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Molecular diversity of oligotrophic and neurotropic members of the black yeast genus *Exophiala*, with accent on *E. dermatitidis*

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Abstract

Analysis of ITS rDNA of the black yeast *Exophiala dermatitidis* revealed a close phylogenetic relationship to the meristematic fungus *Sarcinomyces phaeomuriformis*. As most strains of *S. phaeomuriformis* have a yeast-like phenotype corresponding to the anamorph genus *Exophiala*, a new combination in *Exophiala* is proposed. On the basis of ITS sequence, M-13 fingerprint and SSU intron data, two main entities could be distinguished within *E. dermatitidis*. One of these (B) contained prevalently strains from environmental sources, while the other (A) mainly comprised strains from clinical sources. This may be due to a difference in virulence. All strains from severe brain and disseminated infections in East Asia clustered in group A. However, strains of group A caused a relatively mild fungemia in patients outside East Asia.

Introduction

Neurotropism of the black yeast *Exophiala dermatitidis* (Kano) de Hoog has been proven by animal experiments (Nishimura and Miyaji 1983; Dixon et al. 1992). The species is distributed world wide, but nevertheless cerebral cases are restricted to East Asia (Horré and De Hoog 1999). The rarity of the fungus hampers statistical confirmation of this phenomenon: twelve neurological cases were listed by Hiruma et al. (1993), Matsumoto et al. (1993), while Chang et al. (2000) described a further case from Korea. Only two cases have been reported from outside the Far East. Ajanee et al. (1996) isolated the species from a fatal brain infection in an immunocompetent patient in Pakistan, and Kenney et al. (1992) described an infection with neurological involvement from the U.S.A. Both patients were non-Asian. However, the strains involved have not been preserved and hence verification of their identity is impossible. Outside Asia, infections are mainly sequelae of trauma (Crosby et al. 1989), although subclinical pulmonary colonizations of patients with cystic fibrosis are also elucidated (Haase et al. 1991). Disseminated cases do occur in immunocompromised patients outside Asia (Weissbrodt et al. 1994; Kabel et al. 1994; Blaschke-Hellmessen et al. 1994), but in none of these cases is any neurotropism seen.

The apparent uneven geographical distribution of neurotropism in *E. dermatitidis* remains difficult to explain. Matos et al. (2002) investigated the possibility of unequal exposure to the fungus, due to differences in bathing culture between Asia and elsewhere. The fungus, however, was found to be abundant in the commonly visited public steam baths in Northwest Europe as well as in Asia. Although no quantitative data are available, differences in exposure are not a likely cause of the uneven distribution of cerebral cases.

Uijthof et al. (1994, 1998) investigated whether

local Asian populations of *E. dermatitidis* with different neurotropic pathology exist. One of their populations, defined by RAPD profiles, contained cerebral Asian strains as well as subclinical European strains from cystic fibrosis (CF) patients, and thus the possibility that the former strains were distinct was not supported. Statistical support for their conclusions remained insufficient because the number of strains studied was low.

In recent years a larger set of strains has become available. These have been isolated from diverse environments. The habitats involved are by no means randomly distributed. This suggests the presence of a special, as yet undisclosed ecological niche of the fungus. Matos et al. (2002) were able to partially characterize the preferred niches as low-nitrogen environments. Growth in such habitats may be combined with occasional passage through the intestinal tract of animals. In the present paper we report on the occurrence of genotypes within the species, as a further step towards understanding the behaviour of the latter in nature. We used ITS sequencing, fingerprinting and SSU rDNA intron detection. A comparison is made with *Sarcinomyces phaeomuriformis*, a closely related thermotolerant black yeast which is known from very similar environments. The relationship of the two species to the teleomorph genus *Capronia* (Ascomycota, Chaetothyriales, Herpotrichiellaceae) is considered.

Material and Methods

Strains and culture conditions. Strains studied are listed in Table 1 and were grown on Potato Dextrose Agar (PDA) slants for 2–3 wk at 24 °C. The presence or absence of extracellular polysaccharides (EPS) was verified microscopically in Indian Ink.

DNA extraction. About 1 g of mycelium was transferred to a 2:1 mixture of silicagel and Celite 545 with 300 μL CTAB-buffer (Tris.HCl, 200 mM, pH 7.5; Na-EDTA, 200 mM; NaCl 8.2%; CTAB 2%). The material was ground with a micropestle (Eppendorf, Hamburg, Germany). After adding 200 μL CTAB-buffer and vigorous shaking, the sample was incubated for 10 min in a 65 °C water bath. 500 μL Chloroform was added, followed by vortexing and centrifugation for 5 min at 14,000 r.p.m. The aqueous supernatant was transferred to a new Eppendorf tube, 2 volumes (~800 μL) ethanol 96%, −20 °C were added and mixed gently. The DNA was precipitated at −20 °C for at least 30 min. The pellet, obtained by centrifugation for 5 min at 14,000 r.p.m., was washed twice with 500 μL ethanol 70% at −20 °C. DNA was dried overnight at room temperature and suspended in 97.5 μL TE-buffer (10 mM Tris, 10 mM Na-EDTA, pH 8.0) with 2.5 μL RNAse-solution (10 mg pancreatic RNAse 20 U/mg in 1 mL 0.01 M Na-acetate; pH 7.4). Samples were incubated for 5–30 min at 37 °C, cleaned over GFX® columns (Amersham Biosciences) and stored at 4 °C.

