Molecular Characterization and Application of Random Amplified Polymorphic DNA Analysis of *Mrakia* and *Sterigmatomyces* Species

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The qualitative and quantitative monosaccharide spectra of purified yeast cell walls revealed that there are three phylogenetically distinct lineages of sterigma-forming basidiomycetous yeasts: (i) *Kurtzmanomyces* and *Sterigmatomyces* species, which contain high levels of mannose; (ii) *Tilletiopsis* species, which contain glucose, galactose, and small amounts of mannose; and (iii) *Fellomyces*, *Kockovaella*, *Sterigmatosporidium*, and *Tsuchiyaea* species, which appear to be closely related on the basis of their high levels of glucose and the presence of xylose. The yeast cell wall neutral sugars of *Sporobolomyces antarcticus* and *Sterigmatomyces aphidis* were similar to those of members of the genus *Tilletiopsis*. However, the possibility that these taxa are conspecific was eliminated by the results of a random amplified polymorphic DNA (RAPD) analysis. The conspecificity of *Mrakia frigida* and *Mrakia nivalis*, the conspecificity of *Mrakia gelida* and *Mrakia stokesii*, and the conspecificity of *Sterigmatomyces halophilus* and *Sterigmatomyces indicus* were confirmed by RAPD analysis results. RAPD analysis was found to be a simple and highly sensitive method which can be used to differentiate species at the DNA level; it can replace nuclear DNA-nuclear DNA hybridization experiments for species identification, characterization, and delimitation.

The genus *Mrakia* was proposed for *Leucosporidium frigidum* as a new genus of the Filobasidiaceae by Yamada and Komagata (51). The following four species that formerly had been placed in the genus *Leucosporidium* were transferred to the genus *Mrakia*: *Mrakia frigida*, *Mrakia gelida*, *Mrakia nivalis*, and *Mrakia stokesii* (7, 8, 51). A homothallic, apomictic life cycle, self-sporulating simple holobasidia bearing terminal, sessile basidiospores, fermentation of sugars, the presence of ubiquinone Q-8, production of extracellular amyloid compounds, and yeast cell walls containing xylose are the most distinctive features of *Mrakia* species (14, 26, 32, 51). The fact that the genus *Mrakia* should be separated from phragmobasidial, smut-like *Leucosporidium* species (31) has been corroborated by the results of determinations of parts of the 18S and 26S rRNA sequences (6, 9, 48) and ultrastructural septal pore data (37).

The *Mrakia* species originally defined on the basis of their ability to ferment glucose and their ability to utilize nitrate or various carbon compounds (cellobiose, maltose, maltose, melibiose, raffinose) for growth (7, 8) turned out to be questionable taxa after investigations of the electrophoretic enzyme patterns (39, 52) and parts of the 18S and 26S rRNA sequences (6, 9, 48). The electrophoretic enzyme patterns (52) suggested that *M. nivalis* and *M. stokesii* are synonyms of *M. frigida* and *M. gelida*, respectively.

The genus *Sterigmatomyces* was introduced by Fell (5) for nonfilamentous, yeast-like fungi that are characterized by a unique method of cell division. The yeast cells produce one or more sterigmata, each of which gives rise to a single conidium. The conidium is disjointed at a septum in the middle of the sterigma. New conidia propagate in a similar way. In contrast

to *Mrakia* species, sexuality has not been reported in *Sterigmatomyces* species.

The mode of conidium separation, the ubiquinone system, and the presence or absence of xylose in yeast cell walls have been shown to be useful characteristics for defining different genera of sterigma-producing yeasts. Ubiquinone Q-9 was found in all *Sterigmatomyces* species whose conidia separate by cleavage of sterigmata at the midpoints (40, 46). On the basis of the presence of xylose in the cell walls the following two genera were proposed: *Sterigmatomyces* (xylose negative) and *Tsuchiyaea* (xylose positive) (49). *Sterigmatomyces* species that have conidia which separate at the distal ends of sterigmata and contain ubiquinone Q-10 have been transferred to the genera *Fellomyces* (xylose positive) and *Kurtzmanomyces* (xylose negative) (47, 49). *Sterigmatosporidium polymorphum* (17) is the only teleomorphic species that corresponds to an anamorphic *Fellomyces* (20) counterpart.

The proper generic assignment of another *Sterigmatomyces* species, *Sterigmatomyces aphidis*, remains a problem. There is considerable morphological similarity among *Sterigmatomyces aphidis* (15), *Trichosporon oryzae* (16), and *Sporobolomyces antarcticus* (11). Phenotypically, these three taxa are not like members of the genera *Sterigmatomyces* and *Fellomyces*. DNA comparisons (20) revealed that these three taxa are conspecific, although the reduced level of complementarity exhibited by *Sterigmatomyces aphidis* suggested that this organism is near the limit of relatedness exhibited by members of a species (21).

