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Yeasts Associated with Termites: A Phenotypic and Genotypic Characterization and Use of Coevolution for Dating Evolutionary Radiations in Asco- and Basidiomycetes

HANSJÖRG PRILLINGER[1], ROBERT MESSNER[1], HELMUT KÖNIG[2], ROBERT BAUER[3], KSENIJA LOPANDIC[1], ORSOLYA MOLNAR[1], PETRA DANGEL[2], FRANZ WEIGANG[4], THOMAS KIRISITS[5], TAKASI NAKASE[6], and LYNNE SIGLER[7]

[1] Institut für Angewandte Mikrobiologie, UNI-BOKU Wien, A-1190 Wien, Austria
[2] Angewandte Mikrobiologie, Universität Ulm, D-89069 Ulm, Germany
[3] Institut für Spezielle Botanik/Mykologie, Universität Tübingen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany
[4] Analytische Messtechnik, Hewlett-Packard GmbH, A-1222 Wien, Austria
[5] Institut für Forstentomologie, Forstpathologie und Forstschutz, UNI-BOKU Wien, A-1190 Wien, Austria
[6] Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN), Wako 351-01, Japan
[7] Microfungus Collection and Herbarium T6G 2E1 Devonian Botanic Garden, University of Alberta, Edmonton Alberta, Canada

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Summary

Thirtynine yeast isolates or dimorphic fungi were obtained from the hindgut of the lower termites *Mastotermis darwiniensis* (Mastotermitidae), *Zootermopsis angusticollis, Z. nevadensis* (Hodotermitidae), *Neotermes jouteli* (Kalotermitidae), *Reticulitermes santonensis, Heterotermes indicola* (Rhinotermitidae) and the roach *Cryptocercus punctulatus*. Using RAPD-PCR the 39 yeast isolates were assigned to 13 different species. Commonly yeast species were specific to the termite species isolates from. There were only two yeast species which were found in different species of lower termites. Based on phenotypic characters *Debaryomyces hansenii* showed a high score in four species. The qualitative and quantitative yeast cell wall monosaccharide composition, the ubiquinone system, partial sequencing of 18S ribosomal DNA (bases 1273 to 948; numbering according to the gene of *Saccharomyces cerevisiae*), and the ultrastructure of septal pores indicate that 11 yeast species belong to the Endomycetales. Although ascospores were lacking, two of these species were identified to belong to the genus *Debaryomyces*. One remaining yeast isolate was identified as a *Sporothrix* anamorph representative for the filamentous Ascomycetes (Ophiostomataceae s. str.); the second species showed affinities to the Basidiomycetes in particular to the genus *Trichosporon*. Comparing an additional 18S rDNA fragment (bases 595 to 993) and RAPD-PCR data using different species type strains of the genus *Sporothrix*, the filamentous ascomycete was genotypically identified as *Sporothrix albicans. Sporothrix. albicans*, although phylogenetically closely related to *S. schenckii* var. *schenckii* and *Ophiostoma stenoceras* remains genotypically distinct. An emended species description of S. albicans is presented. Evidence is provided that the yeasts isolated from the hindgut can be considered symbionts.

Key words: *Sporothrix albicans* – *Ophiostoma* – *Debaryomyces* – Termites – Yeasts – Endomycetales – Coevolution-fungi-arthropods – Fungus evolution – Ascomycete phylogeny – Basidiomycete phylogeny – Genotypic identification

Introduction

An amalgamation of molecular characteristics with fossil evidence and/or ages of fungal host is a new and useful approach to trace the phylogeny of fungi (*Gottschalk* and *Blanz*, 1985; *Prillinger* et al., 1990a,b, 1991a,b, 1993b; *Berbee* and *Taylor*, 1993). On the basis of host plants of different *Protomyces* and *Taphrina* strains and yeast cell wall sugars, we have shown that filamentous Ascomycetes and Basidiomycetes may have evolved from a *Protomyces-*

or *Schizosaccharomyces*-like ancestor via genetic integration of representatives of the *Saccharomyces* type during the carboniferous periode (*Prillinger* et al., 1990a, 1993a,b). The presence of glucose (Glc), mannose (Man), galactose (Gal), and rhamnose (Rha) within yeast cell walls of *Taphrina* strains on ferns and different lineages of woody angiosperms as well as *Protomyces* species on Apiaceae and Asteraceae suggests that *Protomyces*- and *Taphrina*-like ancestors already exist before the dichotomy of gymnosperms and angiosperms. *Troitsky* et al. (1991) deduced from ribosomal RNA sequence comparisons that both gymnosperms and angiosperms are monophyletic groups. Their data suggest that the divergence of all the main groups of extant gymnosperms occurred after the branching off of the angiosperm lineage. From the fact that at least some of these gymnosperm taxa are traceable back to the early Carboniferous, *Troitsky* et al. (1991) conclude that the genealogical splitting of gymnosperm and angiosperm lineages occurred before this event, at least 360 million years ago. This is much earlier than the first angiosperm fossils were dated (*Crane* et al., 1995).

There is molecular evidence that the simple-pored Heterobasidiomycetes diverged into two different lineages before the monocots and eudicots separated in early angiosperm evolution (*Gottschalk* and *Blanz,* 1985, *Prillinger* et al., 1990b, 1991a, 1993b; *Qiu* et al., 1993, *Crane,* 1995). Obligate parasitic Heterobasidiomycetes representing the "Glc-Man-Gal" pattern – *Ustilago* type – are known from the Laurales (*Exobasidium lauri; Prillinger* et al., 1993b), Nymphaeales (*Rhamphospora nympheae; Lopandic* et al., 1995), and the monophyletic monocots (Graphiolales and many additional smut fungi: *Prillinger* et al., 1990b, 1993b). The presence of perithecia-like fruiting bodies within the Graphiolales, primitive representatives of the *Ustilago* type, may indicate that evolution of filamentous Ascomycetes antedates the radiation of the monocotyledonous smut fungi (Ustilaginales s. str.). Based on the fossil record and 18S rDNA sequences, *Berbee* and *Taylor* (1993) gave a different interpretation and considered the phragmobasidial smut fungi as primitive Basidiomycetes.

Recently, *Nishida* and *Sugiyama* (1994) presented evidence that *Mixia osmundae* morphologically placed within the Protomycetaceae has to be included in the Heterobasidiomycetes due to 18S ribosomal DNA sequence information.

No consensus, however, exists with respect to the phylogenetic age of the Endomycetales. Based on the fossil record and 18S rDNA sequences, *Berbee* and *Taylor* (1993) regarded this group as a sister group from the filamentous Ascomycetes. Considering a new concept of sexuality and mating type evolution (*Prillinger,* 1987, *Prillinger* et al., 1989, 1993a) and the carbohydrate pattern of purified yeast cells, *Prillinger* et al. (1993b) interpreted the Endomycetales as primitive fungi which have lost their filamentous stage based on the extintion of the hosts (*Messner* et al., 1995). *Kurtzman* (1993) added further arguments to the second interpretation.

In the present paper we have investigated yeast isolates from the hindgut of lower termites and a wood-feeding roach. There is a general consensus that roaches, mantids, and termites represent a monophyletic grouping (*Hennig,* 1969, *Thorne* and *Carpenter,* 1992, *DeSalle* et al., 1992). The roaches are well documented from the Carboniferous (*Hennig,* 1969). According to *Carpenter* (1934), cockroaches appeared to be the most important group of insects during the Carboniferous. Although fossil reports of termites exist only from the Cretaceous (*Emerson,* 1967; *Jarzembowski,* 1981, 1984; *Lacasa-Ruiz* and *Martinez-Delclos,* 1986; *Krishna,* 1990) – the oldest, *Meiatermes bertrani,* was found in a limestone deposit in Spain dating approximately 130 million years – *Martynov* (1937) considered the Isoptera with homonomous wings the ancestral condition in winged insects. *Martynov* (1937) proposed a phylogeny and evolutionary time frame in which roaches and termites diverged in the late Devonian or early Carboniferous, and mantids radiated from roaches in the mid-Carboniferous (*Thorne* and *Carpenter,* 1992).

Although intimate associations are known between Agaricales (*Termitomyces*) or filamentous Ascomycetes (Laboulbeniales: *Laboulbenia;* Pyrenomycetes: *Cordycepioideus*) and higher termites (Termitidae) (*Sands,* 1969; *Becker,* 1976; *Amburgey,* 1979; *Oertel* and *Schäller,* 1984; *Breznak* and *Brune,* 1994), there is no information with respect to the yeast mycoflora in termites and its usefulness to trace the phylogeny of Asco- and Basidiomycetes. If *Martynov's* (1937) speculation about the evolution of roaches and termites is correct, coevolution between yeasts and termites may be extremely useful to trace the phylogeny of filamentous Asco- and Basidiomycetes. Based on the fact that morphology may have dramatically changed in closely related fungi during evolutionary periods as indicated by *Graphiola phoenicis* (*Prillinger* et al., 1990b), *Exobasidium lauri* (*Prillinger* et al., 1993b), and *Mixia osmundae* (*Nishida* and *Sugiyama,* 1994), we have tried to resolve two questions in the present paper:

1. Is coevolution between yeasts and arthropods dated by the fossil record of the arthropod a better indicator for the phylogeny of fungi than the rather poor fungal fossil record?

2. Whether a new polyphasic genotypic approach using RAPD-PCR, cell wall sugars, the ubiquinone system, and partial ribosomal DNA sequences is reliable in the identification of yeasts or yeast-like fungi from nature.

Materials and Methods

Insects. Neotermes jouteli (Banks), *Zootermopsis angusticollis* (Hagen), *Z. nevadensis* (Hagen), and *Mastotermes darwiniensis* (Frogatt) were obtained from the "Bundesanstalt für Materialforschung und Materialprüfung" (Berlin). *Reticulitermes santonensis* (Feytaud) was collected on the ile d'Oleron (France). *Heterotermes indicola* (Wasmann) was obtained from "DESOW-AG Materialschutz GmbH" (Krefeld). The termites were kept at 28 °C and fed on pine, beech and poplar wood. The wood-feeding roach *Cryptocercus punctulatus* (Scudder) was a gift of C. A. Nalepa (Raleigh, N. C.). It was collected in the Appalachian Mountains near Roanoke (Virginia, U.S.A.).

Yeast strains. In order to isolate yeasts from termites, the gut was removed under a dissecting microscope using Inox 5 forceps.

Guts were treated aseptically 30 sec. within a mixture of 0.2% peracetic acid dissolved in 33% (v/v) isopropanol. The solution was prepared immediately before use. Afterwards the gut was tipped in 70% (v/v) ethanol (15 sec.) and finally rinsed with a sterile 1/4 strength Ringer solution (15 sec.). Alternatively termites (worker caste) were surface sterilized by dipping the decapitated animals in 70% ethanol for about 30 sec and rinsing them with sterile water. The gut was removed with sterile forceps. After squeezing one gut/petri dish, streak-cultures were prepared using 3% malt extract agar (M; Merck 5398), Sabouraud-4%-glucose-agar (S; Merck 5438), and glucose (2%) – yeast extract (0.5%) – peptone (1%) (GYP) agar (G). A filter sterilized solution of 100 mg ampicillin (Boehringer) and 100 mg streptomycin-sulfate (Fluka) was added to 1 l agar medium. Three replicates were incubated at 28 °C. A compilation of the isolated yeast strains from different species of the lower termites and *Cryptocercus punctulatus* is given in Table 1. Species used for genotypic identification by Random Amplified Polymorphic DNA (RAPD) PCR-analysis are shown in Table 2. Cryoconservation of yeast strains was performed in liquid nitrogen (10% glycerol) and in a Forma Scientific –86 °C Freezer using 14% (w/v) sucrose and 1% peptone as a cryoprotecant.