PCR fingerprinting. The core sequence of the phage M13 (5′-gAg ggT ggC ggT TCT; Gräser et al. (1999b)) was used as a single primer. Amplification was performed in 50 μL volumes containing 25 ng of template DNA, 5 μL reaction buffer (0.1 M Tris-HCl [pH 8.0], 0.5 M KCl, 15 mM $MgCl_2$, 0.1% gelatin and 1% Triton X-100), 200 μM of each dATP, dCTP, dGTP, dTTP (Pharmacia LKB Biotechnology Inc., NJ, USA) and 2.0 U *Taq* DNA polymerase (ITK Diagnostics, Leiden, The Netherlands). The primer was added at a final concentration of 25 pmol. Samples were amplified through 40 cycles in a Gene Amp thermocycler, as follows: predwell 94 °C, 2′, denaturation (94 °C, 20″), annealing (50 °C, 1′), elongation (72 °C, 20′), postdwell (72 °C, 6′). Amplicons were electrophoresed in 1.2% agarose gels (Gibco BRL, Life Technologies) for 7 h at 60 V in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3). Gels were stained with ethidium bromide and photographed for further analyses. All tests were done in duplicate.

Comparison of fingerprinting data. Gels were imported in BioNumerics version 2.5 (Applied Maths, Kortrijk, Belgium) and compared using the GelCompar option. Trees were made using the UPGMA algorithm with Jaccard's correction and 5% band position tolerance; uncertain bands were ignored.

Sequencing. rDNA ITS domains were amplified with 30 cycles in an Amplitron thermocycler using primers V9G and LS266 (De Hoog and Gerrits van den Ende 1998), as follows: predwell (94 °C, 5′), denaturation (94 °C, 45′), annealing (52 °C, 30′), elongation (72 °C, 2′), postdwell (72 °C, 6′). One unit Super-Taq polymerase (SphaeroQ, Leiden, The Netherlands) was used per reaction. Sequencing primers were ITS1, ITS4 and ITS5 (White et al. 1990) and amplification was done as follows: 96 °C, 10″; 50 °C, 5″; 60 °C, 4′ (25 cycles), carried out with 15–50 ng of DNA for a 10 μL reaction mixture including 4 pmol primer and 4 μL BigDye RR Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Table 1. Strains investigated, listed according to populations based on ITS sequencing, M-13 fingerprint, RAPD and SSU-introns.

Isolate	Alternative identification	Source	Origin	No. of nucl. diff.	M-13 group	RAPD group[1]	SSU length
CBS 578.76	CDC B-3567 NCMH 1224 KMU 1405	**Brain,** pancreas, liver, ileum, lymph nodes, meninges	Taiwan	0	A	1	3000
CBS 207.35	UTMB 3827 NCMH 252 ATCC 28869 WD 252	Skin	Japan	0	A	1	3000
CBS 581.76		**Brain?**	Japan	0	A	-	3000
CBS 153.94	CDC B-3554 NCMH 1211 KMU 1308	Liver	Japan	0	A	-	3000
NCMH 1213	CDC B-3556 KMU 1166	Disseminated	Japan	-	A	-	3000
NCMH 1214	CDC B-3557 KMU 1948	Skin	Japan	-	A	-	3000
CBS 709.95		Subcutaneous	Korea	0	A	-	3000
NCMH 1388		Subcutaneous cyst	USA	-	A	-	3000
NCMH 1218	CDC B-3561 KMU 1616	**Brain,** skin, lung, lymph nodes	Japan	-	A	-	3000
CBS 109154	DH 11215	**Brain**	Korea	-	A	-	-
NCMH 1210	CDC B-3553 KMU 1309 IFM 4837	Disseminated	Japan	-	A	-	3000
CBS 292.49		Faeces	Brazil	0	A	1	3000
DH 11380		Sputum, CF	Finland	0	A	-	3000
DH 10486		Skin	Germany	0	-	-	3000
CBS 632.69		Unknown	Germany	0	A	-	3000
BS 100339	RKI 4672/92 BlschVa3048/1	Blood	Germany	0	A	2	3000
CBS 748.88		Sputum, CF	Norway	0	A	2	3000
UAMH 4268		Sputum	Canada	0	-	-	-
DC B-1781	UAMH 3691	*Brain*	Japan	0	-	-	-
CDC B-2099	UAMH 3694	Oral mucosa		0	-	-	-
NCPF 2758		Cyst elbow	UK	0	-	-	-
NCPF 2926		BAL AIDS	UK	0	-	-	-
GHP 1689		Sputum, bronchiectasy	Germany	0	-	-	-
CBS 154.90		Sputum, CF	Germany	0	A	2	3000
CBS 338.90	OMH F9191.89	Onychomycosis	Mauritius	0	A	2	3000
CBS 577.76	CDC B-1782 DUKE 3381 VGH 625551	**Brain**	Taiwan	0	A	2	3000
CBS 525.76	NCMH 253 UTMB 253 ATCC 34100	Sputum	Japan	0	A	2	3000
CBS 100338	IFM 4826 MM-7	Humidifier	Japan	0	A	-	3000
CBS 100342	IFM 4958	Public bath	Japan	-	A	-	3000
CBS 109145	T-18	Steam bath (AMS2)	Netherlands	0	A	-	3000
CBS 109148	T-21	Faeces	Netherlands	0	A	-	3000
CBS 218.88		Faeces	France	0	A	2	3000
CBS 552.90		Sputum	Germany	-	-	-	3000
CBS 550.90		Sputum	Germany	-	A	-	-
CBS 156.90		Sputum, CF	Germany	-	-	2	3000
CBS 153.90		Sputum, CF	Germany	-	A	2	3000
CBS 213.90		Sputum, CF	Germany	0	-	2	3000
CBS 148.90		Sputum, CF	Germany	0	A	2	3000
IFM 4833		Unknown, diploid	Unknown	-	A	-	3000
IFM 41488		Unknown, diploid	Unknown	-	-	-	-
CBS 100337		Sputum, CF	Sweden	0	A	2	3000
CBS 109141	T-14	Steam bath (L-yeast)	Netherlands	-	A	-	-
CBS 109140	T-12	Finn. sauna (L-dry)	Netherlands	0	A	-	3000
CBS 109143	T-16	Shower (L-dry)	Netherlands	-	A	-	-
CBS 686.92	Bautsch	Blood	Germany	0	A	2	3000
NCMH 1215	CDC B-3558 KMU 1183	Skin	Japan	-	-	-	3000
NCMH 1204	CDC B-3547 DUMC 3379	**Brain,** cervical lymph nodes	Japan	0	A	-	3000
CBS 109153	DH 10050	Ear	Finland	0	-	-	1800
CBS 639.96		Keratitis	France	0	A	-	2100
NCMH 1217	CDC B-3560 KMU 1581	**Brain,** liver, lymph nodes, lung	Japan	-	A	-	2100
DH 9883		Dried fruit	Germany	2	A	-	2100
CBS 151.93	Schroer 661	Plant root	Germany	3	A	-	1800

