzien et al., 1996), *Phaeosclera* Sigler et al. (1981) and *Botryomyces* de Hoog & Rubio (1982). Some show additional budding and are regarded as black yeasts (Gorbushina et al., 1993; Wollenzien et al., 1995; Urzì et al., 1995; Sterflinger, 1995). Often a conversion of various forms of meristematic, filamentous and yeast-like growth is observed (Yoshida et al., 1996; Figueras et al., 1996). This morphological plasticity greatly hampers microscopic identification

(Minter, 1987; de Hoog & McGinnis, 1987). Currently several molecular methods are applied, such as mtDNA RFLP (de Cock, 1994), rDNA RFLP (Uijthof & de Hoog, 1995) and partial or complete 18S rDNA sequencing (Berbee & Taylor, 1992; Spatafora et al., 1995; Haase et al., 1995). With these techniques the MCF can be classified more reliably.

Several MCF have been isolated from marble monuments of the Greek museum island Delos. One of these (CBS 885.95) was reported to cover nearly all the monuments on Delos with a black, clumping growth and was recognized to be the main agent of biopitting of Mediterranean marbles (Sterflinger & Krumbein, 1997). This strain, as well as the less frequently isolated strain CBS 352.97, is judged to represent two hitherto undescribed species (Sterflinger & Gorbushina, 1996). In the present paper we clarify their taxonomic and phylogenetic position on the basis of 18S rDNA sequencing and ITS Restriction Fragment Length Polymorphism (RFLP) data.

Material and methods

Isolation and morphological characterization

Strains DE7a (CBS 885.95) and DE 11 (CBS 352.97) were isolated and purified by methods described before (Wollenzien et al., 1995; Sterflinger & Krumbein, 1995). Strains of *Monodictys castaneae* (Wallr.) S. Hughes (CBS 100.07) from wood, *Phaeosclera dematioides* Sigler et al. (CBS 157.81) from wood and *Coniosporium* sp. (CBS 665.80) from rock were included for comparison.

For morphological studies the fungi were grown in Petri dishes and as slide cultures on 2% malt extract agar (MEA), on potato dextrose agar (PDA) and on Czapek agar (CzA) and incubated at 18.5°C and 27°C. Mounts were made in lactophenol.

Scanning electron microscopy

For SEM analysis fragments of mycelia were fixed in 4% glutaraldehyde (2 hours), dehydrated in an ethyl alcohol series: 15% (30 min), 30% (30 min), 50% (30 min), 70% (over night), 80% (60 min), 90% (60 min) and 100% (60 min, two times) ethanol and dried to the critical point.

DNA extraction

Fungi were grown in liquid 2% malt medium, filtered and washed with buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5). Mycelia were stored at -20°C until DNA-extraction. Frozen cultures were ground in a mortar in liquid N_2. One volume (v/w) of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulphate and 1% β-mercaptoethanol, pH 7.5) was added, and shaken in a water bath (62.5°C, 1 hour). Subsequently one volume of chloroform/phenol (1:1, v/v) was added, followed by centrifugation (2 x, 10,000 rpm, 15 min). DNA was precipitated with 0.02 vol. 3 M sodium-acetate-buffer and 0.54 vol. isopropanol. After centrifugation (10,000 rpm, 15 min) the supernatant was decanted, the pellet was washed with 70% ethanol, speed-vacuum dried and dissolved in TE-buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8). Pancreatic RNases were added (50 mg$\cdot$ml^{-1}) and the solution was incubated at room temperature for one hour. Precipitation of DNA with sodium-acetate-buffer and isopropanol was repeated, DNA was washed and dissolved in TE-buffer. DNA concentration was determined by measuring the OD_{260} as well as on 1% agarose gels with TAE-buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualised with ethidium-bromide stain (0.5 mg$\cdot$ ml^{-1}).

PCR reactions

PCR was performed in 100 μl volumes containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, 100 pmol of each primer, 200 pmol of each deoxynucleotide triphosphate, 2.5 U Taq DNA polymerase and 200 ng of template DNA. The mixture was amplified by 30 PCR-cycles as follows: denaturation at 94°C for 60 sec., annealing at 49°C for 60 sec., extension at 72°C for 1 min 30 sec. First denaturation step was 94°C for 3 min, last extension was 72°C for 10 min. The PCR product was purified using QIAquick columns (Qiagen, Hilden, Germany).

Two different PCR reactions using the same forward-primer but different backward-primers were done. The forward primer was located at the beginning of the 18S rDNA, one backward primer at the end of the 18S rDNA molecule and the other in a conserved region of the 28S rDNA.

Forward:		5′>3′ CTGGTTGAT(C/T)CTGCCAGT
Backward:	(18S)	5′>3′C(A/G)GTAGGTGAACCTGC(C/T)G
	(28S)	5′>3′ ATATGCTTAA(A/G)TTCAGCGGGT

Table 1. List of 16 oligonucleotides complementary to conserved regions in the eukaryotic 18S rDNA used to determine the sequence of both strands of the 18S rDNA of the fungi presented in this paper

Sequence[1]	Strand[2]	Corresponding position in the 18S rDNA of *Coniosporium sp.*
CTGGTTGATYCTGCCAGT	R	4–22
GAAACTGCGAATGGCTCATT	R	86–106
AATGAGCCATTCGCAGTTTC	C	106–86
AGGGYTCGAYYCCGGAGA	R	373–391
TCTCCGGRRTCGARCCCT	C	391–393
TCTCAGGCTCCYTCTCCGG	C	400–384
ATTACCGCGGCTGCTGGC	C	584–568
CGCGGTAATTCCAGCTCCA	R	577–595
TTGGYRAATGCTTTCGC	C	960–944
TTRATCAAGAACGAAAGT	R	972–990
CCGTCAATTYYTTTRAGTTT	C	1159–1139
AATTTGACTCAACACGGG	R	1192–1209
ATAACAGGTCTGTGATGCCC	R	1430–1449
TTTGYACACACCGCCCGTCG	R	1637–1656
GACGGGCGGTGTGTRC	C	1640–1655
CYGCAGGTTCACCTACRG	C	1801–1783

[1] 'R' indicates sequence positions where both purines (A and G) can be present; 'Y' indicates sequence positions with pyrimidines (C and T).
[2] Primers with a sequence corresponding to that of the RNA-like strand are indicated by 'R', those whose sequence is complementary to it by 'C'.

Cloning of the PCR product

The PCR products of strains CBS 885.95 and CBS 352.97 were cloned in the EcoRI site of an *E. coli* PCR vector using the Invitrogen TA Cloning Kit according to the instructions of the manufacturer.

Sequencing reaction

Sequencing reactions were performed by the dideoxynucleotide method using the 'Thermo sequenase fluorescent labelled primer cycle sequencing kit' (Amersham) according to the instruction of the manufacturer. For strains CBS 885.95 and CBS 352.97 sequencing was performed on the PCR plasmide derived from a single clone, for the 3 reference strains the PCR-product was sequenced directly. Sequence assays were analysed on an automated DNA Sequencer ALFexpress (Pharmacia Biotech). Sequence assembly was done using the Seqman program (DNASTAR Inc.). The 18S rDNA primers used are listed in Table 1.