Species delimitation. To identify different yeast species the RAPD-PCR procedure was used as described in *Messner* et al. (1994). Extraction of DNA from the yeast strains was performed by the hexadecyltrimethylammonium bromide (CTAB) method only. The DNA of filamentous fungi was isolated after grinding in liquid nitrogen by the phenol method as described by *de Graaff* et al. (1988). PCR conditions and separation of RAPD-PCR fragments were according to *Messner* et al. (1994). The following primers were employed: Decamer 2: TGCCGAGCTG (*Caetano-Anolles* et al., 1992), Decamer 3: TGCAGCGTGG, Decamer 4: GGGTAACGCC. Primers were synthesized by Codon Genetic Systems (Vienna, Austria) with a model 392 DNA synthesizer (Applied Biosystems, Foster City, Calif.). The levels of similarity between individual lanes were calculated as described by *Nei* and *Li* (1979).

Phenotypic characterization. The physiological properties of the yeast isolates were investigated according to the methods described by *van der Walt* and *Yarrow* (1984). Fermentation tests were carried out for 3 weeks at 28 °C using Durham tubes. The carbon compound assimilation tests were performed on a shaker for 21 days at 28 °C and in API 50 CH plates (*Laaser* et al., 1988; *Hoffmann* pers. comm.). API strips incubated at 25 °C and read daily for 7 d. Readings which were negative after 3 d but positive by 7 d were recorded as delayed (D) positive. Utilization of nitrogen sources was examined in liquid medium and in culture tubes with Yeast Carbon Base agar slants containing bromcresol purple (pH 6.5; Scheide pers. comm.). Vitamin requirements were tested after at least 3 consecutive inoculations on vitamin-free medium. To identify the yeast isolates according to phenotypic characteristics the Yeast Identification PC Program (Version 3) *Barnett* (1994) was used.

Test for anaerobic growth. For anaerobic cultures the medium was supplemented with a resazurin solution (0.1%; 1 ml/l) and Na_2S (0.1 g/l). N_2/CO_2 (80:20) was used as a gas phase. The test was performed in GYP-medium.

Genotypic characterization. To identify the yeast genus genotypically, a polyphasic molecular approach used the following methods:

1. Qualitative and quantitative monosaccharide pattern of purified yeast cell walls. The neutral sugar composition was determined after trifluoracetic acid hydrolysis according to the method of *Dörfler* (1990) with some modifications as described in *Messner* et al. (1994).
2. Ubiquinone analysis (*Messner* et al., 1994). For the high-per-

Table 1. List of the yeast isolates from different lower termites and a wood-feeding cockroach. Strain designation number includes the yeast isolation medium: M: 3% malt extract, S: Sabouraud-4% glucose agar, G: glucose peptone yeast extract agar

Yeast isolates Termites:	Collection number IAM Vienna	Strain designation Ulm	Year of isolation
Mastotermitidae:			
Mastotermis darwiniensis			
M. d. 1	HA 167	MM 1	1993
M. d. 2	HA 168	MS 2	1993
M. d. 3	HA 169	MS 3	1993
M. d. 4	HA 170	MS 4	1993
M. d. 5	HA 615	MYG 4	1994
M. d. 6	HA 616	MYG 5	1994
M. d. 7	HA 618	MYG 7	1994
M. d. 8	HA 619	MYG 8	1994
M. d. 9	HA 620	MYG 9	1994
M. d. 10	HA 612	MYG 1	1994
M. d. 11	HA 613	MYG 2	1994
M. d. 12	HA 614	MYG 3	1994
M. d. 13	HA 617	MYG 6	1994
Hodotermitidae:			
Zootermopsis angusticollis			
Z. a. 1	HA 598	ZAG 1	1994
Z. a. 2	HA 599	ZAG 2	1994
Z. a. 3	HA 600	ZAG 3	1994
Z. a. 4	HA 601	ZAG 4	1994
Z. a. 5	HA 602	ZAG 5	1994
Z. a. 6	HA 603	ZAG 6	1994
Z. a. 7	HA 604	ZAG 7	1994
Z. a. 8	HA 606	ZAG 8	1994
Z. a. 9	HA 605	ZAG 9	1994
Z. a. 10	HA 607	ZAG 10	1994
Zootermopsis nevadensis			
Z. n. 1	HA 162	ZM 1	1993
Z. n. 2	HA 163	ZM 2	1993
Z. n. 3	HA 164	ZM 3	1993
Z. n. 4	HA 165	ZM 5	1993
Z. n. 5	HA 166	ZS 5	1993
Kalotermitidae:			
Neotermes jouteli			
N. j. 1	HA 171	NM 1	1993
N. j. 2	HA 172	NS 1	1993
Rhinotermitidae:			
Reticulitermes santonensis			
R. s. 1	HA 173	RM 1	1993
R. s. 2	HA 174	RS 2	1993
R. s. 3	HA 175	RM 3	1993
R. s. 6	HA 176	RM 6	1993
R. s. 7	HA 177	RM 7	1993
Heterotermes indicola			
H. i. 1	HA 608	HG 1	1994
Roaches:			
Cryptocercus punctulatus			
C. p. 1	HA 609	C G 1	1994
C. p. 2	HA 610	C G 2	1994
C. p. 3	HA 611	C G 3	1994

Table 2. List of strains used for genotypic identification. ^{T} = Indicates the type strain

Species Teleomorph/ Anamorph	Strain		Habitat	Isolation
	IAM no.	other designation		
Hyphozyma roseonigra de Hoog & M. Smith	HA 680^{T}	UAMH 7240^{T}, CBS 514.83^{T}	Soil; New Jersey	leg. *M. Farbood*
Sporothrix albicans S. B. Saksena	HA 654^{T}	MUCL 19327^{T} *S. schenckii* var. *schenckii*	Soil (Kettering loam); England	leg. et det. *S. B. Saksena*; Nov. 1964
	HA 898^{T}	CBS 302.73^{T}	as above	as above
S. curviconia de Hoog	HA 652^{T}	JCM 2933^{T}, CBS 959.73^{T}	*Terminalia invorens*; Foret de Kouin Ivory Coast	leg. *J. Devois*
S. ghanensis de Hoog & Evans	HA 650^{T}	JCM 2925^{T}, CBS 755.73^{T}	On spider with eggs on leaf of *Theobroma cacao*; Tafo, Ghana	leg. *H. C. Evans*
S. inflata de Hoog	HA 649^{T}	JCM 2920^{T}, CBS 239.68^{T}	Soil (Wheat-field), Germany	leg. *W. Gams*
S. insectorum de Hoog & Evans	HA 651^{T}	JCM 2918^{T}, CBS 756.73^{T}	Mt. Atewa, Ghana	leg. *H. C. Evans*; Jan. 1973
S. ranii Moustafa	HA 647^{T}	JCM 2917^{T}, CBS 119.81^{T}	Tidal salt marsh; Kuwait	leg. et det. *A. F. Moustafa*
S. sclerotialis de Hoog	HA 648^{T}	JCM 2913^{T}, CBS 312.77^{T}	*Lolium perenne* (roots); The Netherlands	leg. et det. *de Hoog*
S. schenckii Hektoen & Perkins	HA 646^{T} (possible type culture)	JCM 2915^{T}		leg. *D. J. Davis*
	HA 653^{T}	CBS 359.36		as above
Ophiostoma bicolor Davidson & Wells/ Hyalorhinocladiella	HA 103	IMI 3633172; 14/1	*Picea abies*: phloem; "Rothwald", Austria	leg. et det. *T. Kirisits*; Apr. 1992
O. ips (Rumbold) Nannfeldt/ no Sporothrix	HA 96	ATCC 90961; IMI 363176 MA 19	*Ips sexdentatus*; Austria	leg. et det. *T. Kirisits*; Sep. 1992
O. quercus (Georgév.) Nannfeldt/ Graphium, Sporothrix	HA 207		*Quercus* sp.; Gralla, Austria	leg. *E. Halmschlager*; Apr. 1993; det. *R. Messner & H. Prillinger*
	HA 367	W 3	Quercus robur; Gralla, Austria	as above
O. piceae (Münch) H. & G. Sydow/ Graphium, Sporothrix	HA 377	15811	*Picea abies*; Ojcow, Poland	leg. et det. *T. Kowalski*
O. stenoceras (Robak) Melin & Nannfeldt/Sporothrix	HA 899	CBS 237.32 AUT	Wood pulp; Norway	leg. et det. *H. Robak*
O. ulmi (Buisman) Nannfeldt/ Graphium, Sporothrix	HA 97		*Ulmus minor*; Laaerberg, Wien, Austria	leg. et det. *T. Kirisits*; Nov. 1992
	HA 209		*Scolytus scolytus*, Laaerberg, Wien, Austria	from ascospores; leg. et det. *T. Kirisits*; May 1992
	HA 210		*Ulmus minor*; Laaerberg, Wien, Austria	from ascospores; leg. et det. *T. Kirisits*; Sep. 1992
Ophiostoma sp. 1; cf. stenoceras/ Sporothrix	HA 206		*Quercus petraea*, "Hochleithenwald", N.Ö. Austria	from ascospores; leg. *E. Halmschlager*; Dec. 1992
	HA 395	RA 10	*Castanea sativa*; Loipersbach-Ratterstorf, Bgld. Austria	leg. *E. Wilhelm*; Mar. 1990
Ophiostoma sp. 2; cf. piceae Sporothrix	HA 208		*Picea abies*: phloem; Austria	leg. *T. Kirisits*; Dec. 1992

formance liquid chromatography (HPLC) analysis we used a Hewlett-Packard Series 1050 HPLC pump, a model 1050 diode array detector, and a model 1050 automatic sampler (Hewlett-Packard GmbH, Waldbronn, Germany).

3. Partial sequencing of ribosomal DNA. PCR amplification of a 1kB section of the 18S rDNA and direct sequencing of the PCR fragment was performed as described by *Messner* et al. (1994, 1995). The sequencing primers were GGTAATTCCAGCTCCAAT (primer 18/2) for determination of the bases 595 to 993 and AGGTCTCGTTCGTTA for the bases 1273 to 948 (primer 18/3; numbering according to the 18S ribosomal gene of S. cerevisiae). Primer 18/3 was successfully employed to identify the genus genotypically. For species identification both primers were used.

In order to identify related strains or to prove singularity of the sequences determined, a fast homology search was performed at the "blast" E-Mail server of the National Center of Biotechnology Information / National Library of Medicine (blast @ncbi.nlm.nih.gov). The algorithm of the program BLASTN (*Altschul* et al., 1990) was designed for locating stretches of 100% homology between the query sequence and the entries in the specified database.