Table 1. (*continued*)

Isolate	Alternative identification	Source	Origin	No. of nucl. diff.	M-13 group	RAPD group[1]	SSU length
CBS 424.67		Skin	Germany	0	A*	-	3000
CBS 736.87		Beer	Ireland	2+1−1	A*	-	2100
IFM 45986		Tap water	Japan	2+1−1	-	-	-
CBS 109139	T-10	Steam bath (L)	Netherlands	2+1−1	-	-	2100
CBS 109136	T-7	Steam bath (H)	Netherlands	3	B	-	2100
CBS 109149	T-22	Steam bath	Slovenia	3	B	-	2100
CDC B-1778	UAMH 3700	Blood	USA	3	-	-	-
NCPF 2924		Sinus	UK	3−1	-	-	-
CBS 109142	T-15	Fruit	Netherlands	3	B	-	-
GHP 944		Ceiling	Germany	3	-	-	-
CBS 109144	T-17	Steam bath (AMS1)	Netherlands	3	-	-	1800
CBS 109138	T-9	Hall sauna (H)	Netherlands	3	B	-	-
CBS 109134	T-1	Steam bath (H)	Netherlands	3	B	-	-
DH 11823	T-5	Steam bath (H)	Netherlands	-	B	-	-
CBS 549.90		Sputum	Germany	-	-	-	-
CBS 971.87		Unknown	Iraq	-	B	5	1800
CBS 100341	RKI 4669/92 BlschVa300/8	Blood	Germany	3	B	6	1800
CBS 100340	IFM 4848 INPA 109	Liver of bat	Brazil	3	B	-	1800
CBS 106.92	DBVPG 4256	Grape	Italy	3	B	7	1800
CBS 149.90		Sputum, CF	Germany	1	B	4	1800
CDC B-4584		Pinus	Unknown	1	-	-	-
IFM 41823	CBS 109152	Cactus	Venezuela	−1	C	-	1800
CBS 150.90		Sputum	Netherlands	30	D	3	1800

- = not determined; [1]Data taken from Uijthof et al. (1998). Numbers of ITS nucleotide differences are compared with CBS 207.35 (T) and are limited to phylogenetically informative sites which are identical in each group. + and − are additional differences or deletions. Ams1 and 2 = two sauna complexes in Amsterdam; H = Hilversum; L = Laren.

Amplicons were purified using GFX-columns (Pharmacia, Freiburg, Germany), eluted according to protocols given by the supplier, and collected with TE-buffer. Concentrations of amplicons were estimated by comparison with SmartLadder markers (Eurogentec, Seraing, Belgium) on 1% agarose gels. Subsequently DNA was precipitated with ethanol and sequenced using an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems).

Alignment and phylogenetic analysis. Sequences were adjusted using SeqMan of Lasergene software (DNASTAR, Madison, Wisconsin) and aligned using BioNumerics (Applied Maths, Kortrijk, Belgium). A distance tree was constructed with Neighbor-joining with Kimura correction (transition:transversion = 0.5) using the TREECON (version 1.3b) software package (Van de Peer and De Wachter 1993) also using published *Capronia* sequences. Bootstrap values were calculated from 100 resampled datasets. Not all identical genotypes are included in the phylogenetic analyses; for additional data on strains, see Table 1.

Statistics. Results were verified with χ^2 tests using $P<0.05$ confidence interval.