Species of the genera *Mrakia* and *Sterigmatomyces* are recognized as basidiomycetes because of the lamellar nature of their cell walls as determined by electron microscopy (18) and because of their positive color reactions with diazonium blue B (1). The distant phylogenetic relationship between these two genera (12, 13, 30, 32, 48, 50) and the availability of molecular information (e.g., nuclear DNA [nDNA]-nDNA reassociation data for *Sterigmatomyces* strains and electropherograms for *Mrakia* strains) make *Mrakia* and *Sterigmatomyces* species

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useful for evaluating the significance of random amplified polymorphic DNA (RAPD) analysis for species identification and the applicability of this technique for species delimitation. This rather simple and highly specific method, which was recently introduced independently by two groups of workers (43, 45), is very useful for molecular identification of plant-parasitic fungi (36).

On the basis of qualitative and quantitative yeast cell wall monosaccharide patterns and the ubiquinone isoprenologs, we tried to determine the proper genus for *Sterigmatomyces aphidis*.

MATERIALS AND METHODS

Yeast strains. The strains which we studied are listed in Table 1. All of these strains are maintained in the culture collection of the Institute of Applied Microbiology, Vienna, Austria.

Yeast cell wall analysis. Organisms were cultivated and yeast cell walls were purified as described by Prillinger et al. (33). To disintegrate yeast cells, we used a Vibrogen Cell Mill (Tübingen, Germany) and 0.5-mm-diameter glass beads (yeast pellet, distilled water, and glass beads, 1:1:3, wt/wt). The disintegration time used depended on the strain and was commonly between 20 and 30 min for a level of breakage of 90 to 95% (as determined by light microscopy). A 2-mg portion of powdered cell walls was suspended in 0.5 ml of 2 M trifluoroacetic acid, overlaid with gaseous nitrogen, and hydrolyzed for 4 h at 100°C. The sediment was separated by centrifugation at $4,400 \times g$ for 10 min, and 5 μ l of the supernatant containing 2 μ g of *myo*-inositol (as an internal standard) was used for further investigation. The trifluoroacetic acid was removed by evaporation in a water bath at 35°C under a stream of gaseous nitrogen. After two additions of 200 μ l of methanol, the blow-off procedure was repeated.

Alditol acetates were prepared in two steps (35). During the first step the liberated monosaccharides were reduced by adding 70 μ l of 1 M ammonia and 70 μ l of 4% NaBH₄. The reaction mixture was incubated overnight at room temperature. The remaining sodium borohydride was decomposed by two additions of 50 μ l of 2 M acetic acid, two additions of 200 μ l of 1% acetic acid in methanol, and two additions of 200 μ l of pure methanol. Each addition was followed by evaporation to dryness under a stream of nitrogen. The second step, acetylation of the alditol derivatives, was performed as described by Dörfler (4), with minor modifications (we used 100 μ l of acetic acid anhydride instead of 400 μ g; the preparation was incubated for 1 h at 100°C and was extracted; 500 μ l of dichloromethane was used to dissolve nitrogen-dried residues; and 1 ml of distilled water was used for extraction). The dried residues were dissolved in 50 μ l of dichloromethane immediately before a gas-liquid chromatography-mass spectrometry analysis was performed, in which 0.5 to 2 μ l was used routinely.

Gas-liquid chromatography was carried out with an Erba Instruments model HRGC 5300 Mega Unit gas chromatograph equipped with a Finnigan MAT ion trap detector which was operated in the electron impact mode. Samples were injected (split, 1:5) into a type DB 225 capillary column (30 m by 0.25 mm; film thickness, 0.15 μ m; J & W Scientific). The oven temperature was programmed to increase from 160 to 200°C at a rate of 20°C/min and then to 240°C at a rate of 3°C/min. Helium was used as the carrier gas at a pressure of 1.4×10^5 Pa. Ion masses between 41 and 240 were monitored. Parallel preparations were checked with a Hewlett-Packard model 5890 Series II gas chromatograph equipped with a hydrogen flame ionization detector and a type Rtx-2330 cap-

illary column (60 m by 0.25 mm; film thickness, 0.1 μ m; Restek Corp. Bellefonte, Pa.). The oven temperature was programmed to increase from 160 to 200°C at a rate of 20°C/min and then to 270°C at a rate of 3°C/min. Nitrogen was used as the carrier gas at a pressure of 1.6×10^5 Pa.

Ubiquinone analysis. A ubiquinone analysis was performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen method of Tindall (38), with some modifications (we used 500 mg of freeze-dried yeast cells grown in glucose-peptone-yeast extract medium to the early stationary phase; the preparation was stirred for 60 min in the dark with 4.5 ml of hexane-methanol [1:2] and was extracted with 1.5 ml of ice-cold hexane; and silica gel plates were developed in benzene). For the high-performance 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). The column was a Hypersil 100*2.1mm octyldecyl silane column. The solvent was HPLC grade acetonitrile (E. Merck). The standards used were Q-9 and Q-10 obtained from Sigma-Aldrich, Vienna, Austria. The instrument conditions were as follows: isocratic system at a flow rate of 0.4 ml/min; oven temperature 70°C; and injection volume, 2.5 μ l. UV detection was at 275 nm (band width 2 nm), and no reference wavelength was used. Spectra were determined for entire peaks to determine peak purity.