Sequence alignment and phylogenetic tree construction

The sequences published in this paper were aligned taking into account the secondary structure of the molecule, as described by De Rijk & De Wachter (1993). The phylogenetic tree was constructed with the TREECON software package (Van de Peer & De Wachter, 1994) using the neighbour-joining method (Saitou & Nei, 1987), based on a dissimilarity matrix corrected for multiple mutations at any given site in the molecule (Jukes & Cantor, 1969). The basidiomycete *Agaricus bisporus* was chosen as an outgroup organism. Confidence values for individual branches were determined by bootstrap analysis in which 100 bootstrap trees were generated (Felsenstein, 1985). The sequences have been deposited in the Antwerp databank on small ribosomal subunit RNA and are available through anonymous ftp on the server rrna.uia.ac.be or by World Wide Web at URL http://rrna.uia.ac.be/rrna/ssuform.html (Van de Peer et al., 1996). They have also been deposited at EMBL under the following numbers: *Coniosporium apollinis* Y11713, *C. perforans* Y 11714, *Coniosporium* species

Y11712, *Monodictys castaneae* Y11715, *Phaeosclera dematioides* Y11716.

Restriction analysis

Primers NS1, NS24, ITS1 and ITS4 (White et al., 1990) were employed. Forty amplification cycles were performed: 94°C, 1 min; 48°C, 2 min; 74°C, 3 min (with initial and terminal delay of 1 min) in a Biomed thermocycler (type 60). The amplicons were digested with restriction enzymes (1 U) for at least 2 h. The following enzymes were used: MspI, HaeIII, RsaI and DdeI. Digests were subjected to electrophoresis on 1.4% agarose gels (TAE buffer) containing ethidium bromide. Resulting banding patterns were transformed to estimated molecular weights using the ImageMaster (Pharmacia Biotech) software package.

Results

Sequence alignment

The sequences of the rRNA of CBS 885.95, CBS 352.97, *Coniosporium* sp., *Monodictys castaneae* and *Phaeosclera dematioides* are shown in the form of an alignment in Figure 1. The 3′ and 5′ termini of the 18S rDNA molecule of the fungi presented here were located on the basis of similarity with those of other 18S rRNA genes of fungi. In the alignment the sequence of the ascomycete *Neurospora crassa* (Sphaeriales) was chosen as the leading sequence, followed by the black yeast *Exophiala dermatitidis* (Chaetothyriales, Herpotrichiellaceae). The size of the 18S rDNA molecule was 1.8 kb for the strains CBS 885.95 (EMBL No. Y11714), CBS 352.97 (EMBL No.11713), *Coniosporium* sp. (EMBL No. Y11712), *Sarcinomyces crustaceus* (EMBL No. Y11355) and *Phaeosclera dematioides* (EMBL No. Y11716). The 18S rRNA gene of *Monodictys castaneae* (EMBL No. Y11715) was 2232 base-pairs long, having an intron of 432 bases located downstream from base No. 1177, the sequence of which is given in Figure 2. Because the presence of introns is variable, even within one single species, they are assumed to have little taxonomic value (Uijthof, 1996). Thus, for sequence alignment the presence of an intron was not taken into account.

With reference to the sequence of *N. crassa* substitutions occur mainly in between the positions 180-268, 337-390, 646-715, 756-801, 1057-1127, 1220-1272, 1361-1382, 1455-1521 and 1686-1746. The deviations occurring among the strains CBS 885.95, CBS 352.97 and *Coniosporium* sp. are less than 1% of the whole sequence.

Phylogenetic analysis

Figure 3 shows a phylogenetic tree including 80 ascomycetous fungal species. The results of this study suggest that the strains analyzed cluster in 3 different orders of the Euascomycetes. *Coniosporium* sp., CBS 885.95, CBS 352.97 and *Phaeococcomyces catenatus* form a sister group to the Herpotrichiellaceae within the Chaetothyriales. The clade is supported by a high bootstrap value (100%). *Coniosporium* sp. and CBS 885.95 and CBS 352.97 separate from *Ph. catenatus* to form another significant branch with a bootstrap value of 97%. Distance estimation suggests a very close relationship between *Coniosporium* sp. and the two new isolates from Delos; the similarity values are more than 99%. *Phaeosclera dematioides* clusters in the order Dothideales. Judging from the 18S rDNA sequence data, *Monodictys castaneae* is a member of the order Pleosporales.

Restriction analysis

Molecular weights of fragments of rDNA RFLP of ITS of stone inhabiting fungi are presented in Table 2, in which fragments of comparable lengths are put on the same height. The two analyzed strains of *Sarcinomyces petricola* are strictly identical with HaeIII, DdeI and RsaI but CBS 725.95 shows a small additional band when digested with MspI; CBS 885.95 shares two patterns with the *S. petricola* strain CBS 726.95. CBS 352.97 is different with any of the enzymes used.

Coniosporium perforans Sterflinger, spec. nov. – Figure 4

Thallus saxa calcarea inhabitans, nigricans, e cellulis globosis constat, 20–100 μm diametro, sporulatio abundus. Coloniae in agaro maltoso post 2 menses ad 16–25 mm diametro, colore variabili luteovirides, olivaceo-brunneae, nigricantes, marginibus plerumque submersis, in medio pulverulentae, 27°C nigrae. Maturitate centralis pars elevata. Hyphae plerumque e cellulis globosis vel ellipsoideis, 2–4 μm diametro, symmetricis, constant et nonnumquam e cellulis elongatis 3–10 μm x 2–3 μm. Cellulae gemmantes praesentes. Conidia non catenulata, simul terminalia, simul intercalaria, primo globosa 5–10 μm diametro, denique