Results and Discussion

Phylogenetic relationships of *E. dermatitidis*

Using rDNA ITS sequence data, only a small number of described *Capronia* species could confidently be aligned with the *E. dermatitidis* complex (Figure 1). *Capronia fungicola* (Samuels & E. Müller) E. Müller et al. was found to be among the nearest teleomorph neighbours of *E. dermatitidis*, and was the most distantly related species for which ITS sequences could be aligned with those of *E. dermatitidis*. It has a *Ramichloridium* anamorph in addition to a poorly differentiated *Exophiala* type of conidiation (Samuels and Müller 1978) and is unable to grow at 37 °C (Untereiner et al. 1999). Other *Capronia* species found in the *E. dermatitidis* complex (*C. coronata* G.J. Samuels, *C. epimyces* M.E. Barr, *C. mansonii*

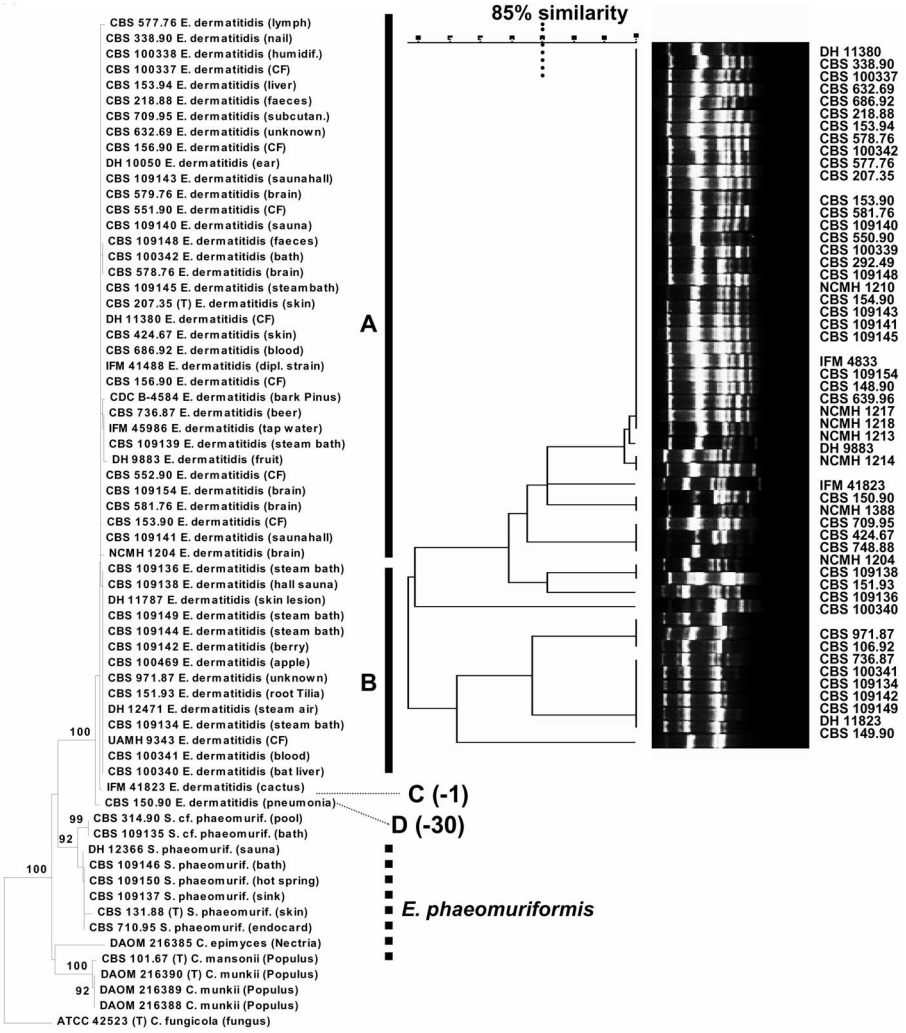

Figure 1. Distance tree of the *Exophiala dermatitidis* complex based on confidently aligned ITS rDNA sequences, constructed with Neighbor joining with Kimura correction, taking *Capronia epimyces* as outgroup. Bootstrap values >90 of 100 resampled datasets are shown. Summarized ecological source of each strain is listed in brackets. Populations with numbers of ITS changes are listed as A-D. Strains with conflicting fingerprint data are mentioned in italics. The right panel shows the M-13 fingerprint data of the *E. dermatitidis* strains, with listing of populations in italics. Strains with conflicting ITS sequencing data are mentioned left of the vertical bars.Abbreviations of names: C = *Capronia*, E = *Exophiala*, S = *Sarcinomyces*

(Schol-Schwarz) E. Müller et al. and *C. munkii* Untereiner) are all relatively close to *Sarcinomyces phaeomuriformis* Matsumoto et al. The anamorph of *C. epimyces* is an *Exophiala* species (Untereiner 1995) and has a phialidic synanamorph (Untereiner and Naveau 1999). *Capronia coronata, C. mansonii* and *C. munkii* have only *Exophiala* anamorphs. *Capronia epimyces* and *C. munkii* are the only *Capronia* species able to grow at 37 °C (Untereiner et al. 1999).