Denaturing protein electrophoresis. Samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (23) in a precast NOVEX gradient gel (8 to 16% acrylamide; Novel Experimental Technology, San Diego, Calif.) under the conditions described in the manufacturer's instructions. The molecular weight standard used was prestained SDS-PAGE standard-low range (Bio-Rad Laboratories, Richmond, Calif.). Protein samples were prepared by heating 5 mg of lyophilized yeast cell wall powder (33) at 97°C for 10 min in SDS buffer (100 μ l of 1 M Tris-HCl buffer [pH 6.8]), 200 μ l of 100% glycerol, 200 μ l of 10% SDS, 20 μ l of mercaptoethanol, 100 μ l of distilled water). Electropherograms were developed for 180 min by using settings of 125 V and 30 mA.

DNA extraction. Chromosomal DNA was extracted rapidly by a modified hexadecyltrimethylammonium bromide method (24). A 50-mg portion of wet matter was harvested from solid medium and suspended in 0.4 ml of hexadecyltrimethylammonium bromide buffer (2% [wt/vol] hexadecyltrimethylammonium bromide, 0.1 M Tris-HCl [pH 8.0], 1.2 M NaCl, 20 mM EDTA, 5 mM dithiothreitol) in a 1.5-ml Eppendorf cup. The suspension was frozen at -20°C and thawed three times and then incubated at 65°C for 30 min. The suspension was vortexed for 1 min after 0.4 ml of chloroform was added. The resulting mixture was centrifuged at $15,500 \times g$ for 20 min, and 0.3 ml of the clear upper phase was precipitated with 0.45 ml of propane-2-ol. The precipitate formed during 30 min at 0°C was collected by centrifugation at $15,500 \times g$ for 15 min, and the pellet was washed exhaustively with 1 ml of 70% (vol/vol) ethanol at room temperature. The pellet was dried under a vacuum and dissolved in 50 μ l of TE buffer. A 20- μ l portion of each preparation was checked for purity and RNA content, and concentrations were estimated by electrophoresis. Preparations chosen for RAPD analysis were diluted with TE buffer to a concentration of approximately 5 ng/ μ l compared with a control consisting of lambda DNA and were stored at -20°C.

RAPD analysis. A 3- μ l portion of a diluted DNA preparation containing 10 to 20 ng of DNA was subjected to RAPD amplification in 50 μ l (final aqueous volume) of a solution containing 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris (pH

8.8), 4.5 mM MgSO₄, 0.1% (wt/vol) Triton X-100, each nucleotide triphosphate (Promega, Madison, Wis.) at a concentration of 0.2 mM, 30 ng of primer, 0.2 µg of bovine serum albumin fraction V per ml, and 0.8 U of *Taq* DNA polymerase (homologous protein; supplied by Biomedica, Vienna, Austria). The mixture was overlaid with 50 µl of light mineral oil (Sigma Chemie GmbH, Diesenhofen, Germany) and processed with a Trio-Thermoblock TB1 thermocycler (Biometra, Göttingen, Germany). The analysis program used was as follows: denaturation at 98°C for 15 s, annealing at 50°C for 60 s, and extension at 72°C for 100 s. A total of 40 cycles were used for 15-mer and 16-mer primers. For decamer primers annealing was performed at 32°C for 90 s, and the number of cycles was reduced to 35. Primers were synthesized by Codon Genetic Systems (Vienna, Austria) with a model 392 DNA synthesizer (Applied Biosystems, Foster City, Calif.). The reactions were stopped by adding EDTA to a concentration of 10 mM, and 20 µl of each mixture was analyzed in a 1.3% (wt/vol) agarose gel in 0.5× TBE buffer supplemented with 0.5 µg of ethidium bromide per ml at 4°C with electrophoresis at 5 V/cm. DNA fragments were visualized by transillumination and were photographed with a Polaroid system. The levels of similarity between individual lanes were calculated as described by Nei and Li (29).

PCR fingerprinting. Ribosomal DNA (rDNA) sequences were amplified either with primers ITS1 and ITS4 (44) (yielding both internal transcribed spacers and the 5.8S rDNA gene) or with primers GGTAGGAATACCCGCTG and GGCTTAATCTCAGCAGATCG (yielding the entire 26S rDNA gene).

To amplify the 3-kb fragment containing the 26S rDNA, we used the proofreading, more thermostable VENT DNA polymerase (NEB, Beverly, Md.). The conditions used were the same as those used for the RAPD PCR (see above). A 20-ng portion of each primer and 0.5 U of VENT DNA polymerase per vial were used. To avoid nonspecific products, the PCR program had to be altered according to the manufacturer's guidelines as follows: 98°C for 15 s, 60°C for 30 s, and 72°C for 5 min. A total of 35 cycles were used. The approximately 0.6-kb fragment between primers ITS1 and ITS4 was amplified by using 2 U of *Taq* DNA polymerase per vial, the MgSO₄ concentration was reduced to 2 mM, and 10 ng of each primer was included. The PCR program was as follows: 98°C for 15 s, 55°C for 1 min, and 72°C for 2 min. A total of 40 cycles were used. A portion (10%) of each PCR fragment was checked for amount and purity by standard agarose electrophoresis, and the rest was precipitated with 2 volumes of ethanol. The pellets were washed in 70% ethanol, dried, and digested with *Hinf*I, *Hae*III, *Taq*I, *Alu*I, or *Msp*I. After digestion at 37°C overnight, the restriction fragments were subjected to agarose electrophoresis. The conditions used to separate the fragments from the 26S rDNA were the same as those described above for the RAPD analysis. The fragments from the region of the internal transcribed spacers were separated with the same buffer system, except that 2% agarose (Seakem NuSieve GTG) was used.