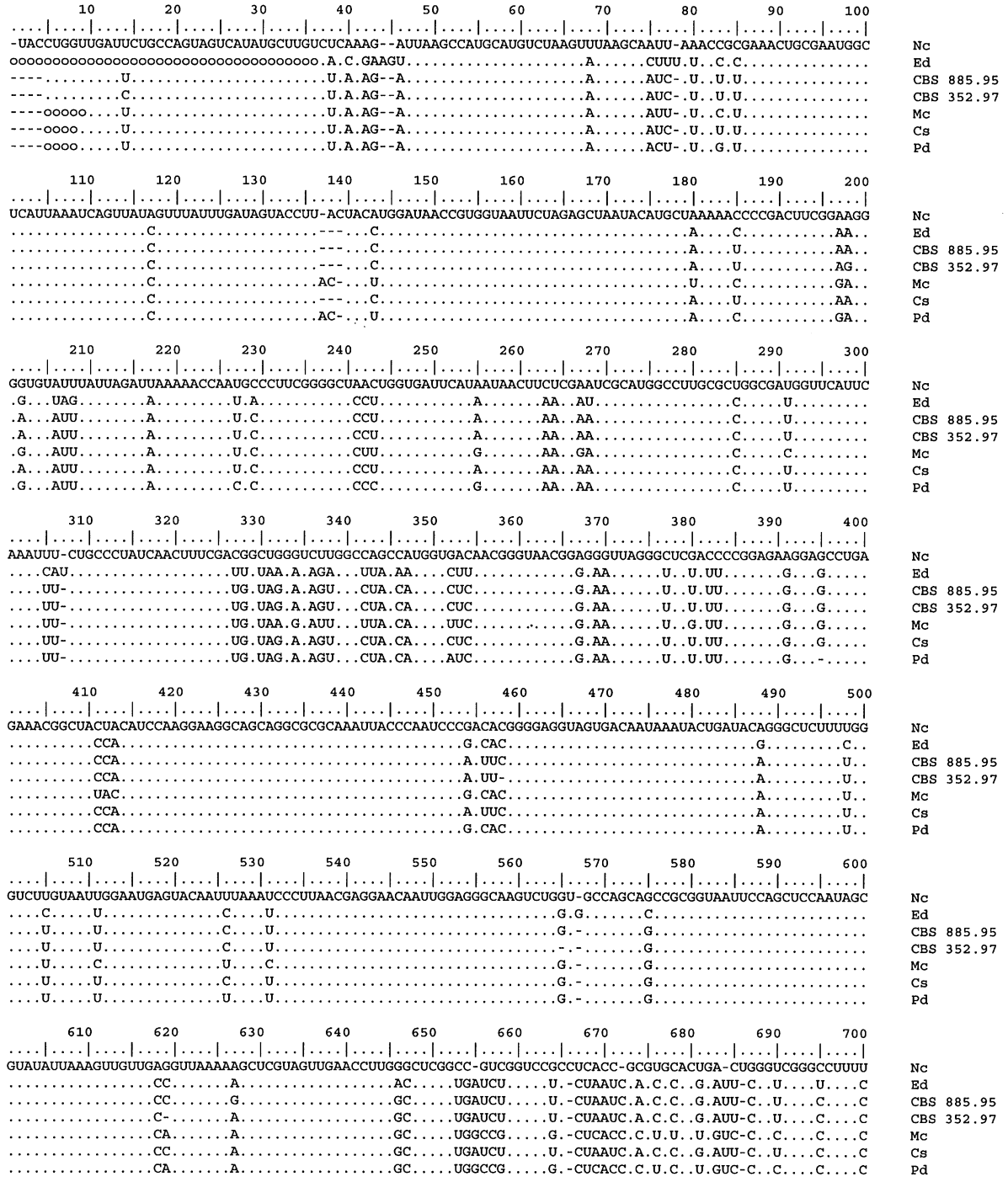

Figure 1.

plus minusve irregularia et 1–4-cellularia septis longitudinalibus et transversalibus, 10–18 μm diametro. Conidia non verrucosa. Pseudosclerotia tarde formata et pauca. Cristalla nulla. Reproductio ascosporica non observata.

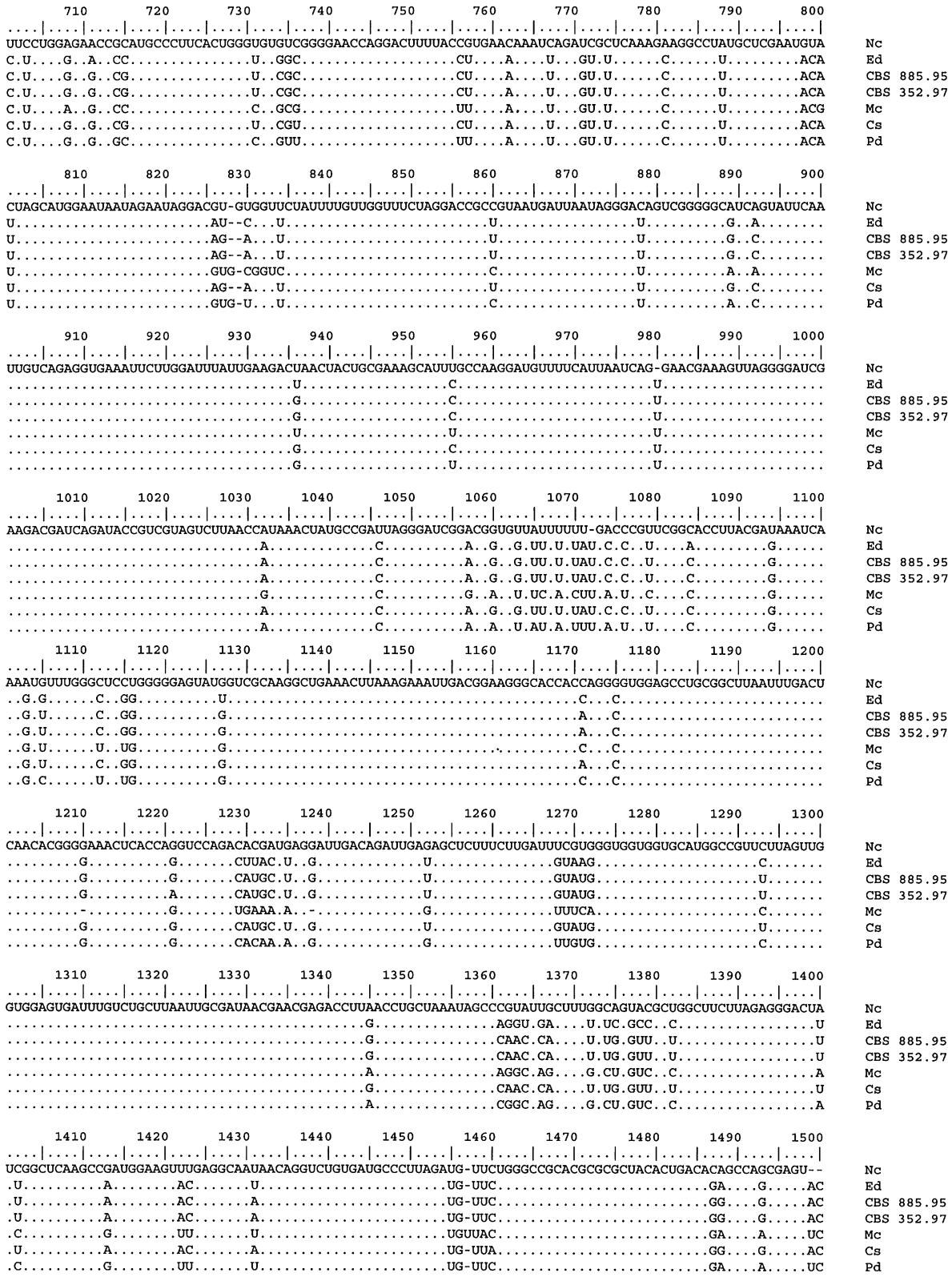
710 720 730 740 750 760 770 780 790 800
UUCCUGGAGAACCGCAUGCCCUUCACUGGGUGUGUCGGGGAACCAGGACUUUUACCGUGAACAAAUCAGAUCGCUCAAAGAAGGCCUAUGCUCGAAUGUA Nc
C.U....G..A..CC.............U..GGC.................CU....A....U...GU.U.....C.....U.........ACA Ed
C.U....G..G..CG.............U..CGC.................CU....A....U...GU.U.....C.....U.........ACA CBS 885.95
C.U....G..G..CG.............U..CGC.................CU....A....U...GU.U.....C.....U.........ACA CBS 352.97
C.U....A..G..CC.............C..GCG.................UU....A....U...GU.U.....C.....U.........ACG Mc
C.U....G..G..CG.............U..CGU.................CU....A....U...GU.U.....C.....U.........ACA Cs
C.U....G..G..GC.............C..GUU.................UU....A....U...GU.U.....C.....U.........ACA Pd
810 820 830 840 850 860 870 880 890 900
CUAGCAUGGAAUAAUAGAAUAGGACGU-GUGGUUCUAUUUUGUUGGUUUCUAGGACCGCCGUAAUGAUUAAUAGGGACAGUCGGGGGCAUCAGUAUUCAA Nc
U.....................AU--C...U.....................U.............U.........G..A........ Ed
U.....................AG--A...U.....................U.............U.........G..C........ CBS 885.95
U.....................AG--A...U.....................U.............U.........G..C........ CBS 352.97
U.....................GUG-CGGUC.....................C.............U.........A..A........ Mc
U.....................AG--A...U.....................U.............U.........G..C........ Cs
U.....................GUG-U...U.....................C.............U.........A..C........ Pd
910 920 930 940 950 960 970 980 990 1000
UUGUCAGAGGUGAAAUUCUUGGAUUUAUUGAAGACUAACUACUGCGAAAGCAUUUGCCAAGGAUGUUUUCAUUAAUCAG-GAACGAAAGUUAGGGGAUCG Nc
.................................U.................C.....................U.................... Ed
.................................G.................C.....................U.................... CBS 885.95
.................................G.................C.....................U.................... CBS 352.97
.................................U.................U.....................U.................... Mc
.................................G.................C.....................U.................... Cs
.................................G.................U.....................U.................... Pd
1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
AAGACGAUCAGAUACCGUCGUAGUCUUAACCAUAAACUAUGCCGAUUAGGGAUCGGACGGUGUUAUUUUUU-GACCCGUUCGGCACCUUACGAUAAAUCA Nc
.............................A.............C..........A..G..G.UU.U.UAU.C.C..U....A.........G...... Ed
.............................A.............C..........A..G..G.UU.U.UAU.C.C..U....C.........G...... CBS 885.95
.............................A.............C..........A..G..G.UU.U.UAU.C.C..U....C.........G...... CBS 352.97
.............................G.............C..........G..A..U.UC.A.CUU.A.U..C....C.........G...... Mc
.............................A.............C..........A..G..G.UU.U.UAU.C.C..U....C.........G...... Cs
.............................A.............C..........A..A..U.AU.A.UUU.A.U..U....C.........G...... Pd
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
AAAUGUUUGGGCUCCUGGGGGAGUAUGGUCGCAAGGCUGAAACUUAAAGAAAUUGACGGAAGGGCACCACCAGGGGUGGAGCCUGCGGCUUAAUUUGACU Nc
..G.G......C..GG..........U.....................................C...C..................... Ed
..G.U......C..GG..........G.....................................A...C..................... CBS 885.95
..G.U......C..GG..........G.....................................A...C..................... CBS 352.97
..G.U......U..UG..........G.....................................C...C..................... Mc
..G.U......C..GG..........G.....................................A...C..................... Cs
..G.C......U..UG..........G.....................................C...C..................... Pd
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
CAACACGGGGAAACUCACCAGGUCCAGACACGAUGAGGAUUGACAGAUUGAGAGCUCUUUCUUGAUUUCGUGGGUGGUGGUGCAUGGCCGUUCUUAGUUG Nc
.........G..........G.......CUUAC.U..G.............U..............GUAAG.....................C....... Ed
.........G..........G.......CAUGC.U..G.............U..............GUAUG.....................U....... CBS 885.95
.........G..........A.......CAUGC.U..G.............U..............GUAUG.....................U....... CBS 352.97
.........-..........G.......UGAAA.A..-.............G..............UUUCA.....................C....... Mc
.........G..........G.......CAUGC.U..G.............U..............GUAUG.....................U....... Cs
.........G..........G.......CACAA.A..G.............G..............UUGUG.....................C....... Pd
1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
GUGGAGUGAUUUGUCUGCUUAAUUGCGAUAACGAACGAGACCUUAACCUGCUAAAUAGCCCGUAUUGCUUUGGCAGUACGCUGGCUUCUUAGAGGGACUA Nc
....G..............AGGU.GA....U.UC.GCC..C..............U Ed
....G..............CAAC.CA....U.UG.GUU..U..............U CBS 885.95
....G..............CAAC.CA....U.UG.GUU..U..............U CBS 352.97
....A..............AGGC.AG....G.CU.GUC..C..............A Mc
....G..............CAAC.CA....U.UG.GUU..U..............U Cs
....A..............CGGC.AG....G.CU.GUC..C..............A Pd
1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
UCGGCUCAAGCCGAUGGAAGUUUGAGGCAAUAACAGGUCUGUGAUGCCCUUAGAUG-UUCUGGGCCGCACGCGCGCUACACUGACACAGCCAGCGAGU-- Nc
.U.........A........AC.......U.....................UG-UUC.....................GA....G.....AC Ed
.U.........A........AC.......A.....................UG-UUC.....................GG....G.....AC CBS 885.95
.U.........A........AC.......A.....................UG-UUC.....................GG....G.....AC CBS 352.97
.C.........G........UU.......U.....................UGUUAC.....................GA....A.....UC Mc
.U.........A........AC.......A.....................UG-UUA.....................GG....G.....AC Cs
.C.........G........UU.......U.....................UG-UUC.....................GA....A.....UC Pd