The distance between *E. dermatitidis* and *S. phaeomuriformis* was considerable (26 bases; Uijthof et al. (1998)), but nevertheless the two species could be confidently aligned over the entire spacer domain. In the two ex-type strains, the SSU rDNA genes, including the introns, were identical (Haase et al. 1999). Some physiological features considered to be diagnostic for *E. dermatitidis*, such as growth with nitrate, nitrite, creatine and creatinine, are also characteristic of *S. phaeomuriformis* (Uijthof et al. 1998). The latter species was described originally (Mat-

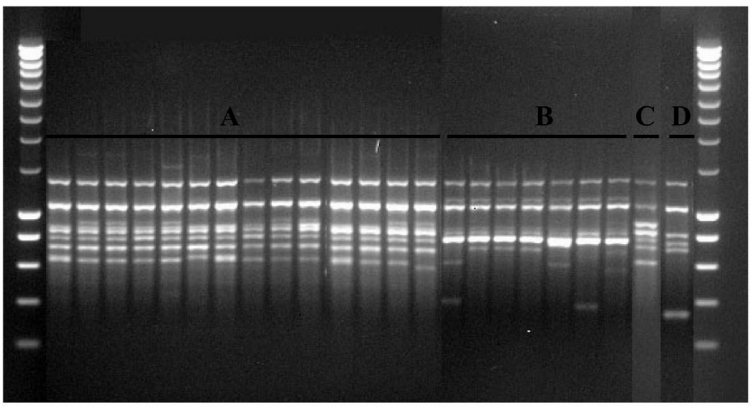

Figure 2. Example of populations of *Exophiala dermatitidis* as distinguished by PCR-fingerprinting with primer M-13. A: CBS 581.76, CBS 100339, CBS 639.96, CBS 100342, NCMH 1217, CBS 109148, CBS 578.76, NCMH 1210, CBS 154.90, CBS 109143, CBS 109141, CBS 153.94, CBS 577.76, CBS 109145; B: CBS 100341, DH 11823, CBS 109134, CBS 109142, CBS 109149, CBS 100340, CBS 106.92; C: IFM 41823; D: CBS 150.90

sumoto et al. 1986) as having very limited expansion growth and a thallus consisting of slowly inflating, thick-walled, spherical cells that gradually disarticulated into smaller units (De Hoog et al. 2000). But as noted earlier by Uijthof et al. (1998), as well as in the present study, strains of *S. phaeomuriformis* mostly have a yeast-like appearance. Such strains closely resemble *E. dermatitidis* in cultural characteristics, and have yeast cells that are on average only slightly larger. Meristematic growth in *S. phaeomuriformis* is actually exceptional. *E. dermatitidis* also occasionally shows meristematic growth (Nishimura et al. 1998). The meristematic synanamorph tends to be induced under acidic conditions (De Hoog et al. 1994) and some strains remain in a similar isodiametric growth form under all known culture conditions, as exemplified by a strain that was ascribed to the meristematic genus *Botryomyces* and was revealed to be *E. dermatitidis* only after sequencing (De Hoog and Horré 2002). Since the most common morphology of *S. phaeomuriformis* is yeast-like rather than meristematic, we propose a new combination in *Exophiala* below.

Matos et al. (2002) encountered both species in public bathing facilities and found them to differ slightly in ecological preferences: *E. dermatitidis* occurred in hot steam baths, whereas *S. phaeomuriformis* was encountered in the warm but less extreme environments of adjacent halls and bathrooms. A primary culture of *S. phaeomuriformis*, DH 12471, from steam bath air, showed only very limited, heaped growth, which consisted entirely of densely packed phialides (Figure 4) similar to the phialidic bodies reported in some *E. dermatitidis* strains on low-nutrient media by De Hoog et al. (1994). *E. dermatitidis* and *S. phaeomuriformis* differ significantly in their maximum temperatures for growth (42 and 36 °C, respectively; Matos et al. (2002)). Like *E. dermatitidis*, *S. phaeomuriformis* can be involved in superficial and systemic infections in humans (Mori et al. (1961), as *Hormiscium dermatitidis*; Gold et al. (1994), as *Exophiala castellanii*).

Exophiala phaeomuriformis (Matsumoto, Padhye, Ajello & McGinnis) Matos, Haase & De Hoog, **comb. nov.** – Figure 3.

Basionym: *Sarcinomyces phaeomuriformis* Matsumoto, Padhye, Ajello & McGinnis – J. Med. Vet. Mycol. 24: 396, 1986.

Colonies of *yeast / hyphal variant* attaining about 5 mm in 10 d, smooth when young, often somewhat slimy, then becoming tough, black to brownish- or olivaceous-black. Budding cells initially subhyaline, smooth, thin-walled, ellipsoidal, about 4 × 2 μm, later swelling to become 'germinating cells' which are olivaceous-brown, smooth, thick-walled, broadly ellipsoidal, about 5–6 × 4 μm. Submerged hyphae pale olivaceous, thin-walled. Aerial hyphae robust, olivaceous, with thicker walls, about 2.0–2.5 μm wide, often starting with a series of ellipsoidal cells (torulose hyphae). Annellated zones produced on germinating cells, mature budding cells and hyphae, very short and inconspicuous, non-elongating; annelloconidia 3–4 × 1.5–1.8 μm. Phialides occasionally produced in large amounts, then often preponderant and clustered in dense aggregates, (sub)spherical, with large, subspherical to vase-shaped collarettes;