RESULTS AND DISCUSSION

Tables 1 and 2 show the phylogenetic relationships of members of the genera of basidiomycetous yeasts which we investigated, based on the qualitative and quantitative monosaccharide patterns of purified yeast cell walls and some additional characteristics. The high levels of glucose and the presence of xylose in yeast cell wall hydrolysates of members of the genera *Fellomyces*, *Mrakia*, *Kockovaella*, *Sterigmatosporidium*, and *Tsuchiyea* suggested that these organisms are

related to the tremelloid fungi (*Tremella* type) (32, 33). The presence of simple holobasidia (32), the ability to ferment carbohydrates (Table 1) (1), the presence of ubiquinone Q-8, and the presence of fucose in yeast cell walls (Table 2) (33) suggested that *Mrakia* species occupy a rather primitive and distinct position within the *Tremella* type; the results of partial sequence determinations of 18S and 26S rRNAs have corroborated this interpretation (6, 9, 12, 48, 50).

Whereas the genera *Fellomyces*, *Kockovaella*, *Sterigmatosporidium*, and *Tsuchiyea* form a homogeneous cluster within the *Tremella* type (Table 2) (9, 28), *Sterigmatomyces* and *Kurtzmanomyces* species appear to be only distantly related to this group (53). On the basis of qualitative and quantitative cell wall neutral sugar data, the genera *Kurtzmanomyces* and *Sterigmatomyces* occupy a distinct position within the *Microbotryum* type (Table 2) (31, 33). An absence of fucose, which is often characteristic of representatives of the *Microbotryum* type indicates that there may be different phylogenetic lineages within this yeast type (31, 33). Although we were originally inclined to place *Sterigmatomyces halophilus* in the *Ustilago* type (30) on the basis of balanced amounts of glucose and mannose, the results obtained for another species in this study did not support this interpretation. The high levels of mannose in *Sterigmatomyces elviae* and both *Kurtzmanomyces* species are consistent with placement of these organisms in the *Microbotryum* type, which has been considered ancestral to the *Ustilago* and *Tremella* types (30, 33). The cell wall carbohydrate compositions of *Mrakia* species may indicate that there is an relationship between the *Microbotryum* and *Tremella* types, and the data for *Sterigmatomyces halophilus* and *Sterigmatomyces indicus* (Table 2) suggest that there is a similar relationship between the *Microbotryum* and *Ustilago* types.

The cell wall monosaccharides of *Sterigmatomyces aphidis* CBS 6821^T (T = type strain) and HB 54 and *Sporobolomyces antarcticus* CBS 5955^T exhibit the characteristic *Ustilago* type pattern; namely, there are high levels of glucose and low levels of mannose, galactose is present, and no additional differentiating monosaccharides such as rhamnose, fucose, and xylose, are present (30). A positive diazonium blue B test (Table 1) eliminates the possibility that these organisms are related to ascomycetous yeasts and yeast stages containing galactose in their yeast cell walls (e.g., *Schizosaccharomyces* species) (33). In their quantitative glucose/mannose ratios and proportions of galactose, respectively, *Sporobolomyces antarcticus* and *Sterigmatomyces aphidis* strains resemble some *Exobasidium* species (e.g., *Exobasidium symploci-japonicae*) (30), *Ustilago* species (e.g., *Ustilago grandis*) (30), and *Tilletiopsis* species (30). The presence of ubiquinone Q-10 (Table 2) supports this interpretation. On the other hand, different *Sporobolomyces* species and *Rhodotorula* species exhibit a yeast cell wall monosaccharide pattern which is similar to the pattern exhibited by smut fungi of dicotyledons (31). The 5S rRNA sequence comparisons of Walker and Doolittle (42) and Blanz and Gottschalk (2) revealed a similar relationship. Both of these investigations placed *Sporobolomyces antarcticus* in a group that includes *Tilletia controversa* and *Exobasidium vaccinii*. The possibility that these organisms are phylogenetically related to the type species of the genus *Vanrija*, *Vanrija humicola* (Daszewska) Moore (25), can be eliminated. *Vanrija* (*Cryptococcus*) *humicola* contains significant levels of xylose in its cell walls (32). Partial large-subunit rRNA sequences (9, 12, 54) revealed that *V. humicola* is distinct from *Cryptococcus neoformans*, the neotype species of the genus *Cryptococcus*. However, *V. humicola* cannot be excluded from the genus *Trichosporon*. Nakase et al. (26) rejected the genus *Vanrija* because it apparently includes various basidiomycetous ana-

TABLE 1. Strains studied, their habitats, and selected characteristics that have phylogenetic importance (as determined by standard yeast characterization tests)