Figure 1.

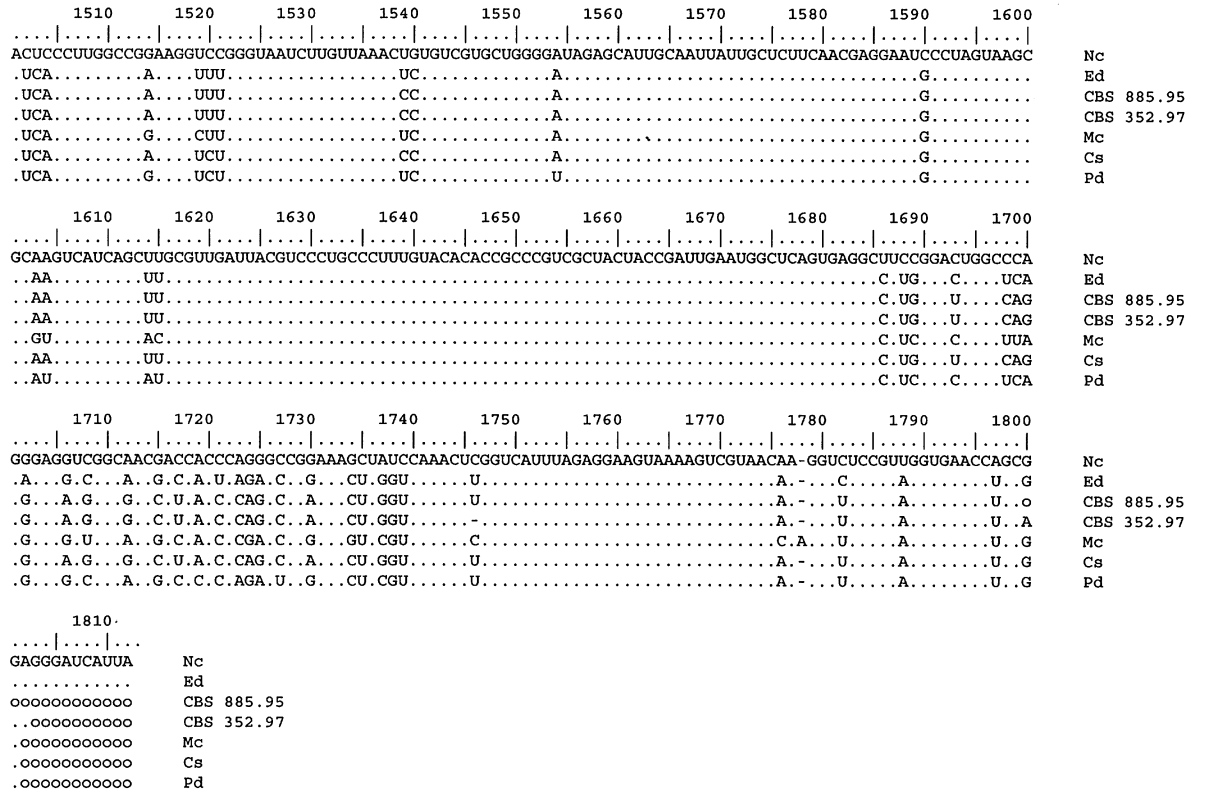

Figure 1. 18S rDNA sequences of *Neurospora crassa* (Nc), *Exophiala dermatitidis* (Ed), *Coniosporium perforans* (CBS 885.95), *C. apollinis* (CBS 352.97), *Monodictys castaneae* (Mc, CBS 100.07), *Coniosporium sp.* (Cs, CBS 665.80) and *Phaeosclera dematioides* (Pd, CBS 157.81).