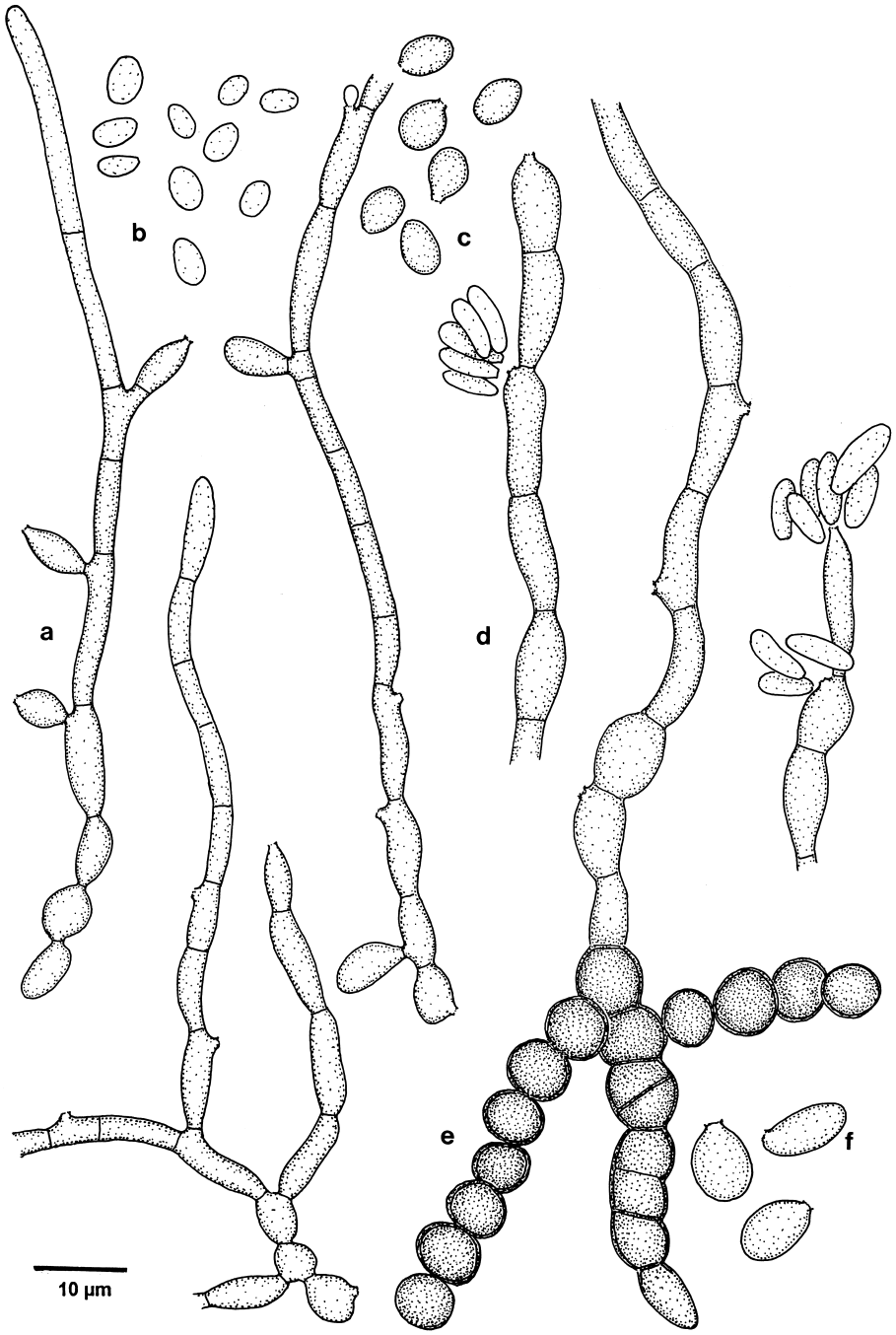

Figure 3. Hyphal / yeast-like morphology *Sarcinomyces phaeomuriformis*. a-c. DH 12366, slide culture of 5-day-old culture on PDA. a. Hyphae with non-elongating annellated zones; b. budding cells; c. germinating cells. d-f. CBS 109135, one-month-old slant culture on PDA. d, e. Hyphae with non-elongating annellated zones and packets of conidia; f. torulose hyphae converting into meristematic cells. For meristematic morphology of CBS 131.88, see De Hoog et al. (2000)

phialoconidia short-cylindrical, 2.5–3.0 × 1.2–1.5 µm. Colonies of *meristematic variant* are black, with very slow growth, forming an irregular, folded, mulberry-like mass. Yeast-like cells soon converting into spherical sclerotic cells with several cross walls, then slowly swelling and disarticulating; maximum diam-

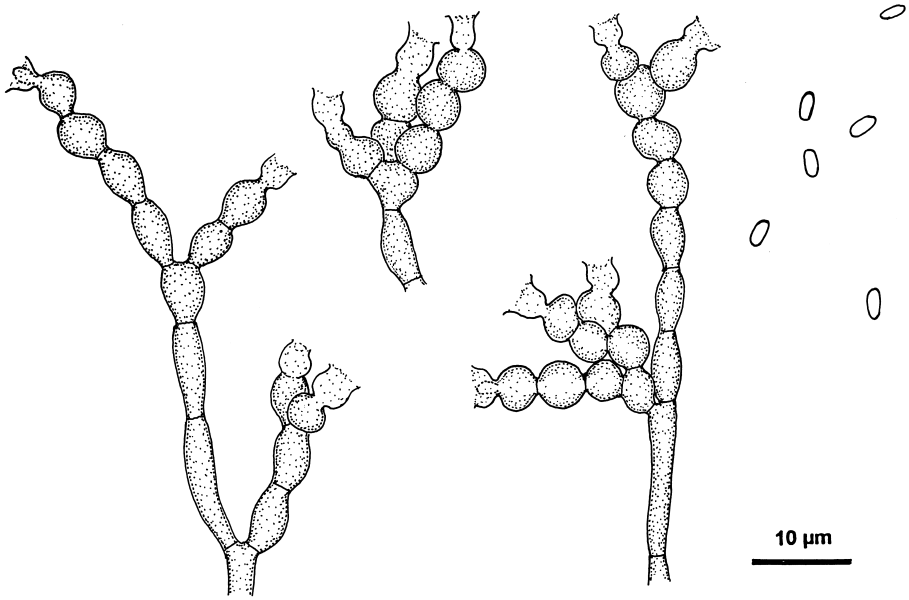

Figure 4. Phialidic synanamoph of *Sarcinomyces phaeomuriformis*

eter of cell clumps 12–20 µm. Budding multilateral. Cells thick-walled, olivaceous-black. Chains of budding cells at broad bases frequently present.