Ultrastructure. Agar blocks containing mycelium and/or yeast cells were fixed overnight in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature. Following six transfers in 0.1 M sodium cacodylate buffer, samples were postfixed in 1% osmium tetroxide in the same buffer for 2 h in the dark, washed in distilled water, and stained in 1% aqueous uranyl acetate for 1 h in the dark. After five transfers in distilled water, samples were dehydrated in acetone, using 10 min changes at 25%, 50%, 70%, 95%, and 3 times in 100% acetone. Samples were embedded in Spurr's plastic (*Spurr,* 1969).

Serial sections (65–75 nm) were cut using a Reichert-Jung Ultracut E (Leica, Nußloch) equipped with a diamond knife. Sections were mounted on formvar-coated single-slot copper grids, stained with lead citrate (*Reynolds,* 1963) at room temperature for 3–5 minutes, and washed again with distilled water. Loaded grids were examied with a Zeiss EM 109 transmission electron microscope at 80 kV.

Table 3. Species assignment after RAPD-PCR analysis (column 1); cell wall monosaccharide composition (glucose: Glc, mannose: Man, galactose: Gal, xylose: Xyl, rhamnose: Rha, fucose: Fuc); hydrolysis by urease (UREA); Diazonium blue B test (DBB); formation of extracellular amyloid compounds (EAC, starch test); fermentation of glucose (FERM); major ubiquinone system (UBI)

Species	Yeast isolate	Collection no.	Cell wall monosaccharide composition (mol%)						UREA	DBB	EAC	FERM	UBI
			GLC	MAN	GAL	XYL	RHA	FUC					
1	M. d. 1	HA 167	30	56	14	–	–	–	–	–	–	–	9
	M. d. 2	HA 168	40	48	12	–	–	–	–	–	–	–	9
	M. d. 3	HA 169	42	46	12	–	–	–	–	–	–	–	9
	M. d. 4	HA 170	39	48	13	–	–	–	–	–	–	–	9
	M. d. 5	HA 615	51	38	11	–	–	–	–	–	–	–	9
	M. d. 9	HA 620	41	48	11	–	–	–	–	–	–	–	9
2	M. d. 10	HA 612	52	48	–	–	–	–	–	–	–	+	9
	M. d. 11	HA 613	50	50	–	–	–	–	–	–	–	+	9
3	M. d. 12	HA 614	60	48	–	–	–	–	–	–	–	+	9
4	M. d. 13	HA 617	59	16	–	20	–	–	+	+	–	–	9
5	Z. a. 1	HA 598	55	45	–	–	–	–	–	–	–	+	9
	Z. a. 6	HA 603	51	49	–	–	–	–	–	–	–	+	9
6	Z. a. 8	HA 606	47	53	–	–	–	–	–	–	–	+	9
7	Z. n. 1	HA 162	38	33	2	–	21	–	+	–	–	–	10 H_2
	Z. n. 2	HA 163	39	33	2	–	26	–	+	–	–	–	10 H_2
	Z. n. 3	HA 164	41	34	2	–	23	–	+	–	–	–	10 H_2
	Z. a. 10	HA 607	52	25	2	–	21	–	+	–	–	–	10 H_2
8	Z. n. 4	HA 165	42	52	–	–	–	–	–	–	–	+	9
	Z. n. 5	HA 166	52	48	–	–	–	–	–	–	–	+	9
9	H. i. 1	HA 608	42	39	19	–	–	–	–	–	–	+	9
10	N. j. 1	HA 171	30	56	14	–	–	–	–	–	–	–	9
	N. j. 2	HA 172	34	51	15	–	–	–	–	–	–	–	9
	R. s. 7	HA 177	36	47	17	–	–	–	–	–	–	–	9
11	R. s. 1	HA 173	40	60	–	–	–	–	–	–	–	+	9
	R. s. 6	HA 176	46	54	–	–	–	–	–	–	–	+	9
12	R. s. 2	HA 174	37	49	14	–	–	–	–	–	–	–	9
	R. s. 3	HA 175	36	59	5	–	–	–	–	–	–	–	9
13	C. p. 1	HA 609	38	62	–	–	–	–	–	–	–	+	9
	C. p. 3	HA 611	58	42	–	–	–	–	–	–	–	+	9

Results

Species assignment

Yeasts isolated from the hindgut of the lower termites *Mastotermis darwiniensis* (Mastotermitidae), *Zootermopsis angusticollis, Z. nevadensis* (Hodotermitidae), *Neotermes jouteli,* (Kalotermitidae), *Heterotermes indicola, Reticulitermes santonensis* (Rhinotermitidae), and the roach *Cryptocercus punctulatus* (Table 1) were assigned to 13 different species using RAPD-PCR analysis (Table 3). From *M. darwiniensis* 4 distinct species were isolated. Species 1 represented by the isolates *M. d.* 1–9 was common in two subsequent isolations 1993 and 1994. Three other species (2, 3, 4; Table 3) were detected after a more thorough investigation in 1994. The hindgut of *Zootermopsis angusticollis* harbours at least three different yeasts or yeast stages of filamentous fungi (species 5, 6, 7; Table 3). From *Z. nevadensis* two genotypically distinct yeasts or yeast stages were isolated (species 7, 8; Table 3). One appeared to be conspecific with an isolate from *Z. angusticollis* (species 7; Fig. 1, lanes: 6–9, Fig. 2 lanes: 5–8). The yeasts isolated from *Neotermes jouteli* (*N. j.* 1 and *N. j.* 2; species 10: Table 3) were conspecific with one yeast isolate from *Reticulitermes santonensis* (*R. s.* 7). From *R. santonensis* two further species were obtained (species 11, 12). The three yeast isolates from the roach *Cryptocercus punctulatus* were found to be conspecific representing one species only (species 13; Table 3).

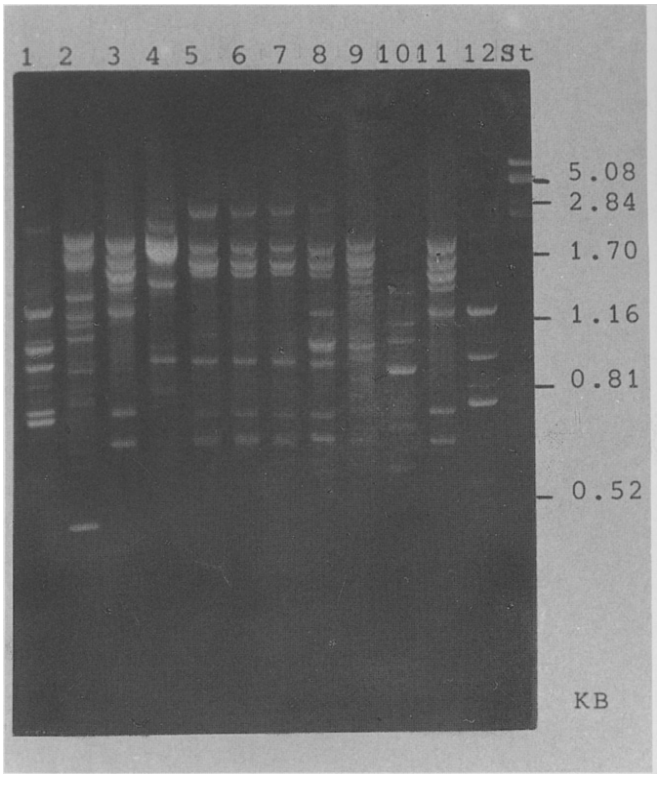

Fig. 1. Species assignment for species 7 (*Z. n.* 1, *Z. n.* 2, *Z. a.* 10) and species identification using RAPD pattern generated by Decamer 2 (primer: TGCCGAGCTG; *Caetano-Anolles* et al., 1992). Lane 1: *Ophiostoma* sp. 1 (HA 206), 2: *Ophiostoma* sp. 1 (HA 395), 3: *O. ulmi* (HA 209), 4: *O. ips* (HA 438), 5: *Sporothrix ghanensis* (HA 650), 6: *Z. n.* 1 (HA 162), 7: *Z. n.* 2 (HA 163), 8: *Z. a.* 10 (HA 607), 9: *S. albicans* (HA 654^T), 10: *O. quercus* (HA 367), 11: *O. piceae* (HA 377), 12: *S. schenckii* (HA 646), St.: lambda DNA digested by *PstI*).

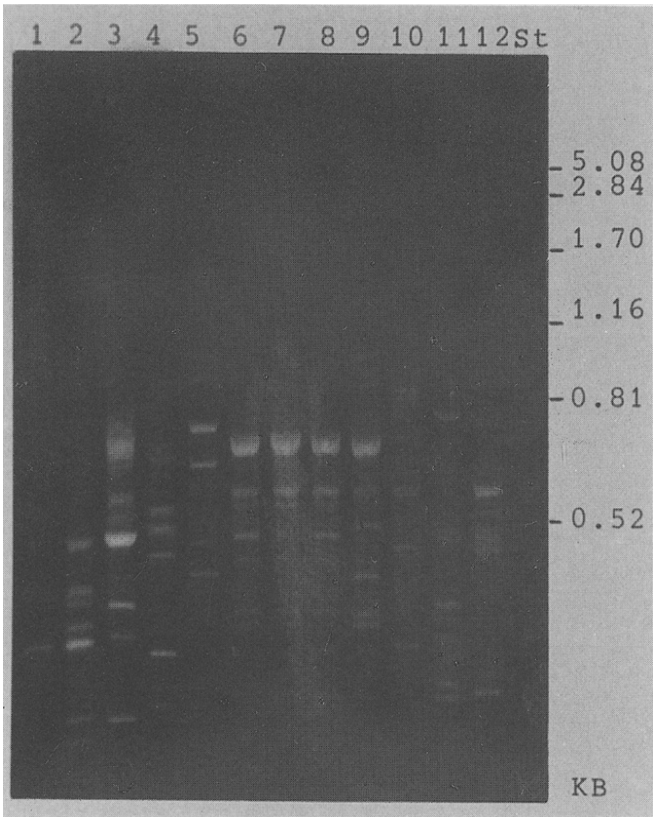

Fig. 2. Species assignment for species 7 (Z. n. 1, Z. n. 2, Z. a. 10) and species identification using RAPD pattern generated by Decamer 3 (primer: TGCAGCGTGG). Lane 1: *Sporothrix inflata* (HA 649, CBS 239.68), 2: *S. curviconica* (HA 652, CBS 959.73), 3: *S. schenckii* var. *schenckii* (HA 653, CBS 359.36), 4: *S. ghanesis* (HA 650, CBS 755.73), 5: Z. n. 1 (HA 162), 6: Z. n. 2 (HA 163), 7: Z. a. 10 (HA 607), 8: *S. albicans* (HA 654, MUCL 19327), 9: *S. insectorum* (HA 651, CBS 756.73), 10: *O. sp. 1.* (HA 206), 11: *S. schenckii* (HA 646, CBS 359.36), 12: *S. scelerotialis* (HA 648, CBS 312.77), St.: lambda DNA digested by *PstI*).

Phenotypic identification

Fermentation and assimilation profiles and some additional physiological characteristics as requested for the PC Yeast Identification Program (Version 3) from *Barnett* (1994) are given in Tables 3 to 6. None of the 13 yeast species of lower termites and a roach agreed completely with the data sets for known yeast species. There was at least a deviation in 2 tests, commonly in 7 to 11 tests. Species 1 from *M. darwiniensis* shows affinities to *Candida blankii* Buckley & van Uden. *C. blankii* differs from this species by 7 tests {fermentation: + (weak and delayed); assimilation: melibiose: –, lactose: +, nitrate: –, nitrite: –, cadaverine: +, T at 40 °C: +}. Further characteristics not shown in Tables 3–6: The streak culture on GYP-agar is white to cream, smooth, and shiny; vegetative

reproduction by budding, elaborate pseudohyphae and/or septate hyphae, especially on PDA; no sexual reproduction, no chlamydospores or arthrospores.