AAACGCATTCGACTTGCATGGCGCTTCACCGCGCCGTCGCTAGGCTCTGCGCCAGAAAGCAGCCCGAAAGGTGAG
TGGTTGCCGTCCTTAAATGCTAGTCGCCCCCGGCGACCCCCTCCGTTGGGGCCCCGGGGCGGCGGCGAGATGCCT
AAATTGCGGGAAACTCGCGAAGTGCTCCACCACCAAGCTGCGCCCCGAAAGGGGTGCAGTTGGCCAGGGTAATGA
CCTCGGGTACGGTAACAGCGTGGAGCATATGTGACAATCCGCAGCCAAGCCCCTCTATGGCCAGCGCCAGTGGGG
AADGTTCAGAGACTATAATGGCGTCGGTTGGCTATATCGAACCGTACGGATCGCTGAAATATAGCGGGCTTAATG
TATAGTCCATGCCCACGCCGAAAGGGTGGGATTGACCTTAAAACGGTTCAAGTTTTA

Figure 2. Intron sequence of *Monodictys castaneae*. The 432 bases intron is located downstream of base No. 1177 of the 18S rDNA.

Holotypus: Delos 7, ex marble, Dioscourion, Delos, Graecia, in Geomikrobiologie ICBM, Oldenburg praeservatur; cultura viva DE 7a (= CBS 885.95).

Description on rock surface

Thallus moriform (Figure 4A), meristematic, 20–100 μm diam, frequently located in pits reflecting the size and shape of the colony (Figure 4B). Single cells 3–5 μm wide. As recently described by Sterflinger and Krumbein (1997), fungal colonization is initiated with adherence to the rock surface, subsequently narrow penetration hyphae are formed which dwell in fissures between rock crystals and in internal caves of the rock and internal satellite colonies are formed. In some cases ellipsoidal penetration pegs were observed at the hyphal tips (Figure 4C).

Description in culture

Colonies on 2% MEA or PDA at 18.5°C effuse, black, blackish-brown, greenish-blue or olivaceous, attaining 8–12 mm diam in 4 weeks, on CzA 4–7 mm diam. The mycelium on 2% MEA is partially immersed, with local hyphae deeply penetrating the medium. Colony margin sharp with submerged fimbriae composed of threads of moniliform hyphae. Aerial mycelium in the central part of the colony lanose, sparse, pale oliva-

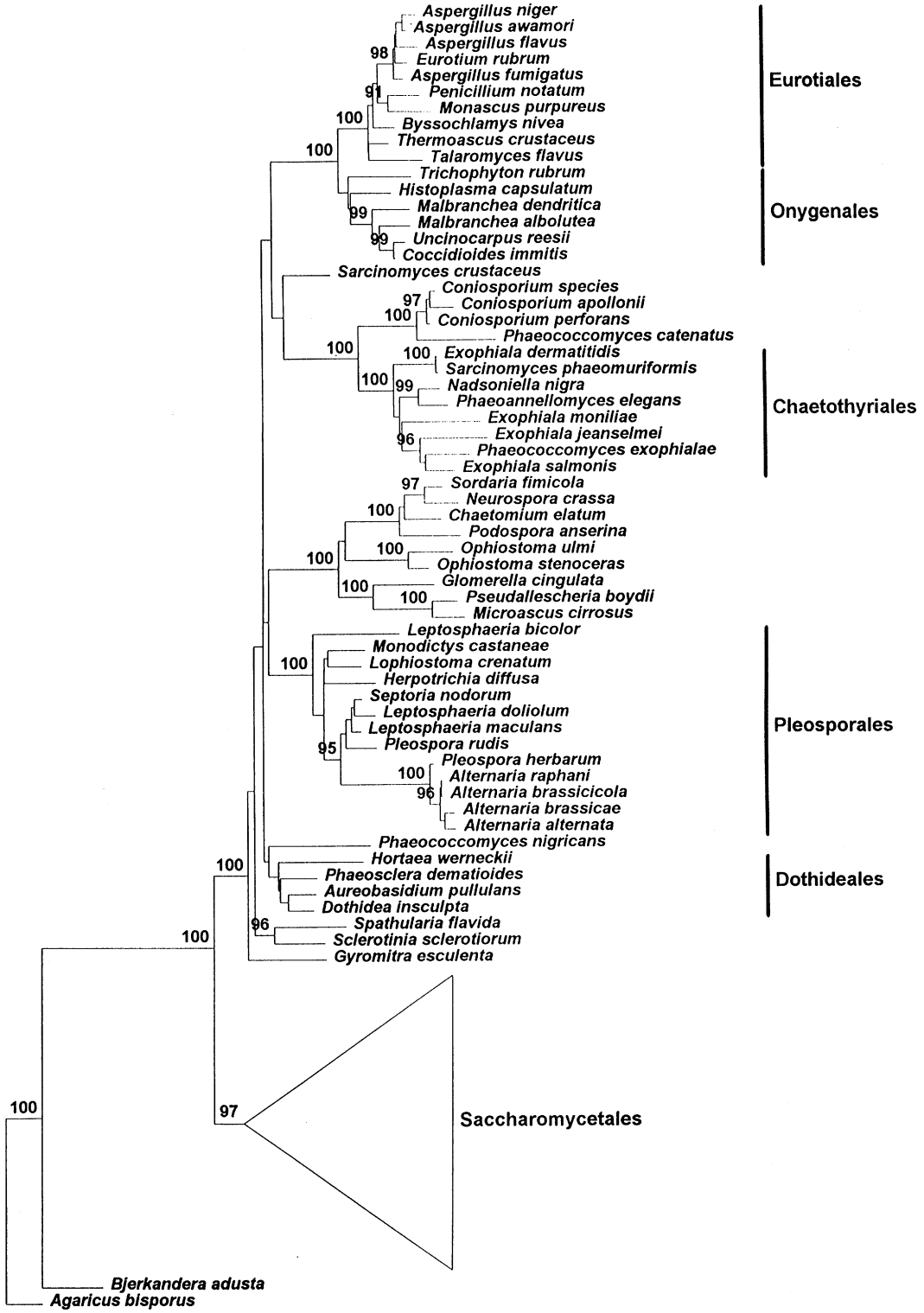

Figure 3. Similarity tree based on 18S rDNA sequencing data of 80 fungal species.