Ex-type culture: CBS 131.88 = CDC B-3558 = KMU 1303 = NCMH 1215 = UAMH 4278, cutaneous mycosis of 48-year-old female patient, Japan (Mori et al. 1961; Matsumoto et al. 1986).

Additional strains examined:

CBS 314.90, tile floor of swimming pool, Germany.

CBS 710.95 = UAMH 6533 = FR 2450.89 = GHP 1821, endocarditis of 61-year-old female patient, Canada (Gold et al. (1994), case 1).

CBS 109135 = Matos T-6, bathroom tap, Hilversum, The Netherlands.

CBS 109137 = Matos T-8, bathroom sink, Baarn, The Netherlands.

CBS 109146 = Matos T-19, bathroom tap, Baarn, The Netherlands.

CBS 109150 = Matos T-23, natural hot spring, Slovenia.

DH 12366, sauna, hall, Vienna, Austria.

GHP 227, external ear channel in case of otitis externa of female patient, Aachen, Germany.

GHP 622, bath, Germany.

GHP 815, oral mucosa, Frankfurt, Germany.

GHP 946, roof of car parked under tree (*Acer* sp.), Aachen, Germany.

GHP 981 = M4Q, sent out as *E. jeanselmei* for proficiency testing, U.S.A.

GHP 1011 and GHP 1023, sputum of CF-patient, Heidelberg, Germany.

GHP 1096 = M 248/1995, nail, The Netherlands.

GHP 1224, ex sputum, bronchiectasis of 75-year-old female, Aachen, Germany.

CBS 110171 = GHP 1603, external ear channel in case of otitis externa, Düren, Germany

CBS 110170 = UAMH 7048 = GHP 1822, ex sputum, female 62 yr, Sarnia, Ontario, Canada.

UAMH 8111 = GHP 1823, ex sputum, female 66 yr with bronchiectasis, Germany, P. Gibsey.

CBS 146.97 = CCMF 11195 = GHP 1376 = dH 10807, moist air of grotto (20 °C), Punkevn Caves, Blansko, Czech Republic, 1994, L. Marvanová.

GHP 1162 = bathroom, Aachen, Germany.

Two strains attributed to *S. phaeomuriformis*, CBS 314.90 and 109135, deviate from the remaining strains (Figure 1) by eight mutations and two Indels in ITS1 and three Indels in ITS2 (Table 3). The distance between is 2.3% of the ITS domain (including the 158 bp long 5.8S rDNA). Strain GHP 622 showed considerable deviation near the end of the ITS1 spacer. A local blast search of about 1000 black yeast sequences held at CBS did not reveal any species closer than the ones included in the tree. The taxonomic significance of such localized ITS variation is as yet difficult to

evaluate. A similar phenomenon was noted in *Aureobasidium pullulans*, where ITS2 was found to be almost six times more variable than ITS1 (Yurlova et al. 1999).

Infraspecific typing of *E. dermatitidis* strains

The present study included all *E. dermatitidis* strains from cerebral cases that are known to be preserved. A further strain published as *Phialophora dermatitidis* by Jotisankasa et al. (1970) from brain in Japan, CBS 579.76, was found to have an ITS sequence identical to that of *Exophiala* sp., CBS 725.88, reported by Tintelnot et al. (1991) from a fatal cerebral infection in a patient from Germany. This taxon will be described as a new species elsewhere (G.S. de Hoog, unpublished data). Based on fingerprinting data, nearly all 75 strains definitely assigned to *E. dermatitidis* could be attributed to one of two main groups A and B (Table 1). The profiles closely resembled each other (Figure 2). Only three strains, CBS 151.93, IFM 41823 and CBS 150.90, were clearly different, having only few bands in common. These strains also deviated in their ITS sequences: CBS 150.90 had a deletion of 26 bp, IFM 41823 lacked a base on position 184 and CBS 151.93 had 3 substitutions. The latter mutations were shared by all members of group B (Figure 1). Remarkably, CBS 151.93 (ITS group B) had an M-13 A-profile. Profile A was also found in CBS 109139 and DH 9883, which deviated by three mutations. The incongruences show that members of groups A and B are very closely related. Some additional single nucleotide polymorphisms (SNPs) were occasionally noted in ITS. They were not connected to differences in M-13 banding profiles. Attribution of strains to groups A or B is mostly possible by either fingerprinting or by ITS sequencing. In this way we were able to categorize nearly all 75 strains listed in Table 1, even though not all methods were applied to all strains. The patterns seem to be stable, as can be concluded from the fact that recent sauna strains from The Netherlands, as well as a strain, CBS 100340, isolated 25 years previously from bat liver in Brazil (Mok and Luizão 1981), were identical. Uijthof et al. (1994) found identical RAPD patterns in strains isolated at time intervals of 15 to 50 yr (RAPD group 1).