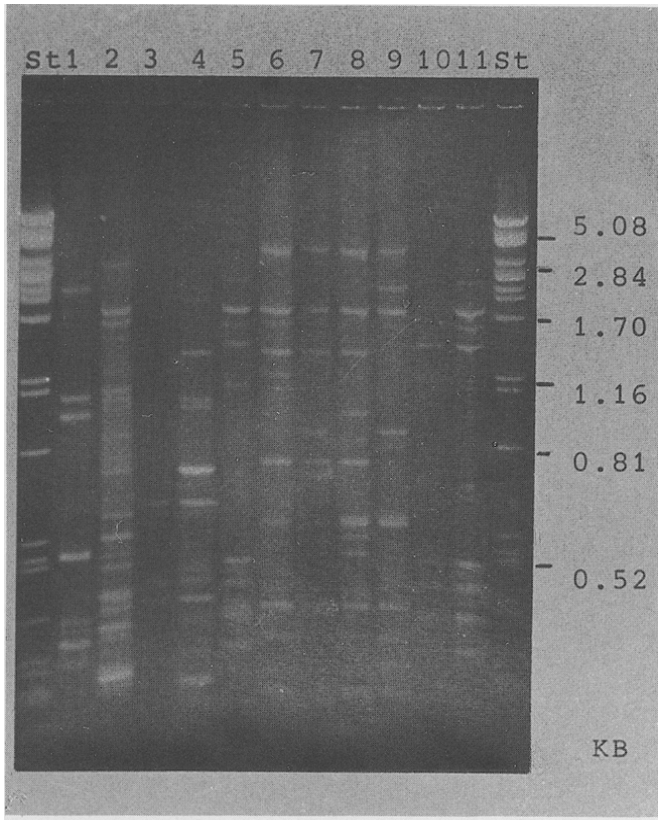

Fig. 3. Species assignment for species 7 (Z. n. 1, Z. n. 2, Z. a. 10) and species identification using RAPD pattern generated by Decamer 4 (primer: GGGTAACGCC). St.: lambda DNA digested by *PstI*). Lane 1: *Sporothrix inflata* (HA 649), 2: *S. curviconia* (HA 652), 3: *Ophiostoma* sp. 1 (HA 206), 4: *Ophiostoma* sp. 1 (HA 395), 5: *Sporothrix schenckii* (HA 653), 6: Z. n. 1 (HA 162), 7: *Z. n.* 2 (HA 163), 8: *Z. a.* 10 (HA 607), 9: *S. albicans* (HA 654^T), 10: *S. schenckii* (HA 646^T), 11: *S. schenckii* (HA 653^T).

Species 2 from *M. darwiniensis* comes very close to *Debaryomyces hansenii* var. *fabryii* (Ota) Nakase & Suzuki. *D. hansenii* differs in three results only (assimilation: xylitol: +, note weak + reaction from *M. d.* 10; ethylamine: +; cadaverine: +). Further characteristics: The streak culture on GYP-agar is white to cream, butyrous and shiny, a filamentous margin containing simple pseudohyphae may develop; ascospores so far not found on acetate agar, vegetative reproduction by budding; arthrospores and true hyphae absent. The species grows under strictly anaerobic conditions.

Similarly species 3 from *M. darwiniensis* comes very close to *Debaryomyces hansenii* var. *fabryi.* Again three tests appeared to be different (assimilation: xylitol: +, cadaverine: +, creatinine: −). Further characteristics as described for species 2 except that no pseudohyphae were found. Growth without oxygen is again characteristic.

Species 4 from *M. darwiniensis* is a basidiomycete (Table 3. DBB: +). Although it was originally identified as an ascomycete based on a negative DBB test, the presence of xylose (see below) in yeast cell walls suggested a reinvestigation was necessary. Prolonged preincubation (2 month 25 °C) and incubating the plates at 55–60° for 16 hours before applying the DBB reagent succeded in a positive result. Species 4 differs from *Cryptococcus humicolus* (Daszewska) Golubev as suggested by the PC identification program in the presence of arthroconidia and the assimilation of nitrate. Further characteristics: Cream to tan colonies with a sparse whitish aerial mycelium, colonies with a flat mycelial margin; vegetative reproduction by budding and splitting, simple to elaborate pseudohyphae and/or septate hyphae, no sexual reproduction.

From *Zootermopsis angusticollis* three (species 5–7; Table 3) and from *Z. nevadensis* two different yeast species were isolated (species 7, 8). One dimorphic species was found in both termites. The yeast isolates *Z. a.* 1–6 and *Z. a.* 9 were conspecific. The closest yeast species suggested by PC identification program, *Debaryomyces hansenii* var. *fabryi,* differs only in three tests from species 5 (assimilation: nitrate: −, ethylamin: +, creatinine: −).

Table 4. Fermentation profile of yeast isolates from lower termites and a cockroach. V variable response (either + or −). Species 8: Z. n. 4, 5; species 5: Z. a. 1–7, 9; species 6: Z. a. 8; species 11: R. s. 1, 6; species 9: H. i. 1; species 2: M. d. 10–11; species 3: M. d. 12; species 13: C. p. 1–3

Fermentation	Yeast-Isolates							
	Z. n. 4, 5	Z. a. 1–7, 9	Z. a. 8	R. s. 1, 6	H. i. 1	M. d. 10, 11	M. d. 12	C. p. 1–3
D-Glucose	+	+	+	+	+	+	+	+
D-Galactose	+	V	+	+	+	+	−	+
Maltose	+	−	−	−	−	−	−	−
Me-α-D-glucoside	−	−	−	V	−	−	−	−
Sucrose	−	V	+	+	−	+	+	−
α,α-Trehalose	+	V	+	V	+	+	+	+
Melibiose	−	V	+	+	+	+	+	−
Lactose	−	−	−	−	−	−	−	−
Cellobiose	−	V	−	−	−	+	+	+
Melezitose	−	−	−	−	−	−	−	−
Raffinose	−	V	+	+	−	+	+	−
Inulin	−	V	+	+	−	−	−	−
Starch	−	−	−	−	−	−	−	−
D-Xylose	+	−	−	−	−	−	−	−

Note a remarkable variability in the fermentation of different carbohydrates if 8 different isolates were investigated (Table 4). Further characteristics: white to cream, butyrous colonies with a wrinkled surface, margin often filamentous; simple pseudohyphae especially on corn meal agar, septate hyphae absent, vegetative reproduction by budding, ascospores not observed.

A second yeast species from the hindgut of *Z. angusticollis* was isolated only once so far (species 6). Again *Debaryomyces hansenii* var. *fabryi* has the best score in the PC identification program. It differs by 5 tests from the termite isolate (fermentation: inulin, assimilation: nitrate: −, ethylamine: +, cadaverine: +, creatinine: −). Further characteristics: white to cream, butyrous and shiny colonies, vegetative reproduction by budding; simple pseudohyphae on corn meal agar; sexual reproduction not observed; growth under strictly anaerobic conditions.

A third yeast isolate from *Z. angusticollis*, species 7

Table 5. Oxidative degradation (assimilation profile) of carbon and nitrogen compounds of yeasts from lower termites and a roach. V: variable response, w: only weak growth was detected, D: readings were negative after 3 d but positive after 7 d

Assimilation	Yeast-Isolates															
	Z.n. 1–3	Z.a 10	Z.n. 4,5	Z.a. 1–7,9	Z.a. 8	R.s. 1,6	R.s. 2,3	H.i. 1	M.d. 1–4	M.d. 5–9	M.d. 10,11	M.d. 12	M.d. 13	N.j. 1,2	R.s. 7	C.p. 1–3
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	+	+	−	+	+	+	V	+	+	+	+	+	+	+	+	+
D-Glucosamine	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
D-Ribose	−	+	−	V	−	−	−	+	V	+	w	−	+	−	+	−
D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Arabinose	−	+	V	−	+	+	−	+	−	+	+	−	+	−	−	−
L-Arabinose	V	+	V	+	+	+	+	+	+	+	+	+	+	+	+	−
L-Rhamnose	+	+	−	−	+	+	−	+	+	+	−	−	+	V	+	−
Sucrose	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
α,α-Trehalose	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Me α-D-Glucoside	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−
Arbutin	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	V
Melibiose	−	−	−	+	+	+	−	+	+	+	+	+	+	+	+	−
Lactose	−	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−
Raffinose	−	−	−	+	+	+	−	+	+	+	+	+	+	+	+	−
Melezitose	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−
Inulin	−	−	−	w	−	−	−	−	−	−	−	−	+	−	−	−
Starch	+	+	+	−	−	−	−	+	V	+	w	−	+	−	−	−
Glycerol	+	+	+	+	+	+	−	+	−	+	+	w	+	−	−	+
Erythritol	+	+	−	+	−	−	+	+	+	+	+	+	w	+	+	+
Xylitol	V	+	−	+	+	+	−	+	+	+	V	+	w	+	+	−
L-Arabinitol	−	+	−	+	+	+	−	+	+	+	+	w	w	+	+	−
D-Glucitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactitol	−	+	−	+	+	+	−	+	−	+	+	+	+	−	D	−
myo Inositol	+	+	−	−	−	−	w	+	+	+	−	−	+	+	+	−
2Keto-D-gluconate	V	w	+	+	+	+	−	−	+	+	+	+	+	−	+	−
5Keto-D-gluconate	−	−	−	V	−	−	−	−	+	+	−	−	+	−	−	−
D-Gluconate	−	−	−	+	−	−	−	−	+	+	w	+	+	−	−	−
D-Glucuronate	+	+	−	−	−	−	+	+	+	+	w	+	+	−	−	−
DL-Lactat	−	−	−	+	w	−	−	+	−	+	+	+	+	−	−	−
Succinate	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+
Citrate	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+
Methanol	w	−	−	−	−	+	V	−	−	−	−	w	−	−	−	−
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate	+	+	+	+	+	+	+	+	+	+	w	−	−	+	+	−
Nitrite	+	+	w	+	+	w	w	w	V	+	−	−	−	V	−	−
Ethylamine	−	−	−	−	−	−	−	−	−	−	−	w	w	−	−	−
L-Lysine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cadaverine	−	V	−	−	−	−	−	−	−	−	−	−	w	−	−	−
Creatine	+	+	+	+	+	+	+	+	+	−	w	+	w	+	w	−
Creatinine	+	+	w	+	+	w	w	+	+	−	w	+	w	+	w	−

Table 6. Additional tests for phenotypic identification: vitamin requirements, maximum temperature of growth, cycloheximide resistance, osmotolerance