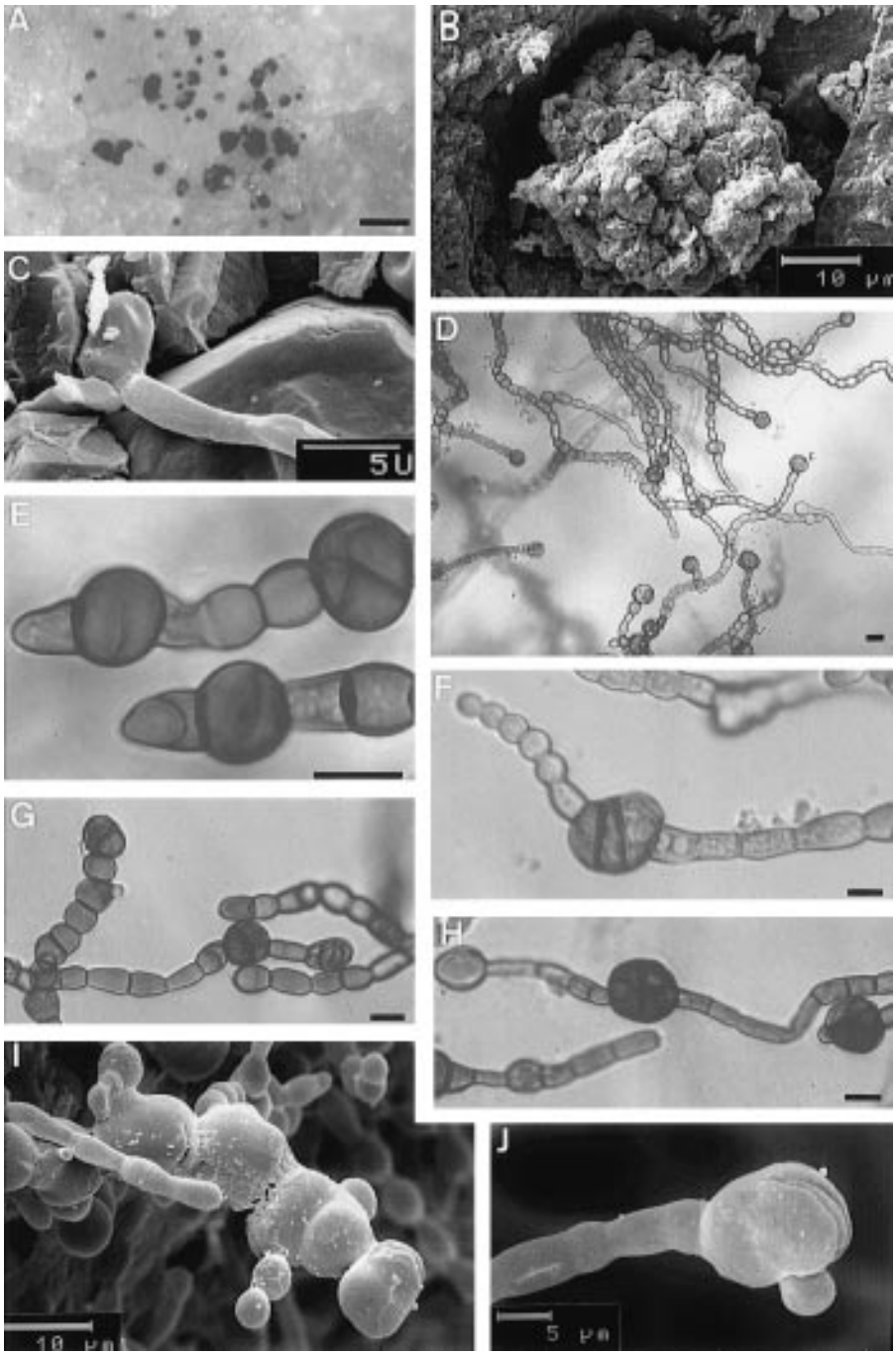

Figure 4. Coniosporium perforans (A) Macrophotograph of microcolonies on a marble surface. (B) SEM of a microcolony in a pit of a marble surface. (C) SEM of an ellipsoidal penetration peg at the end of a hypha. (D) Slide culture showing hyphae of elongated and monilioid cells with young conidia. (E) Mature conidia. (F) Three-celled conidium formed on an elongated hypha and producing a new monilioid hypha. (G) Multicellular conidia and yeast-like budding cell. (H) Young terminal conidium and mature multicellular, intercalary conidium. (I) SEM of conidia and conidial budding. (J) SEM of a terminal conidium producing a daughter cell by budding.

Table 2. Molecular weights [bp] of ITS restriction fragments of *C. apollinis* (CBS 352.97), *Coniosporium* sp. (CBS 665.80), *S. petricola* (CBS 725.95, CBS 726.95) and *C. perforans* (CBS 885.95). Confidence level 5%

ITS x MspI					ITS x Hae III				
352.97	665.80	725.95	726.95	885.95	352.97	665.80	725.95	726.95	885.95
	294				392		375	375	370
		236	236	236		264			
205						177	179	173	
	136								147
127		121	119	117	101		105	103	
98	98								93
		65							
ITS x DdeI					ITS x RsaI				
352.97	665.80	725.95	726.95	885.95	352.97	665.80	725.95	726.95	885.95
		648	648		517	514			
				582			425	422	422
	422					186	187	191	
279					85	85			
199									
	135								
	103								
88									

ceous. When grown at 27°C the fungus forms muriform, multilocular thalli arching up to 3 mm above the agar surface. A stroma is not developed. Setae absent, hyphopodia-like hyphal outgrowths sometimes developed.

The mycelium consists of two types of cells: (A) regular hyphae consisting of pale brown, elongate cells, thin- and smooth-walled, 2–3 μm wide and up to 10 μm long, sometimes with anastomoses; (B) moniliform hyphae (Figure 4D) pale brown, tapering to their ends, consisting of spherical to subspherical cells (2–4 μm). Single, terminal and sometimes lateral, holoblastic budding cells are frequently produced on MEA and PDA in cultures older than 4 weeks (Figure 4F, G–J), sometimes adhering in short moniliform branches.

Conidia (Figure 4D-J) terminal, sometimes intercalary on undifferentiated hyphae, spherical, 5–10 μm diam, one-celled and hyaline when young, later developing 1(−3) transverse and 0–1(−2) longitudinal septa and becoming muriform, 10–18 μm diam. Conidia sessile or borne by a subhyaline supporting cell which has a broad diameter at the conidial scar and is connected by a narrow isthmus with the hypha. Conidia occur both on regular and on moniliform hyphae. Mature conidia dry, dark olivaceous-brown, smooth-walled (light microscopy and SEM; Figure 4I, J), mostly single, sometimes in chains of up to four. Frequently enteroblastic budding of conidia can be observed. The outer wall of the conidium forms a pore through which a spherical daughter cell is released that germinates with a hypha. In cultures older than 8 weeks sclerotia are sometimes present.

Cardinal temperatures
Optimum 20°C, maximum 35°C, no growth at 37°C.