Species	Strain ^a		Habitat	Fermentation of glucose	Hydrolysis by urease	Formation of EAC ^b	Diazonium blue B test
	IAM no.	Other designation					
<i>Kurtzmanomyces nectairei</i> (Rodrigues de Miranda) Yamada et al. (1988)	HB 106 ^T	CBS 6405 ^T	Cheese	—	+	—	+
<i>Kurtzmanomyces tardus</i> Giménez-Jurado et van Uden (1990) ^c	HB 268 ^T	CBS 7421 ^T	Distilled water	—	+	—	+
<i>Sterigmatomyces elviae</i> Sonck et Yarrow (1969)	HB 104 ^T	CBS 5922 ^T	Man with seborrheic eczema (groin)	—	+	—	+
<i>Sterigmatomyces halophilus</i> Fell (1966)	HB 100 ^T	CBS 4609 ^T	Air	—	+	—	+
<i>Sterigmatomyces indicus</i> (Fell) Fell (1970)	HB 70 ^T	CBS 5628 ^T	Indian Ocean	—	+	—	+
<i>Sterigmatomyces aphidis</i> Henninger et Windisch (1975)	HB 54		Aphid secretion on leaves of <i>Solanum pseudocapsicum</i>	—	+	—	+
<i>Sporobolomyces antarcticus</i> Goto et al. (1969)	HB 55 ^T HB 56 ^T	CBS 6821 ^T CBS 5955 ^T	Lake Vanda (sediment)	— —	++ +	— —	++ +
<i>Mrakia gelida</i> (Fell et al.) Yamada et Komagata (1987)	HB 76 ^T	JCM 7858 ^T	Antarctic soil	+	+	+	+
	HB 101 ^T	DSM 70882 ^T		+	+	+	+
<i>Mrakia stokesii</i> (Fell et al.) Yamada et Komagata (1987)	HB 77 ^T	CBS 5917 ^T	Antarctic snow	+	+	+	+
<i>Mrakia frigida</i> (Fell et al.) Yamada et Komagata (1987)	HB 103 ^T	CBS 5270 ^T	Antarctic soil	+	+	+	+
<i>Mrakia nivalis</i> (Fell et al.) Yamada et Komagata (1987)	HB 102 ^T	CBS 5266 ^T	Antarctic soil	+	+	+	+
<i>Fellomyces fuzhouensis</i> (Yue) Yamada et Banno (1988)	HB 94	CBS 6133	Mango flower	—	+	+	+
<i>Fellomyces penicillatus</i> (Rodrigues de Miranda) Yamada et Banno (1984)	HB 95 ^T HB 98 ^T	CBS 8243 ^T CBS 5492 ^T	Mango flower Contaminant on agar plate	— —	++ +	++ +	++ +
	HB 99 ^T	CBS 5492 ^T (S-form)		—	+	+	+
<i>Fellomyces polyborus</i> (Scott et van der Walt) Yamada et Banno (1984)	HB 96 ^T	CBS 6072 ^T	Tunnels of <i>Xyleborus torquatus</i> in <i>Cussonia umbellifera</i> Ambregris	—	+	+	+
	HB 97	CBS 6643		—	+	+	+
<i>Kockovaella imperatae</i> Nakase et al. (1991)	HB 58 ^T	JCM 7826 ^T	On leaves of <i>Imperata cylindrica</i>	—	+	—	+
<i>Kockovaella thailandica</i> Nakase et al. (1991)	HB 63 ^T	JCM 7824 ^T	On leaves of <i>Imperata cylindrica</i>	—	+	—	+
<i>Tsuchiyaea wingfieldii</i> (van der Walt et al.) Yamada et al. (1988)	HB 93 ^T	CBS 7118 ^T	Frass of a scolytid beetle on olive plant (<i>Olea europaea</i>)	—	+	+	+
<i>Sterigmatosporidium polymorphum</i> Kraepelin et Schulze (1982)	RBF 977	CBS 8089	Wet log in a mine	—	+	+	+
	RBF 978	CBS 8090	Wet log in a mine	—	+	+	+

^a Strains were obtained from the Centraalbureau voor Schimmelcultures (CBS), the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), the Japan Collection of Microorganisms (JCM), the Raiffeisen Bioforschung (RBF), and the collection of the Institute of Applied Microbiology in Vienna (IAM).

^b EAC, extracellular, amyloid compounds (starch test).

^c Reference 10.

morphic yeasts that supposedly have diverse phylogenetic origins.

The yeast cell wall monosaccharide compositions and the ubiquinone spectra which we determined suggest that *Sporobolomyces antarcticus* and *Sterigmatomyces aphidis* should be placed in the genus *Tilletiopsis* until the proper teleomorphic genus is found. Although ballistoconidia are not present in *Sterigmatomyces aphidis*, there can be no doubt that these two species are closely related. On the basis of DNA relatedness

data Kurtzman (20) postulated that these organisms are conspecific. Our RAPD fingerprinting results support the view that these fungi are genetically distinct (Fig. 1 through 4), although they belong to the same genus. Using partial nucleotide sequencing, Nakase et al. (28) demonstrated convincingly that the mode of vegetative reproduction, such as production of ballistospores, stalked conidia, and budding yeast cells, does not reflect phylogenetic relationships among basidiomycetous yeasts and is a less significant taxonomic criterion than previ-