Additional tests	Yeast-Isolates															
	Z.n. 1–3	Z.a 10	Z.n. 4,5	Z.a. 1–7,9	Z.a. 8	R.s. 1,6	R.s. 2,3	H.i. 1	M.d. 1–4	M.d. 5–9	M.d. 10,11	M.d. 12	M.d. 13	N.j. 1,2	R.s. 7	C.p. 1–3
w/o Vitamins	V	+	+	+	+	+	−	+	+	+	−	−	−	+	+	+
w/o Biotin	+	+	+	+	+	+	−	+	+	+	−	−	+	+	+	+
w/o Thiamin	V	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+
w/o Biotin & thiamin	V	+	+	+	+	+	−	+	+	+	−	−	−	+	+	+
T 25 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T 30 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T 35 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T 37 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
T 40 °C	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
0.01% Cycloheximide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.1% Cycloheximide	+	+	+	−	−	+	+	+	+	+	−	−	+	+	+	−
50% D-Glucose	−	w	+	+	+	+	V	−	−	−	+	+	−	V	−	−
60% D-Glucose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

(Table 3), was detected also in the hindgut of *Z. nevadensis*. Although the PC identification program suggested *Cryptococcus laurentii* as a candidate differing by eight tests, a morphological investigation on 0.2% (w/v) malt extract agar carrying sterilized pieces of wood from oak and spruce revealed a *Sporothrix* anamorph. Growth on 0.1% cycloheximide (*Mariat*, 1975) and an oxidative degradation of creatine and creatinine gave support to the morphological data (*Staib* et al., 1972, 1974) and suggested affinities to filamentous Ascomycetes (Ophiostomatales) for this species. There are only few *Sporothrix* species characterized by physiological characteristics in the literature (*de Hoog* et al., 1985). Based on an absence of fermentation and a broad assimilation pattern (e.g. inositol: +, rhamnose: +, erythritol: +, melibiose: −) *S. schenckii* comes close to the yeasts isolated from *Zootermopsis* (minor differences: assimilation: inulin: +, raffinose: +, ethylamine: +, growth without vitamins: −). A positive urease test (Table 3) and assimilation of creatine and creatinine excludes *S. catenata*, the anamorph of *Stephanoascus ciferrii*, and Endomycetes species with a *Sporothrix* anamorph (*de Hoog* et al., 1985, *de Hoog*, 1993). Further characteristics: cream membranous colonies, vegetative reproduction by budding, septate hyphae, sexual reproduction not observed; additional physiological data: amygdaline, glycogene, β-gentiobiose, D-turanose, L-lyxose, and D-tagatose were assimilated as sole carbon sources; growth on putrescine but not on D-glucosamine as a sole nitrogen source.

Species 8 includes two yeast isolates from *Z. nevadensis* (*Z. n.* 4 and *Z. n.* 5; Table 3). The closest relative yeast species suggested by the PC identification program is *Debaryomyces hansenii* var. *fabryi*. *D. hansenii* differs in 5 tests from the yeast isolate of *Zootermopsis* (fermentation: D-xylose: − assimilation: raffinose: +, xylitol: +, nitrate: −, ethylamine: −). Further characteristics: white to cream, butyrous colonies, septate hyphae and pseudohyphae absent, formation of ascospores not observed on McClary acetate agar.

The yeast species from *Heterotermes indicola* differs from its relative *Debaryomyces hansenii* var. *fabryi* suggested by the PC-program from *Barnett* (1994), in 8 results. *D. hansenii* deviates in the following tests from species 9 (assimilation: myo-inositol: −, 2-keto-D-gluconate: +, nitrate: −, ethylamine: +, cadaverine: +, creatinine: −, 50% D-glucose: +, no septate hyphae). Further characteristics of this remarkable yeast species with fermentative abilities and a very broad oxidative degradation pattern are: wrinkled, whitish colonies with a dense aerial mycelium and a fimbriate margin; vegetative reproduction by budding, elaborate pseudohyphae and/or septate hyphae; no sexual reproduction observed.

The yeast isolates from *Neotermes jouteli* (*N. j.* 1, *N. j.* 2) and one isolate from *Reticulitermes santonensis* (*R. s.* 7) belong to the same species (species 10; Table 3). Phenotypically *Candida edax* van der Walt & Nel comes closest to this yeast species. *Candida edax* differs in at least 8 tests (assimilation: D-arabinose: +, glycerol: +, nitrite: +, ethylamine: +, cadaverine: +, growth at 40 °C: +, presence of a septate mycelium). Further characteristics: white to cream, gleamless colonies, absence of septate hyphae, no sexual reproduction.

A second yeast species from *Reticulitermes santonensis*, species 11, is represent by the isolates *R. s.* 1. and *R. s.* 6. This species differs from *Debaryomyces hansenii* var. *fabryi* as suggested by the PC yeast identification program by 5 results (*D. hansenii:* fermentation: inulin: −; assimilation: methanol: −, nitrate: −, ethylamin: +, cadaverine: +). *Pichia guilliermondii* Wickerham, is a second species suggested by the PC program. It differs by 6 results although with a significantly lower probability (0.01). Further characteristics of species 11 are: white to cream, butyrous and shiny colonies, pseudohyphae rather sparsely developed; ascospores so far not found on acetate agar, vegetative reproduction by budding; arthroconidia and true hyphae absent.

Species 12 includes two further yeast isolates from *R. santonensis* (*R. s.* 2 and *R. s.* 3; Table 3). In comparison

with all the other yeast species isolated from lower termites, this yeast has only a narrow oxidative degradation pattern. It differs from the PC identification suggested *Pichia pini* (Holst) Phaff by 8 tests (*P. pini:* assimilation: D-ribose: +, salicin: +, arbutin: +, glycerol: +, D-glucuronate: −, nitrate: −, creatine: −). Further characteristics: white to cream, butyrous colonies with a distinct coherent filamentous margin; vegetative reproduction by budding, elaborate pseudohyphae and/or septate hyphae; no sexual reproduction observed.

Species 13 summarizes the data of three yeast isolates from the wood-feeding roach *Cryptocercus punctulatus*. *Candida sake* (Saito & Ota) van Uden & Buckley shows the highest probability (0.969) from the PC yeast identification program differing in 5 tests (fermentation: cellobiose: −; assimilation: erythritol: −, 2-keto-D-gluconate: +, cadaverine: +, T 35 °C: −). It is notable, however, that *Debaryomyces hansenii* var. *fabryi* was in the second position differing by 7 tests (probability: 0.017). Further characteristics: white to cream, butyrous colonies with a labyrinthous wrinkled ("daedaloid") surface; simple to elaborate pseudohyphae; septate hyphae and sexual reproduction not observed.

Polyphasic genotypic characterization

To characterize the yeast isolates from lower termites and a wood-feeding roach on the molecular level, we used the qualitative and quantitative monosaccharide patterns of purified and hydrolyzed yeast cell walls, the ubiquinone system (Table 3), partial 18S rDNA sequences (positions 948 to 1273 homologous to the *Saccharomyces cerevisiae* gene). Based on this polyphasic approach it becomes obvious that the majority of yeast species of lower termites and the cockroach comprise a phylogenetically homogeneous group belonging to the Endomycetales (species 1, 2, 3, 5, 6, 8–13). These species are characterized by a ubiquinone Q-9 (Table 3). From the cell wall monosaccharide pattern two distinct groups could be separated. The presence of mannose and glucose ("*Saccharomyces type*") is characteristic for species 2, 3, 5, 6, 8, 11, and 13. A glucose – mannose – galactose pattern was found in species 1, 9, 10, and 12. Based on yeast cell wall sugars, the ubiquinone Q-9 and ribosomal DNA sequence homology of 100% (region 948–1273), species 3 and 11 belong to the genus *Debaryomyces* represented by the type species *D. hansenii* (CBS 789). Species 6 a nd 8 came close to the genus *Debaryomyces* but need further investigations. Species 2 and 5 are congeneric with each other. Similar to species 13 we were not able to identify these yeasts on the genus level so far.

Although the galactose containing group of yeast species 1, 9, 10, and 12 exhibits a closer relationship to the Lipomycetaceae in comparison with species of the Dipodascaceae it was not possible to establish conspecificity with an already known yeast species of the Endomycetales. Species 4 is a heterobasidiomycetous yeast with high sequence homology to the genus *Trichosporon*. *Cryptococcus humicolus* suggested by the PC identification program can be excluded. This is in agreement with the presence of arthroconidia. Species 7 shows a cell wall monosaccharide pattern and ubiquinone system characteristic for filamentous Ascomycetes of the genus *Ophiostoma* s. str. or anamorphic species of the genus *Ophiostoma* (*de Hoog,* 1993) and *Hyphozyma roseonigra* (Table 7). The genus *Hyphozyma* was heterogeneous with respect to the ubiquinone system. Whereas the cell wall monosaccharide pattern exhibiting glucose – mannose – galactose – rhamnose was similar in *H. lignicola* (UAMH 7002^T), *H. roseonigra* (CBS 514.83^T), *H. sanguinea* (CBS 406.52^T), and *H. variabilis* var. *odora* (CBS 328.80^T) only *H. roseonigra* showed the ubiquinone Q-10 H_2 (Table 7). The remaining *Hyphozyma* species are characterized by a ubiquinone Q-10.

Table 7. Cell wall monosaccharide composition of *Hyphozyma roseonigra* and different *Ophiostoma* and *Sporothrix* species

Species	Strain	Cell wall monosaccharide composition (mol%)					
		GLC	MAN	GAL	XYL	FUC	RHA
Ophiostoma							
O. bicolor	HA 103	51	28	5	–	–	16
O. ips	HA 96	51	26	3	–	–	19
O. quercus	HA 207	62	19	3	–	–	16
O. sp. 2	HA 208	61	27	2	–	–	10
O. sp. 1	HA 206	39	31	1	–	–	29
O. ulmi	HA 209	36	33	1	–	–	30
O. ulmi	HA 210	42	30	2	–	–	26
O. ulmi	HA 97	48	28	4	–	–	20
Hyphozyma							
H. roseonigra	HA 680	30	26	–	–	–	44
Sporothrix							
S. schenckii	HA 653^T	58	34	2	–	–	6
S. albicans	HA 654^T.	51	25	3	–	–	21