Coniosporium apollinis Sterflinger, spec. nov. – Figure 5

Thallus saxa calcarea inhabitans, nigricans, irregularis e cellulis globosis constat, 20–100 μm diametro, nonnumquam hyphae leves, sporulatio abundans. Coloniae in agaroso maltoso post 28 dies 17 mm diametro, colore non variabilio, nigro-viride, marginibus plerumque submersis, maturitate centralis pars elevata. Hyphae plerumque e cellulis globosis, 6–23 μm diametro vel ellipsoideo-cylindricis, symmetricis vel basi apiculatis et nonnumquam e cellulis elongatis 10–17 × 6–8 μm. Cellulae gemmantes praesentes. Conidia catenulata et non-catenulata, simul terminalia, et intercalaria, primo

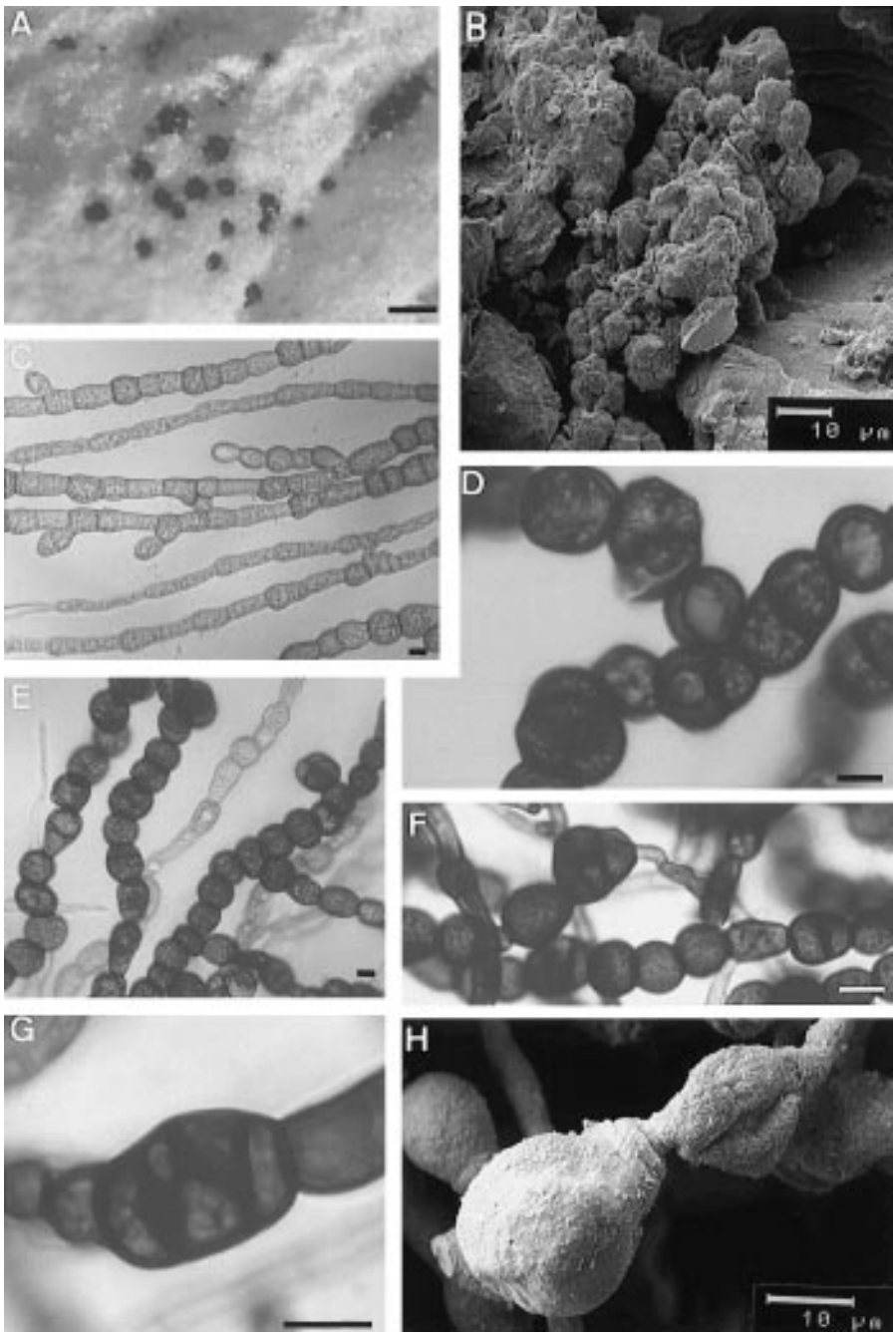

Figure 5. *Coniosporium apollinis* (A) Macrophotography of microcolonies on a marble surface. (B) SEM of a microcolony in a marble fissure. (C) Slide culture showing elongated and monilioid hyphae. (D) and (E) Mature conidial chains. (F) Conidial chains with one-celled and multicellular conidia. (G) Multicellular conidium. (H) SEM-photography of a conidium with a disjunction connecting it with the supporting cell.

globosa et maturitate plus minusve irregularia et 1–6 cellularia septis longitudinalibus et transversalibus, nonnumquam annellata. Conidia maturitate 27–32 μm longa et 27–23 μm lata, verrucosa. Cristalla nulla. Reproductio ascosporica non observata.

Holotypus: Delos11, ex marble, Delos, Graecia, in Geomikrobiologie ICBM, Oldenburg, praeservatur; cultura viva DE11 (= CBS 352.97).

Description on rock surface
Thallus moriform (Figure 5A), meristematic, 20–100 μm diam, frequently located in pits reflecting the size and shape of the colony (Figure 5B). Single cells 3–5 μm wide. Meristematic satellite colonies present. Penetrating hyphae not observed.

Description in culture
Colonies on 2% MEA and CzA at 18.5°C effuse, blackish-green, attaining 17 mm diam in 4 weeks. Colonies on PDA small, punctiform, attaining 2 mm diam in 4 weeks, first black and glistening, becoming lanose and dark green after 4 to 8 weeks. Colony colour stable on 2% MEA, the older colonies with lanose surface appearing lighter in colour than the young ones. On 2% MEA the mycelium is flat and mostly immersed. Colony margin sharp, with submerged fimbriae composed of threads of moniliform hyphae. The surface of young colonies is smooth and glistening although not slimy, in colonies older than 8 weeks the surface can become more lanose, wrinkled and arches over the agar surface with the highest point at the middle of the colony. Aerial mycelium not developed.

The mycelium consists of two types: (A) regular hyphae consisting of pale olivaceous-green, elongate cells, thick- and smooth-walled, 6–8 μm diam and 10–17 μm long, sometimes with anastomoses; (B) moniliform hyphae (Figure 5C) tapering to their ends, consisting of obovoidal cells with maximum width (about 6 μm) at the distal end and up to 23 μm in length.

Conidia (Figure 5D-H) terminal, in long chains, on undifferentiated hyphae, initially one-celled and spherical, 10–23 μm diam, to subspherical, up to 15 $\times$ 23 μm, later becoming more irregular and developing 1–5 transverse and 1–3 longitudinal septa and becoming muriform, up to 27 $\times$ 32 μm. Older conidial chains break up and release conidia either separately or in short chains of 3–5. In some cases disjunctor-like structures between conidium and supporting cell were observed with SEM (Figure 5H). Conidia occur both on regular and moniliform hyphae. Mature conidia are dry, dark greenish-black, thick-walled and smooth-walled in light microscopy but with some roughness in SEM (Figure 5H), with clearly visible oil droplets in each cell. Enteroblastic budding of conidia is rarely observed, taking place by irregular disruption of the outer wall and release of a spherical daughter cell from inside. Sclerotia not observed.