Species	Strain	D-Glucose	D-Mannose	D-Galactose	DL-Xylose	L-Fucose
<i>Microbotryum</i> type						
<i>Kurtzmanomyces nectairei</i>	HB 106 ^T	11	89	tr ^c		10
<i>Kurtzmanomyces tardus</i>	HB 268 ^T	20	80	tr		10
<i>Sterigmatomyces elviae</i>	HB 104 ^T	19	80	1		9
<i>Sterigmatomyces halophilus</i>	HB 100 ^T	50	50	tr		9
<i>Sterigmatomyces indicus</i>	HB 70 ^T	49	48	2		9
<i>Ustilago</i> type						
<i>Sterigmatomyces aphidis</i>	HB 55 ^T	67	10	23		10
	HB 54	62	9	29		10
<i>Sporobolomyces antarcticus</i>	HB 56 ^T	77	7	16		10
<i>Tremella</i> type						
<i>Mrakia gelida</i>	HB 76 ^T	76	11	1	9	4
<i>Mrakia stokesii</i>	HB 77 ^T	89	5		4	2
<i>Mrakia frigida</i>	HB 103 ^T	88	6		4	2
<i>Mrakia nivalis</i>	HB 102 ^T	84	7		6	2
<i>Fellomyces fuzhouensis</i>	HB 94	81	9	1	9	
	HB 95 ^T	77	9		14	
<i>Fellomyces penicillatus</i>	HB 98 ^T	79	11		10	
	HB 99 ^T	80	11		9	
<i>Fellomyces polyborus</i>	HB 96 ^T	70	11		19	
	HB 97	80	10		10	
<i>Kockovaella imperatae</i>	HB 58 ^T	78	10		12	
<i>Kockovaella thailandica</i>	HB 63 ^T	78	12		10	
<i>Tsuchiyaea wingfieldii</i>	HB 93 ^T	83	8	1	8	
<i>Sterigmatosporidium polymorphum</i>	RBF 977	76	14	2	8	
	RBF 978	77	14	2	7	

^a None of the strains contained L-rhamnose.

^b Data from references 46 and 50 and this study.

^c tr, trace (less than 1%).

ously believed. The qualitative and quantitative yeast cell wall monosaccharide data shown in Table 2 support the conclusions of Nakase et al. Stalked conidia, as well as ballistospores, are found in at least three phylogenetically distinct yeast types (the *Microbotryum* type, including *Kurtzmanomyces*, *Sterigmatomyces*, *Sporobolomyces*, and *Sporidiobolus* species [31]; the *Ustilago* type, including *Ustilago* and *Tilletiopsis* species [30]; and the *Tremella* type, including *Fellomyces*, *Tsuchiyaea*, *Sterigmatosporidium*, *Kockovaella*, and *Bullera* species [32, 33]).

Sterigmatomyces aphidis HB 54 and HB 55^T, *Sterigmatomyces elviae* HB 104^T, *Sterigmatomyces halophilus* HB 100^T, *Sterigmatomyces indicus* HB 70^T, *Sporobolomyces antarcticus* HB 56^T, *M. gelida* HB 76^T (= HB 101^T) (type strains obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen and the Japan Collection of Microorganisms, respectively), *M. frigida* HB 103^T, *M. stokesii* HB 77^T, and *M. nivalis* HB 102^T, were assessed for species delimitation by performing a RAPD PCR analysis. Previously, this method has been useful for identifying and differentiating microorganisms (34, 36), and we were interested in comparing the resolving power of RAPD analysis with the resolving power of nDNA reassociation experiments (20) and with the resolving power of enzyme and protein electrophoresis (39, 52). The PCR fragment patterns generated by four different arbitrary primers were studied. The results obtained for the different *Sterigmatomyces* and *Mrakia* species illustrate the resolving power of the RAPD method (Fig. 1 through 4). When the distantly related *Mrakia* and *Sterigmatomyces* species were compared with each other, the background noise inherent in RAPD analysis became apparent.

Rapid DNA extraction without digestion or removal of

RNA yields the template for RAPD analysis, which is routinely used to determine the concentration of chromosomal DNA and the DNA-to-RNA ratio on the basis of the intensities of ethidium bromide-stained bands (Fig. 5). The presence of plasmid DNA in the preparations of two *M. gelida* type strains (Fig. 5, lanes 3 and 4) obtained from different culture collections was evident at five dominant positions at relative mobilities corresponding to fragment lengths of 5 to 0.5 kb. *M. stokesii* (lane 5) did not harbor any plasmid DNA, although conspecificity was proposed previously on the basis of the results of isoenzyme comparisons (52).

All *Mrakia* and *Sterigmatomyces* strains were processed simultaneously, and the PCR products were separated on agarose gels. Since the amplification reaction for RAPD analysis cannot be completely standardized, the absolute numbers and sizes of the fragments formed are by themselves not significant markers for strain differentiation. Therefore, all of the strains that are to be compared must be processed with one stock solution of premixed reagents in one run of the thermocycler used and loaded onto one gel for maximum information output.