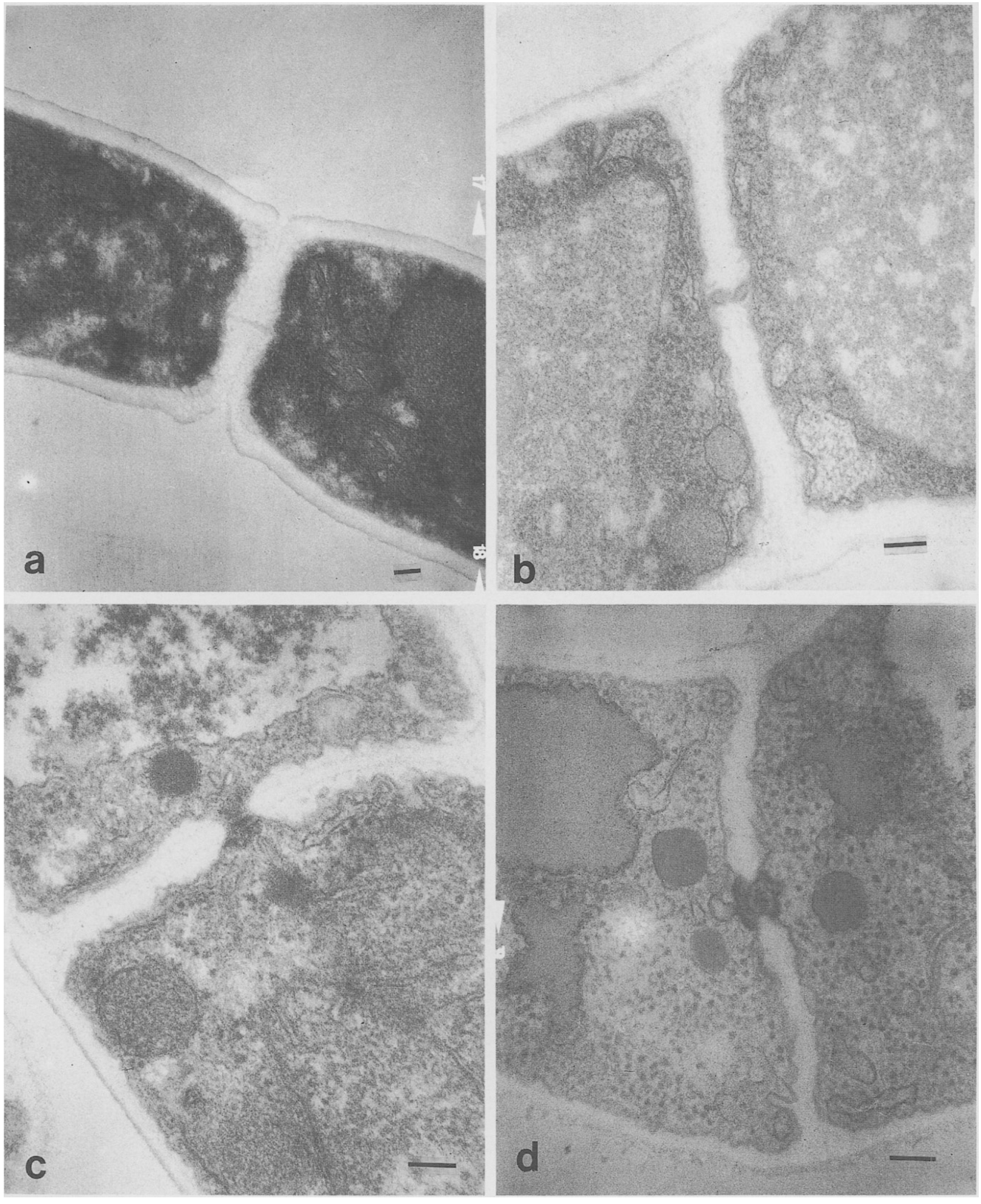

Fig. 4. Ultrastructure of septa from species 12 (HA 174, HA 175) and *Sporothrix albicans* (species 7: HA 162, HA 163; note simple septa with Woronin bodies). A bilayered cell wall suggesting affinities to the Ascomycetes was characteristic for all species except species 4 (Table 3). Magnification 85.000 x; bar indicates 0.1 μm.

Ultrastructure

Species 1–3 and 5–13 revealed a uniform cell wall morphology. Walls were electron-transparent and non-lamellate (Fig. 4). Usually, a small outer electron-dense layer was additionally present (Fig. 4). An electron-dense multilayered lamellar cell wall and an enteroblastic mode of budding was characteristic for species 4 (Fig. 5). Hyphal septa were found in species 1, 3, 4, and 7–12 only. Among these species, a central micropore-like channel was observed in species 1, 3, 8–12 (Fig. 4a, b). Distinct simple pores with Woronin bodies were only present in species 7 (Fig. 4c, d). Dolipores associated with a number of more or less sacculate bodies were characteristic for species 4. Each saccule consisted of an outer limiting membrane which was continous with the endoplasmic reticulum at adseptal base and an inner curved electron-dense layer at the abseptal base (Fig. 5). Thus cell wall ultrastructure indicates that species 4 is a basidiomycete and species 1–3 and 5–13 are ascomycetous. The species 1, 3, 8–12 share the narrow micropores with members of the Endomycetales (*van der Walt* and *von Arx*, 1985). The presence of distinct simple pores with Woronin bodies confirmes the the affinities to filamentous Ascomycetes for species 7 (Fig. 4 c, d; *Smith* and *Batenburg-van der Vegte*, 1985, 1986). The septal pore apparatus of species 4 is essential similar to that of the Tremellales s. str. (Filobasidiaceae included; *Oberwinkler*, 1985;). It is known that the pore-associated saccules vary considerably in length and shape among the Tremellales (*Berbee* and *Wells*, 1988). The saccules of species 4 are similar to those reported for the Tremellales s. str., but they are in contrast to e.g. *Sirobasidium magnum* (*Moore*, 1978) and *Tremella globospora* (*Berbee* and *Wells*, 1988) rather shallow and appear to represent somehow an archaic form of the typical *Tremella* saccules. Nearly all species with this septal pore type are mycoparasites (*Bauer* and *Oberwinkler*, 1990). Similar doliporous septa were shown recently in the genus *Trichosporon* by *Guého* et al. (1992). Although the genus *Trichosporon* is remarkable homogeneous on the basis of partial 26S rRNA sequences, except *T. pullulans*, various septal types are known in different species. The parenthesom could be nearly absent (*T. sporotrichoides*), irregularly vesicular (*T. asahii, T. laibachii*) or consisted of tubuli which in cross-section were seen as small round vesicels with double membranes (*T. asahii, T. coremiforme*; Fig. 5).

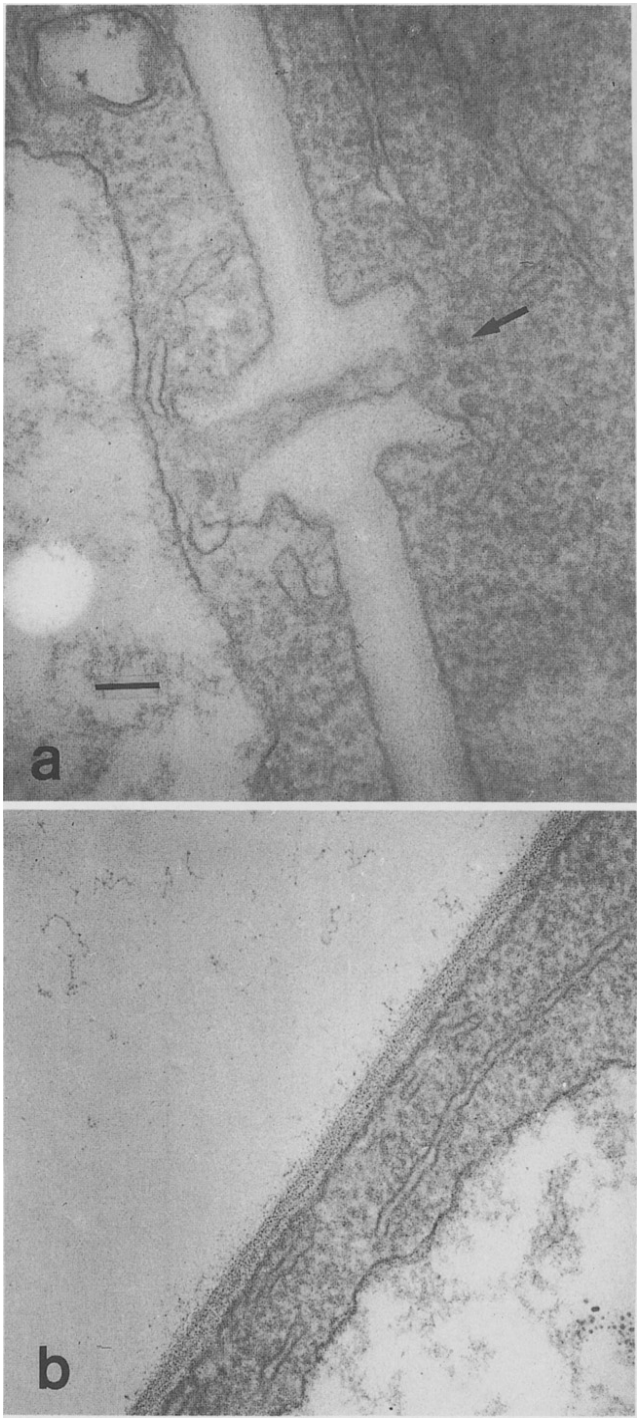

Fig. 5. Ultrastructure of the septenporus (a) and cell wall (b) from species 4. Doliporus associated with sacculate bodies (indicated by an arrow; magnification 81.000 x; bar indicates 0.1 μm).

Genotypic identification of Sporothrix albicans

Two strains HA 163 and HA 607 (compare Table 3) from species 7 were chosen for genotypic identification using ribosomal DNA sequences. The portion of the small ribosomal subunit gene from base 577 to base 1643 was amplified by PCR. The resulting fragment was used to sequence two regions. The upstream region comprises the bases 595 to 993. Within this fragment the region 644 to 742 exhibits the highest variability known for the fungal 18S rDNA (Fig. 6A). The downstream region includes the segment 948 to 1273 already used for genus identification (Fig. 6B). Both partial sequences from the strains HA 163 and HA 607 were identical suggesting conspecificity of the yeast isolates from *Zootermopsis nevadensis* and *Z. angusticollis*.

The two partial sequences (Fig. 6) yielded three identical entries in the National Center of Biotechnology Information. (National Library of Medicine (see Material and Methods) of the highest score: The 18S rDNA sequences from *Sporothrix schenkii* (accession No. M85053),

A:

GCGTATATTA AAGTTGTTGC AGTTAAAAAG CTCGTAGTTG AACCTTGGGC CTGGCTGGCC

GGTCCGCCTC ACCGCGTGCA CTGGTCCGGC CGGGTCTTTC CCTCTGGGGA GCCGCATGCC

CTTCACTGGG TGTGTCGGGG AACCAGGACT TTTACTTTGA AAAAATTAGA GTGTTCAAAG

CAGGCTTATG CTCGGATACA TTAGCATGGA ATAATAGAAT AGGACGTGCG GTTCTATTTT

GTTGGTTTCT AGGACCGCCG TCAATGATTA ATAGGGACAG TCGGGGGCAT CAGTATTCAA

TTGTCAGAGG TGAAATTCTT GGATTTATTG AAGACTAACT ACTGCGAAAG CATTTGCCAA

GGATGTTTTC ATTAATCAGG AACGAAAGTT AGGGGATCGA AG

B:

CCAAGGATGT TTTCATTAAT CAGGAACGAA AGTTAGGGGA TCGAAGCCGA TCAGATACCG

TCGTAGTCTT AACCATAAAC TATGCCGACT AGGGATCGGA CGATGTTATT TTTTGACTCG

TTCGGCACCT TACACGAAAG TACAAGTTTC TGGGTTCTGG GGGGAGTATG GTCACAAGGC

TGAAACTTAA AGAAATTGAC GGAAGGGCAC CACCAGGGGT GGAATCTGCG GCTTAATTTG

ACTCAACACG GGGAAACTCA CCAGGTCCAG ACACGATGAG GATTGACAGA TTGAGAGCTC

TTTCTTGATT TCGTGGGTGG TGGTGCATG

Fig. 6. Partial 18S rDNA sequences of isolates HA 163 and Z10, determined by direct sequencing of a PCR fragment. Panel A shows the region homologous to the positions 595 to 993 from the respective gene in *S. cerevisiae* (J01353). The "fingerprint" section of highest variability is underlined. In panel B the region homologous to bases 948 to 1273 is also given in the sense of the coding strand.