Nearly all members of group A had SSU amplicon lengths of approximately 3000 bp, corresponding with the presence of two introns. Three strains, CBS 639.96, NCMH 1217 and DH 9883, had only a single intron (2100 bp; Table 1). CBS 109153 was the only strain of group A without an intron. Most members of group B lacked introns, some having a single intron and none was observed to have two introns (Table 2). Thus it seems that groups A and B, though closely interrelated, are well-supported.

Group A contained 43 strains (Table 3) originating from humans. Twenty-three of these strains were from symptomatic individuals and 19 were apparently harmless colonizers of lungs and intestinal tract. Group A contained significantly more clinical strains than group B ($P<0.05$). All strains from cerebral, liver and disseminated cases clustered in group A. Twenty-eight of the human-derived strains had two introns (SSU approx. 3000 bp; Table 1), while three had one intron or none. Conversely, of the 9 environmentally isolated strains of group A, four had one or no intron. In contrast, 10 strains of group B are

Table 2. Infraspecific variability in ITS1-2 domains of *S. phaeomuriformis* and *E. dermatitidis*.

Sarcinomyces phaeomuriformis													
	ITS1 (210)										ITS2 (220)		
	8	34	50	59	108	135	146	169	188	189	194	196	203
Sarc*	A	T	T	T	T	A	T	C	A	-	A	-	A
Sarc(T)	C	C	C	C	G	G	C	T	T	T	-	A	-
Exophiala dermatitidis													
ITS1 (210)						ITS2 (220)							
	62	123	162	184	196	16	198						
Ed(T)	A	T	T	-	A	-	A						
1	A	T	T	-	A	C	A						
2	G	C	T	-	A	-	A						
2+1−1	G	C	T	-	A	C	-						
3	A	T	C	T	C	-	A						

ITS1 and 2 domains given with approximate total lengths in brackets. Sarc(T) = *Sarcinomyces phaeomuriformis*, group containing the ex-type strain; Sarc* = *Sarcinomyces cf. phaeomuriformis*, deviating strains. Ed(T) = *Exophiala dermatitidis*, type strain

strictly environmental and significantly ($P<0.05$) have fewer introns than members of group A (Table 1). Group A has a world wide distribution. All strains from Asia are of the A genotype. Members of group B have been found in Europe and the Americas, but not in Asia. The three strains of group B isolated from bat and human blood were involved in asymptomatic or transient cases of fungemia (Mok 1980; Blaschke-Hellmessen et al. 1994). Blaschke-Hellmessen et al. (1994) reported several, clinically identical cases of fungemia from the same clinic. These strains proved to be of different genotypes (CBS 100339 = A, CBS 100341 = B). Patients with underlying disease as reported by Blaschke-Hellmessen et al. (1994) apparently can be infected by both genotypes, with the same clinical outcome. However, in Asian patients all cases of deep infections by members of group A were severely symptomatic. We thus have to conclude that Asian patients are especially susceptible to infection by members of group A.

A further subdivision of the strains of groups A and B is possible using random amplified polymorphic DNA (RAPD). Uijthof et al. (1998) found seven genotypes, five of which belong to the M-13 group B. In contrast, strains of the M-13 group A were very similar, containing strains of two RAPD profiles only. Thus, the environmentally isolated strains show a considerably higher degree of diversity than the human-associated strains. This suggests a recent evolution of the strains of group A. It is tempting to speculate that *E. dermatitidis* has colonized the new environment of the steam bath, and from there has gone through a host shift and acquired elevated virulence for humans. This evolutionary event is likely to have taken place in Asia, where all the severe infections by genotype A are located. Similar suppositions have been expressed concerning dermatophytes like *Trichophyton rubrum* (Castell.) Semon. In the dermatophyte species-complex *Microsporum canis/M. audouinii*, speciation was noted accompanying a host shift from zoo- to anthropophilic behaviour (Gräser et al. 2000).

Table 3. Prevalence of clinical *vs*. environmentally isolated strains in groups A and B. Numbers of strains in each category.

	Group A	Group B
Clinical	42	6
Symptomatic	23	3
Colonization	19	3
Environmental	11	10
Total	53	16
Clinical strains:		
Neurotropic	7	-
having two introns	6	-
having one intron	1	-
Prevalence of introns:		
0	2	5
1	5	2
2	36	-
Total strains analyzed:	43	7

Micro-evolution within *E. dermatitidis* indeed seems to occur within the man-made environment of the bathing facility. Three strains of genotype B in the hall of the Laren sauna facility were dry and unable to grow at 42 °C (T12, 14, 16), while strains with the same genotype in the adjacent steam bath (T10) had the typical phenotype, with slimy colonies and growth at 42 °C. This suggests that dryness is a phenetic expression of characters in strains which are more suited to grow under conditions of reduced humidity. The dry strains from Laren are likely to be local phenotype which has emerged too recently to be expressed with the applied fingerprint method.

In summary, we conclude that the black yeast *Exophiala dermatitidis* exhibits adaptability in some essential virulence factors such as thermotolerance and production of extracellular polysaccharide in the artificial environment of the steam bath. It is likely that steam baths combine factors that the species needs for survival in its as yet unknown natural ecological niche. A further evolution towards human pathogenicity may be expected.

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