Figures 1 through 4 show the results of RAPD analyses when we used primers M13 (GAGGGTGGCGGTTCT) and (GACA)₄ (24) and primers ACGGTCTTGG (36) and TGC CGAGCTG (3). To calculate levels of DNA relatedness among species, bands were checked for reproducibility in parallel experiments. Fragments that had unique sizes (e.g., the fragment in Fig. 4, lane 10, indicated by an arrow) were not considered characteristics that represent differences, even though they were reproducible. While the procedure was kept the same during replicate analyses, very short or very long

FIG. 1. RAPD patterns of *Sterigmatomyces* and *Mrakia* strains primed by M13 consensus sequence GAGGGTGGCGGTTCT. Lane 1, *Sterigmatomyces aphidis* HB 54; lane 2, *Sterigmatomyces aphidis* HB 55^T; lane 3, *Sporobolomyces antarcticus* HB 56^T; lane 4, *Sterigmatomyces indicus* HB 70^T; lane 5, *Sterigmatomyces halophilus* HB 100^T; lane 6, *Sterigmatomyces elviae* HB 104^T; lane 7, *M. frigida* HB 103^T; lane 8, *M. nivalis* HB 102^T; lanes 9 and 10, *M. gelida* HB 76^T and HB 101^T, respectively; lane 11, *M. stokesii* HB 77^T. Lane 12 contained lambda DNA digested with *Pst*I. Note the similarity in the patterns of *Sterigmatomyces halophilus* and *Sterigmatomyces indicus* (lanes 4 and 5), *M. frigida* and *M. nivalis* (lanes 7 and 8), and *M. gelida* and *M. stokesii* (lanes 9 through 11).

fragments that resulted from minor variations in the reaction mixtures could be identified in individual experiments. Therefore, the 2-kb fragment amplified from *M. gelida* HB 101^T by TGCCGAGCTG may not distinguish this strain from *M. stokesii* HB 77^T (Fig. 4). The total number of bands used for similarity calculations depended on the changes in new template-primer combinations. The number of fragments obtained from *M. nivalis* with primer (GACA)₄ (Fig. 2, lane 8) did not meet our expectations. In contrast to the other primers used, only a single band was observed reproducibly in this analysis. Reisolation of template DNA also did not result in any additional PCR products.

Levels of similarity were calculated individually for each primer, and the results were combined in equal proportions (Table 3). The level of background noise was determined from the similarity values obtained for different *Mrakia* and *Sterigmatomyces* species. The average range was about 14%, and no range was greater than 19%. The lowest similarity values which could be considered significant were obtained when we compared *Sporobolomyces antarcticus* HB 56^T with *Sterigmatomyces aphidis* HB 54 (28%) and HB 55^T (33%). The values for all other pairs of strains belonging to different species in the *Sterigmatomyces* cluster (except *Sterigmatomyces indicus*) were at the level of background noise. The conspecificity of *Sterigmatomyces halophilus* HB 100^T and *Sterigmatomyces indicus* HB 70^T, as shown previously by nDNA-nDNA reassociation

FIG. 2. RAPD patterns of *Sterigmatomyces* and *Mrakia* strains primed by (GACA)₄. For the contents of lanes 1 through 11 see the legend to Fig. 1. Compare the patterns in lanes 4 and 5, lanes 7 and 8, and lanes 9 through 11 with the patterns shown in Fig. 1. Lane 12 contained lambda DNA digested with *Pst*I.

(level of DNA complementarity, 98%) (20), was confirmed by the results of the RAPD analysis performed in this study.

Kurtzman demonstrated that utilization of nitrate as a sole source of nitrogen, which has been used to separate *Sterigmato-*

FIG. 3. RAPD patterns of *Sterigmatomyces* and *Mrakia* strains primed by ACGGTCTTGG. For the contents of lanes 1 through 11 see the legend to Fig. 1. Lane 12 contained lambda DNA digested with *Pst*I.

FIG. 4. RAPD patterns of *Sterigmatomyces* and *Mrakia* strains primed by TGCCGAGCTG. For the contents of lanes 1 through 11 see the legend to Fig. 1. Lane 12 contained lambda DNA digested with *Pst*I. The arrow indicates a fragment that had a unique size and was not considered in strain comparisons (see text).

myces halophilus and *Sterigmatomyces indicus*, should not be considered a characteristic that has taxonomic importance (20). Figures 1 through 4 (lanes 4 and 5) show that the overall level of nDNA similarity, as indicated by a common DNA band pattern in RAPD analyses, is about 90% (Table 3). In contrast to the nDNA-nDNA reassociation value of 98% (20), we obtained greater resolution with the RAPD analysis, which can resolve close relationships with a good level of accuracy. Furthermore, a single RAPD comparison never results in a similarity value of more than 100%, in contrast to the results obtained by reassociation methods (27). The visibility of the

FIG. 5. Test for the quality and concentration of nDNA and the presence of plasmids in different *Mrakia* species. We performed agarose gel electrophoresis with extracted whole-cell DNAs from *M. frigida* HB 103^T (lane 1), *M. nivalis* HB 102^T (lane 2), *M. gelida* HB 76^T (lane 3), *M. gelida* HB 101^T (lane 4), and *M. stokesii* HB 77^T (lane 5); 20 µl of extract was used per lane. Lane 6 contained 50 ng of lambda DNA, and lane 7 contained lambda DNA after digestion with *Pst*I. Note the presence of a plasmid in *M. gelida* (arrows).

results compared further increases our confidence in the method.