Ophiostoma stenoceras (M85054) and *Ophiostoma ulmi* (M83271). While no differences were observed between the query and the entries for *S. schenckii* and *O. stenoceras*, the comparison with the data from *O. ulmi* showed 7 bases difference in the upstream and 2 bases difference in the downstream region. The two entries of 100% homology were submitted both by *Berbee* and *Taylor* (1992; pers. comm.).

The use of the full length of the partial sequences for database homology search is not mandatory. If the section of highest variability (bases 644 to 742, underlined in Fig. 6A) is put as a query alone, the response recovers the sequences shown above in addition to a number of short, partial sequences from different *Ophiostoma* species: *O. retusum, O. ips, O. longisporum, O. ulmi, O. parva, O. minuta-bicolor, O. minima, O. piliferum, O. fasciata, O. ranaculosum* and *O. crassivaginata*. None of these, however, reached the maximal score, which accounts to the entries of *S. schenckii* and *O. stenoceras* only.

For accurate identification we have checked the output from database by RAPD-PCR. A number of *Sporothrix* and *Ophiostoma* strains were selected for the high resolution comparison with the isolates HA 162, HA 163, and HA 607 (Species 7). The use of three different primers yielded consistent results (Decamer 2, 3, and 4) which gather the yeast isolates from *Zootermopsis* species 7 and the type strain of *Sporothrix albicans* (MUCL 19327^T, CBS 302.73^T; Fig. 1). Although *de Hoog* (1974) proposed conspecificity between *S. albicans* and *S. schenckii* var. *schenckii* (CBS 359.36; possible type culture) based on morphological observations, levels of similarity did not exceed 50% when RAPD-PCR patterns were compared.

The average range between the type strains of *S. schenckii* var. *schenckii* and *S. albicans* was 20 and 30%. This comes tight to the background noise and may indicate closely related species (Fig. 1. lanes 9 and 12, Fig. 2. lanes 3, 8, 11, Fig. 3. lanes 9, 10, 11). In contrast, similarity levels between *S. albicans* and species 7 calculated individually from three different primers and five separate investigations come close to 65% (Fig. 1. lanes 6–9, Fig. 2. lanes 5–8, Fig.. lanes 6–9). Two *Ophiostoma* strains from deciduous trees (HA 206, HA 395; Table 2) preliminary identified by morphology as *O. stenoceras (Robak) Melin & Nannf.* showed similarity levels below 20% within the range of background noise (Fig. 1. lanes 1, 2, Fig. 3. lanes 3, 4). Although strain HA 206 was isolated from *Quercus petraea* and HA 395 from *Castanea sativa* strains appeared to be conspecific based on the RAPD-PCR data. The authentic strain of *O. stenoceras* HA 899 was isolated from a coniferous tree (*Robak,* 1932). This culture did not produce a teleomorph on different culture media. Its RAPD-PCR pattern was distinct from the two isolates from deciduous trees as well as from *S. albicans*. Further *Ophiostoma* and *Sporothrix* species used for genotypic identification showed distinct banding patterns. *O. ulmi* (HA 209; Fig. 1) may be an exception. Levels of similarity were around 50% with respect to the two isolates from deciduous tree, morphologically identified to belong to *O. stenoceras*.

Discussion

Roaches and termites are well known for specific prokaryotic and eukaryotic gut symbionts (*Douglas,* 1992; *Breznak* and *Brune,* 1994; *Varma* et al., 1994). The greatest density of microorganisms in the alimentary tract of termites is in the proximal portion of the hindgut, known as the paunch (*Douglas,* 1992). In all termites, the paunch harbors bacteria, at 10^9–10^{10} cells per ml gut contents. *Byzov* et al. (1993a,b) detected a similar relationship for a symbiotic yeast community in the hindgut of the diplopod *Pachyiulus flavipes*. It is widely accepted that the symbiotic flagellates (*Yamin,* 1981; *Breznak* and *Brune,* 1994) in *Cryptocercus* and lower termites have been inherited from the common ancestor of the Isoptera and Blattaria, possibly in the Carboniferous or Permian periods (*Thorne* and *Carpenter,* 1992).

In lower termites the association with fungi was exhaustively studied by *Hendee* (1935). Representatives of thirty-three genera of Zygo-, Asco-, Deutero-, and Basidiomycetes and twenty non-sporulating cultures of fungi were isolated from the nests and fecal pellets of termites belonging to the genera *Kalotermes, Reticulitermes,* and *Zootermopsis*. There was no evidence of any specific relation between a given species of termite and any genus of fungus. *Penicillium* and *Trichoderma* were the genera of fungi most frequently isolated from colonies of all three species of lower termites. A comparatively small number of Basidiomycetes was detected. The fungi most commonly associated with the termites showed no apparent correlation with the species of wood in which the termite colonies occurred. The cultures from the wood of the surface of walls of burrows, in general, showed greater amounts of fungal growth and yielded a larger number of different fungi than those from wood 1–2 mm from burrows. *Zoberi* and *Grace* (1990) added some additional genera of Dictyosteliales, Stemonitales, Mucorales, and Hyphomycetes recently for the subterranean termite *Reticulitermes flavipes*. Parasitic interactions between lower termites and fungi are known for *Absidia coerulea* Bainier and some *Entomophthora* species (*Sands,* 1969). Nothing is known about the occurrence of yeasts in the hindgut of lower termites. The species of lower termites chosen for the present study represent the three important habitat types: *Zootermopsis angusticollis* and *Z. nevadensis,* the damp wood termites which live in rotting wet wood in warm temperate regions; *Neotermes jouteli,* represents a dry wood termite, which excavates logs and maintains colonies strictly in wood without necessary soil connections; *Reticulitermes santonensis* and *Heterotermes indicola,* the subterranean termites. *Mastotermis darwiniensis* and the roach *Cryptocercus punctulatus,* we have chosen from the phylogenetic point of view. According to the traditional systematics, the Mastotermitidae are most primitive and closest relatives to the roaches. They had a world-wide distribution in the tertiary period. Today they are restricted to Australia with a single species *M. darwiniensis*. *Reticulitermes santonensis* is a termite species representative for the palearctic region (France; *Krishna* and *Weesner,* 1969). *Heterotermes indicola* is found throughout northern India (oriental region; *Krishna* and *Weesner,* 1969). The two species of *Zootermopsis* and *Neotermes jouteli* are termites from the nearctic region. The *Zootermopsis* species occur in the forested areas along the Pacific coast, in the Cascade and Sierra Nevada Mountains and in some of the inland ranges west of the Continental Divide (*Krishna* and *Weesner,* 1969). *N. jouteli* is a termite of the coastal areas of Florida, Cuba, the Bahamas, and both the east and west coasts of Mexico, as well as Socorro Island (*Krishna* and *Weesner,* 1969).

Using the qualitative and quantitative monosaccharide pattern of purified yeast cell walls, the ubiquinone system, and partial sequences of 18S rDNA (positions 948 to 1273), the majority of yeast isolates from lower termites and the roach *Cryptocercus punctulatus* comprise a homogeneous assemblage of representatives from the Endomycetales (Table 3; note the uniformity in the ubiquinone system; *Kuraishi* et al., 1985, *Yamada* et al., 1987). Whereas the Man – Glc monosaccharide pattern ("Saccharomyces-type") is commonly sufficient to assign a yeast to the Endomycetales this is not true for the Glc – Man – Gal pattern ("Schizosaccharomyces-type"; *Prillinger* et al., 1993b; compare *Kurtzman,* 1993). Additional tests like hydrolysis by urease, formation of extracellular amyloid compounds, DBB-reaction (*Prillinger* et al., 1990a,b) or the ultrastructure of septa are important (*Kreger-van Rij* and *Veenhuis,* 1974; *van der Walt* et al., 1983; *Smith* and *Batenburg-van der Vegte,* 1985). Furthermore the Glc – Man – Gal pattern was detected recently in different orders of the filamentous Ascomycetes (*Prillinger* et al., 1990a; unpublished results). We have found the Glc –

Man – Gal/ubiquinone Q-9 pattern in *Yarrowia, Wickerhamiella, Lipomyces, Dipodascus/Geotrichum* (*Prillinger* et al., 1990a, *Lopandic* and *Prillinger,* unpubl. results), genera not closely related based on partial 18S rDNA sequences. Genus identification in species 1, 10, and 12 therefore needs further 18S rDNA sequence information. Data so far suggest that these species are related to the Lipomycetaceae. The absence of EAC in the termite yeasts, however, does not agree with a new concept of family delimitation within the Lipomycetaceae (*von Arx* and *van der Walt,* 1987; Table 3).

Based on our genotypic approach species 4 was the only basidiomycetous yeast isolated from lower termites. This species belongs to the genus *Trichosporon*. The phenotypic characteristics of this species come close to *Cryptococcus humicolus*. It is noteworthy that *C. humicolus* was the only *Cryptococcus* species found with the *Trichosporon* cluster on the basis of partial large subunit rRNA sequences by *Guého* et al. (1993). Recently, *Byzov* et al. (1993a,b) detected a highly symbiotic yeast community in the hindgut of the diplopod *Pachyiulus flavipes*. Terrestrial arthropods belonging to the Diplopoda are well known from the Paleozoic (*Shear* and *Kukalová-Peck,* 1990). The yeast communities consist almost exclusively of Ascomycetes phenotypically identified to be *Debaryomyces hansenii, Torulaspora delbrueckii,* and *Zygowilliopsis californica* (DTZ-group; *Byzov* et al. 1993a,b). Typical litter yeasts like *Aureobasidum pullulans, Rhodotorula rubra,* and *Candida* sp. found in the food of diplopods were eliminated completely during the passage through the digestive tract. Beside the DTZ-group *Trichosporon cutaneum, Geotrichum candidum,* and *Pichia membranaefaciens* were minor components of the yeast community in the excrement. The cell walls of these yeasts probably resist the enzymatic attack in the digestive tract.

In contrast to lower termites, basidiomycetous yeasts were commonly found in the hindgut of the higher termite *Odontodermes obesus*. From *O. obesus* only, four different species of basidiomycetous yeasts were isolated (*Prillinger* et al. unpublished). Based on yeast cell wall sugars and partial ribosomal DNA sequences one species come close to *Rhodosporidium toruloides* as a representative of the *Microbotryum* type (*Prillinger* et al., 1991a, 1993b). Three other species contained xylose in their cell walls and showed affinities to the genera *Filobasidium* and *Tremella* (*Prillinger* et al. 1991b, 1993b).

The assimilation of NO_3 from species 6, 8, and 11, however, does not agree with the general genus description of *Debaryomyces* (*Barnett* et al., 1990). Ribosomal DNA sequences from *Kurtzman* and *Robnett* (1991) and *Yamada* et al. (1991, 1992) as well as cell wall neutral sugars (*Prillinger* and *Lopandic,* unpublished results) suggest that the genus *Debaryomyces* is presently heterogeneous. We therefore decided to concentrate in species identification to species 7 which is a dimorphic ascomycete.