Cardinal temperatures
Optimum 20°C, maximum 35°C, no growth at 37°C.

Discussion

Morphologically the strains CBS 885.95, CBS 352.97 and CBS 665.80 are attributed to the so-called black yeasts on the basis of their melanized hyphae and the presence of budding cells. They differ from most black yeasts described to date by a predominance of meristematic growth on the natural rock substratum. This condition occurs particularly in epiphytic and epi- and endolithic fungi. The meristematic fungi are known to be unrelated, which was confirmed in the present study by a large phylogenetic distance between *Coniosporium* spp. and *Phaeosclera dematioides* (Figure 3). For meristematic fungi, four main lines of relationship within the Ascomycota have been suggested so far:

(1) Family Herpotrichiellaceae (order Chaetothyriales), through *Sarcinomyces petricola* Wollenzien & de Hoog. On the basis of SSU RFLP this species was supposed to be phylogenetically close to *S. phaeomuriformis* Matsumoto et al. (Wollenzien et al., 1996; Uijthof, 1996). The SSU sequencing data presented here suggest that the type species *Sarcinomyces crustaceus* is not in a close phylogenetic relation to these strains (Figure 3).

(2) Family Dothideaceae (order Dothideales). *Trimmatostroma abietis* Butin & Pehl (Butin et al., 1996) shows a growth phase-dependent conversion from extension growth to meristematic growth (Yoshida et al., 1996; Figueras et al., 1996). SSU RFLP data (G.S. de Hoog, unpublished data) indicate a relationship with *Aureobasidium* Viala & Boyer. *Phaeosclera dematioides* has intercalary, sclerotia-like conidia without budding (Sigler et al., 1981). In 18S phylogeny it was found to cluster in the Dothideales together with, e.g., *Aureobasidium pullulans* (de Bary) Arnaud and *Hortaea werneckii* (Horta) Nishimura & Miyaji (Figure 3).

(3) Family Capnodiaceae (order Capnodiales), through *Capnobotryella renispora* Sugiyama, which occasionally occurs on roof tiles (Titze & de Hoog, 1992). As yet no molecular data are available on this group.

(4) Family Pleosporaceae (order Pleosporales), through *Botryomyces caespitosus* de Hoog & Rubio, which was recently proven to be closely related to *Alternaria* (de Hoog et al., unpublished data); strains morphologically similar to this genus are frequently found on stone (Wollenzien et al., 1995). In the present study *Monodictys castaneae* was found to be a member of the same order (Figure 3). On the natural substratum *Monodictys* species are characterized by blackish-brown, muriform conidia which are lifted slightly above the surface by intercalary extension of a short conidiophore (Rao & de Hoog, 1986), but in culture some species with larger, moriform conidia morphologically resemble *Phaeosclera.*

From the phylogenetic divergence it can be concluded that the ability to grow meristematically has been developed several times in the course of evolution as an adaptation to environmental conditions of stress. On bare rock surface the ecotype consists of moriform, very slow growing thalli (Sterflinger & Krumbein, 1995).

Occasionally ecotypes of what is taken to be a single species show small but significant molecular differences. For example, the meristematic taxon *Sarcinomyces phaeomuriformis* was supposed to be a mutant of the black yeast *Exophiala dermatitidis* (Kano) de Hoog. The ITS sequences of the two entities differ in 31 positions; strains morphologically identical to *E. dermatitidis* may have either one of these types (Uijthof, 1996). The meristematic yeast *Fissuricella filamenta* Pore et al. has been supposed to belong to *Trichosporon asteroides* (Rischin) Ota, although morphologically and culturally the two entities are extremely different. nDNA-DNA reassociation values were around 85% and partial SSU rRNA sequences revealed one base difference only (Guého et al., 1992). An explanation of this striking discrepancy between groups of taxonomic parameters can as yet not be given, but it is tempting to suppose a segregation of ecotypes followed by speciation. It is worth mentioning that such events are more likely to be revealed in fungi than in bacteria, where the rate of morphological differentiation is limited and thus no conflicting datasets are observed.

Molecular phylogeny of these organisms should enable a natural classification of these fungi according to purported teleomorph family relationships. To this aim we need information on the position of the type species of meristematic genera. Wollenzien et al. (1996) classified their stone species *S. petricola* in *Sarcinomyces*, despite the fact that the phylogenetic position of the generic type species, *S. crustaceus* Lindner, could not be established by SSU RFLP due to the occurrence of introns. In the present study *S. crustaceus* was proven to be unrelated (Figure 3). CBS 885.95 and CBS 352.97 probably are phylogenetically close to *S. petricola* as judged from SSU RFLP data (G.S. de Hoog, unpublished data). Morphologically they cannot be classified in *Sarcinomyces* because (a) their multicellular conidia develop singly or in proximal succession on hyphae, whereas free cell packets of *Sarcinomyces* continually enlarge and break up into packets that again start swelling, (b) the resulting multicellular conidia do not disarticulate into separate cells, and (c) their lack of endoconidiation; species *Sarcinomyces* typically release daughter cells by deterioration of the mother cell with remnants of the mother cell wall being attached to the daughter cells. Conidia of CBS 885.95 and CBS 352.97 produce only single budding cells through pores (CBS 885.95) or only local disruption of the conidial wall (CBS 352.97).

Coniosporium species develop catenate, muriform conidia from a central meristem (Ellis, 1971). Most *Coniosporium* species described to date were found on rotten wood. Link's (1809) original specimen of *C. olivaceum* Link:Fr. was isolated from *Pinus maritima* in the Mediterranean area. Thus far no cultured material is available of any *Conisporium* species to allow verification of the identity of species from wood and our stone-inhabiting ecotypes. Although morphological comparison with *Coniosporium* species (Ellis, 1971, 1976) isolated from different substrata allows an assignment of CBS 885.95 and CBS 352.97 to the genus *Coniosporium*, an identification as one of the currently described species is not possible. However, the close relation of the both strains to the genus *Coniosporium* is confirmed by the phylogenetic analysis presented here.

Coniosporium perforans is morphologically reminiscent of the genus *Monodictys*, both having non-catenate, dictyosporous conidia. However, the sequencing data clearly show that *Monodictys* and *Coniosporium* are unrelated. *Coniosporium apollinis* resembles the bulbil-like structures typical for the genus *Phaeosclera*, but also in this case phylogenetic analysis clearly distinguishes both taxa.

The 18S phylogeny of the two stone-inhabiting species and a third *Coniosporium*-like strain suggests that the clade is a sister group to the Herpotrichiellaceae (Figure 3). *Phaeococcomyces catenatus* also belongs to this group, though at a larger distance.

The 18S rDNA sequences of *Coniosporium apollonis* and *C. perforans* were found to differ in 8 positions, which corresponds to a 18S rDNA similarity higher than 99%. Based on species concepts developed in bacteriology, more than 99% similarity of the SSU rDNA corresponds to a nDNA-DNA homology of at least 50% (Fox et al., 1992). A species is defined, albeit quite arbitrarily, as more than 70% DNA-DNA similarity. Thus 18S sequencing data alone are not sufficient to split or lump strains into species. They can be distinguished from each other by stable morphological features and this favours classification into two different species *C. perforans* and *C. apollinis*. This is supported by differences in the restriction fragment polymorphism of ITS shown in Table 2. *C. perforans* might be closely related to *S. petricola* since it has identical ITS restriction patterns with MspI and RsaI.

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