Species 7 was identified genotypically at the species level. Based on a negative DBB reaction, hydrolysis by urease, an absence of fermentation (Table 3), and the broad assimilation pattern of carbohydrates (Table 5), especially an oxidative degradation of inositol, starch, and erythritol *Hyphozyma sanguinea* (*de Hoog* and *Smith,* 1981; *Hutchison* et al., 1993) and *Sporothrix schenckii* (*de Hoog* et al., 1985) became the favourites for species identification. Although rhamnose was detected in the cell wall of all *Hyphozyma* species type strains (Table 7), *H. sanguinea* could be excluded based on a ubiquinone Q-10. According to our ubiquinone data and yeast cell wall analysis the type species *H. variabilis de Hoog* et *M. Th. Smith* of the genus *Hyphozyma* comes close to the genera *Protomyces* and *Taphrina*. *De Hoog* and *Smith* (1981) added further arguments in favour of this phylogenetic relationship. The presence of ubiquinone Q-10 H_2 in *H. roseonigra* makes this yeast-like hyphomycete a candidate for reclassification (*de Hoog* and *Smith,* 1986). Phylogenetic relationships between *H. roseonigra* and the Ophiostomatales as indicated by the ubiquinone system and yeast cell wall sugars await further corroboration.

A Glc-Man-Gal-Rha monosaccharide cell wall pattern, a ubiquinone Q-10 H_2, and a high score of 18S ribosomal DNA sequence homology in the regions 948 to 1273 and 595 to 993 made *Sporothrix schenckii* and *Ophiostoma stenoceras* the only candidates for species identification. RAPD-PCR analysis, however, indicates that it is rather difficult to identify a *Sporothrix* and *Ophiostoma* species with morphological or physiological data only (*Weijman* and *de Hoog,* 1985; *de Hoog,* 1993; *Summerbell* et al., 1993; *Halmschlager* et al., 1995). RAPD-PCR data demonstrate convincingly that the yeast isolates from *Zootermopsis angusticollis* and *Z. nevadensis* identified as species 7 (Table 3) are conspecific with the type strain of *Sporothrix albicans* S. B. Saksena only (Fig. 1, 2, and 3). *S. albicans* was isolated from soil in England and described by *Saksena* (1964). Later *de Hoog* (1974) synonymized *S. albicans* with *S. schenckii* var. *schenckii* (*de Hoog* pers. comm.). *Summerbell* et al. (1993) noticed in cases where *S. schenckii* was isolated from natural materials, non-pathogenic, non-perithecial isolates very similar to *S. schenckii* are also frequently obtained (*Mackinnon* et al., 1969, *Mariat,* 1975). Like *S. albicans* these isolates lack pigmented secondary conidia. In addition, their vitamin requirements may differ from *S. schenckii's* characteristic requirement for thiamine (Table 6; note *Z. n.* 1–3 and *Z. a.* 10; *Mariat,* 1975, *Mariat* et al., 1962). Using type strains and RAPD-PCR analysis we have been able to exclude also conspecificity of *O. stenoceras* with *S. schenckii* (*Summerbell* et al., 1993). Studies from *Ryter* and *Fromentin* (1985) revealed that *S. schenckii,* but not *O. stenoceras,* is able to resist digestion by macrophage cells of the mouse immune system.

Emended species description based on the yeast stage of *Sporothrix albicans* S. B. Saksena:

In liquido "GYP" dicto, post dies 3 ad 25 °C, cellulae sphaericae, ovoideae, ellipsoidales aut elongatae (2.8–3.5) × (3.9–5.5) µm, singulae vel binae. Annulus et sedimentum formantur. In agaro "GYP" post unum mensem 25 °C, cultura margine pilosa. Pseudomycelium et mycelium formantur.

Fermentatio nulla. Glucosum, D-galactosum (lente vel nullum), L- sorbosum, D-glucosaminum, D-ribosum (lente

vel nullum), D-xylosum, D-arabinosum (lente vel nullum), L-arabinosum (lente vel nullum), L-rhamnosum, saccharosum (lente vel nullum), maltosum, trehalosum (lente vel nullum), α-methyl-D-glucosidum, cellobiosum, salicinum, arbutinum, lactosum (lente vel nullum), melezitosum, amylum solubile, glycerolum, erythritolum, xylitolum (lente vel nullum), L-arabinitolum, D-glucitolum, D-mannitolum, galactitolum (lente vel nullum), inositolum, acidum 2-keto-gluconicum (lente et exigue vel nullum), acidum D-glucuronicum, acidum succinicum, acidum citricum (lente vel nullum), methanolum (exigue vel nullum) et ethanolum assimilantur at neque melibiosum, raffinosum, inulinum, acidum 5-ketogluconicum, acidum gluconicum nec acidum DL-lacticum. Kalium nitricum, natrium nitrosum, L-lysinum, cadaverinum (lente vel nullum), putrescinum, creatinum et creatininum assimilantur at neque ethylaminum. Maxima temperatura crescentiae: 37 °C. Ad crescentiam thiaminum (vel nullum) necessarium est. Diazonium caeruleum B non colorat. Ubiquinonum majus: Q-10-H_2. In agaro GYP post 25 dies colonia zymosa alba vel cremea; substantia amyloidea extracellularis haud formatur. Ureasum adest. In medio 0.1% cycloheximido confecto crescit. Paries cellularis hydrolysatus glucosum, mannosum, galactosum et rhamnosum continet.

Teleomorphosis ignota

Holotypus vivus HA 162

Growth in GYP medium: After 3 days at 25 °C, cells are globose, ovoidal, ellipsoidal or elongate (2.8–3.5) × (3.9–5.5) µm commonly single or in pairs. A ring and sediment containing a septate mycelium with lateral blastoconidia present. Blastoconidia borne on denticles. After one month a voluminous sediment, a ring but no pellicle present.

Growth on GYP agar: After one month at 25 °C the streak culture is white to cream, membranous, delicately striated with a filamentous margin.

Dalmau plate culture on corn meal agar: septate mycelium present.

Formation of ascospores not observed.

Fermentation: Absent

Assimilation of carbon and nitrogen compounds see Table 5

Vitamins required see Table 6

Production of starch-like substances: Negative

Growth on 50% (w/w) glucose-yeast extract agar: Negative

Urease: Positive

Growth 0.1% cycloheximide: Positive

Diazonium Blue B reaction: Negative

Maximum growth temperature: 37 °C

Yeast cell wall monosaccharide composition: Glc, Man, Gal, Rha

Major ubiquinone: Q-10 H_2

The species has to be included in the family Ophiostomataceae s. str.

A comparison between the classical phenotypic approach and genotypic identification methods introduced in the present paper reveals a high degree of precision and reliability and additional phylogenetic information for the polyphasic genotypic approach. The variability in the physiological characteristics, especially if more than three strains were investigated, is a serious handicap of phenotypic identification methods (Tables 4 to 6). The main goal of our genotypic approach becomes obvious from the disappearance of the artificial Asco-/Deuteromycetes barrier. There can be not doubt that *S. albicans* belongs to the Ophiostomataceae s. str. (*Berbee* and *Taylor,* 1992). At present, Article 59 of the Botanical Code of Nomenclature makes no provision for including *S. albicans* in the genus *Ophiostoma* (*Hennebert* and *Weresub,* 1977). *Reynolds* and *Taylor* (1991, 1992) therefore suggested a revision of Article 59. Although *S. albicans* lacks a sexual state an important, morphological taxonomic character necessary for generic placement, genotypic identification methods can compensate for missing sexual characters (*Prillinger,* 1987; *Prillinger* et al., 1989, 1993a). We do not agree with *Reynolds* and *Taylor* (1991) who suggest DNA may serve as the type element in modern fungal taxonomy. Our genotypic approach, however, demonstrates convincingly the importance of culture collections having well preserved living fungal cultures available. The advantage of RAPD-PCR analysis in genotypic identification has been discussed in two previous publications (*Messner* et al., 1994, *Mólnar* et al., 1995).

With respect to symbiotic association and coevolution the phylogenetic homogeneity of at least 11 yeast species (Table 3) isolated from the hindgut of lower termites from different habitats and geographical areas (e. g. Australian, Nearctic, Oriental, and Palearctic Region) signals that the yeasts can be considered symbionts that remain in a steady state. A similar symbiotic yeast population consisting of *Debaryomyces hansenii, Torulaspora delbrueckii,* and *Zygowilliopsis californica,* detected recently in the hindgut of the diplopod *Pachyiulus flavipes* (*Byzov* et al., 1993a,b) corroborates our interpretation. Similar to the roaches there is a very rich fauna of carboniferous diplopods known from Central Bohemia (*Kraus,* 1974). The first Diplopoda are known from Late Silurian and Early Devonian deposits (*Almond,* 1985; *Ross* and *Briggs,* 1993). According to *Shear* and *Kukalová-Peck* (1990) nothing suggests that the ecological role of millipeds (Diplopoda) has changed in the more than 400 million years since they first may have appeared.

Kluyveromyces blattae is an other yeast so far only known from the intestinal tract of the cockroach *Blatta orientalis* (*Henninger* and *Windisch,* 1976). *K. blattae* shows the Man-Glc monosaccharide pattern and a ubiquinone Q-6 systeme. Phylogenetically *K. blattae* is very close to *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* (*Messner* et al., 1995; *Prillinger* et al. unpublished results). A specific association of *K. blattae* and *Blatta orientalis,* however, needs further corroboration.

Neither in lower termites and roaches nor in diplopods was there evidence for yeast isolates representing the *Ustilago* type pattern (*Prillinger* et al., 1990b, 1993b). *Berbee* and *Taylor* (1993) considered *Ustilago* and *Tilletia* ancestral to the Endomycetales and filamentous Ascomycetes. The presence of a symbiotic Endomycetes yeast community representing the Man-Glc carbohydrate pattern in di-

plopods and lower termites documents that the "*Saccharomyces* type" is most probably ancestral to the *Protomyces, Microbotryum, Ustilago,* and *Tremella* type as suggested by *Prillinger* et al. (1993b). Recently, we have shown a tight phylogenetic relationship between the classical yeasts *Saccharomyces* and *Kluyveromyces* and primitive filamentous fungi from the borderline between polykaryotic sporangia and uninucleate asci based on ribosomal DNA-sequencing (*Messner* et al., 1995). From the present paper we have to conclude, that the presence of galactose in ascomycetous yeast cell walls ("*Schizosaccharomyces* type") needs additional information to retain phylogenetic importance otherwise this type is heterogeneous (*Prillinger* et al., 1993b).

Based on our preliminary study of yeast communities in the hindgut of roaches and termites we may summarize that an amalgamation of information obtained from obligate parasitic or symbiotic associations, yeast cell wall sugars, the ubiquinone system, fermentation and a broad or rather small assimilation pattern with ribosomal DNA sequencing and the fossil record might be a more reliable indicator for fungal phylogeny than evolutionary trees based on ribosomal DNA sequencing alone to trace fungal phylogeny back to early paleozoic periods. Genotypically similar yeasts in roaches and termites are in favour of the hypothesis from *Martynov* (1937). *Martynov* (1937) proposed an evolutionary time frame in which roaches and termites diverged in the late Devonian or early Carboniferous. This, however, antedates the first fossil record of termites known from 120 million years considerably (250 million years; *Thorne* and *Carpenter*, 1992). Further data, especially on coevolution, therefore may be urgently needed.

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Prof. Dr. *H. König*, Abteilung Angewandte Mikrobiologie, Universität Ulm, D-89069 Ulm, Germany, Tel.: +49(0)731-502-2707, Fax: +49(0)731-502-2719

Prof. Dr. *Hansjörg Prillinger*, Universität für Bodenkultur, Inst. f. Angew. Mikrobiologie, A-1190 Wien, Austria