Our results also show that the two strains of *Sterigmatomyces aphidis* studied, HB 54 and HB 55^T, are conspecific (level of similarity, 94%) (Fig. 1 through 4, lanes 1 and 2) and that the type strains of *M. gelida* obtained from two different culture collections, strains HB 76^T and HB 101^T, are conspecific (level of similarity, 100%) (Fig. 1 through 4, lanes 9 and 10). Within the *Mrakia* cluster, the high levels of similarity between *M. nivalis* and *M. frigida* (70%) (Fig. 1 through 4, lanes 7 and 8) and between *M. gelida* and *M. stokesii* (85%) (Fig. 1 through 4, lanes 9 through 11) are striking. A classification based solely on the RAPD data supports the conclusions that *M. stokesii* and *M. gelida* are conspecific and that *M. nivalis* with *M. frigida* are

TABLE 3. Levels of similarity of the RAPD patterns, as determined with a 1.3% agarose gel

Strain	% Similarity with strain ^a :										
	HB 54 ^T	HB 55	HB 56 ^T	HB 70 ^T	HB 100 ^T	HB 104 ^T	HB 103 ^T	HB 102 ^T	HB 76 ^T	HB 101 ^T	HB 77 ^T
<i>Sterigmatomyces aphidis</i> HB 54	100	94	28	10	20	10	17	12	ND ^b	ND	ND
<i>Sterigmatomyces aphidis</i> HB 55 ^T		100	33	10	22	20	17	12	ND	ND	ND
<i>Sporobolomyces antarcticus</i> HB 56 ^T			100	20	20	16	6	2	ND	ND	ND
<i>Sterigmatomyces indicus</i> HB 70 ^T				100	90	12	13	8	14	12	8
<i>Sterigmatomyces halophilus</i> HB 100 ^T					100	14	13	6	16	17	14
<i>Sterigmatomyces elviae</i> HB 104 ^T						100	19	13	12	12	11
<i>M. frigida</i> HB 103 ^T							100	70	12	12	5
<i>M. nivalis</i> HB 102 ^T								100	14	14	10
<i>M. gelida</i> HB 76 ^T									100	100	85
<i>M. gelida</i> HB 101 ^T										100	85
<i>M. stokesii</i> HB 77 ^T											100

^a Levels of similarity were calculated by the method of Nei and Li (29).

^b ND, not determined.

FIG. 6. PCR fingerprinting of whole 26S rDNAs amplified from the nDNAs of *Mrakia* species. Lanes 1 through 5, DNAs digested with *Hinf*I; lanes 7 through 11, DNAs digested with *Hae*III; lane 6, lambda DNA digested with *Pst*I. Lanes 1 and 7, *M. frigida*; lanes 2 and 8, *M. nivalis*; lanes 3, 4, 9, and 10, *M. gelida*; lanes 5 and 11, and *M. stokesii*.

conspecific because the levels of similarity (70 and 85%) are consistent with the levels of variability observed within a single species. A global investigation of the genus *Trichoderma* by RAPD analysis resulted in levels of similarity in this range for the strains of one species which were isolated in one geographical area (19). An antarctic habitat and low cultivation temperatures (15°C) are common to all *Mrakia* species (7).

A fifth primer, (GTG)₅, yielded results that were partially consistent with the results obtained with the primers used in the experiments shown in Fig. 1 through 4 (data not shown). Heavy development of the background smear and the smaller average size of the fragments formed made it possible to identify only a few bands for similarity calculations. The huge number of small bands generated were not sufficiently resolved by electrophoresis. Therefore, the data obtained with this primer were not included in Table 3.

Our RAPD analysis data corroborate the data obtained from an electrophoretic comparison of enzymes performed by Yamada and Matsumoto (52). On the basis of identical electrophoretic mobilities of glucose-6-phosphate dehydrogenase, malate dehydrogenase, and fumarase in *M. frigida* and *M. nivalis* and in *M. gelida* and *M. stokesii*, only *M. frigida* and *M. gelida* were placed in the genus *Mrakia*. Because the descriptions of *M. frigida* and *M. gelida* were published before the descriptions of *M. nivalis* and *M. stokesii* (7, 51), *M. nivalis* and *M. stokesii* are synonyms of *M. frigida* and *M. gelida*, respectively.

FIG. 7. SDS-PAGE of the cell wall proteins of four *Mrakia* species. Lane 2, *M. frigida* HB 103^T; lane 3, *M. nivalis* HB 102^T; lanes 4 and 6, *M. gelida* HB 101^T; lane 5, *M. stokesii* HB 77^T. Protein bands which differentiate *M. frigida* and *M. nivalis* from *M. gelida* and *M. stokesii* are indicated by arrows (see text).

A comparison of the results of the RAPD PCR method with the results of rDNA restriction fragment analysis ("PCR fingerprinting") (Fig. 6) (22, 41) and electropherograms of yeast cell wall proteins (Fig. 7) (4), two methods previously used for species identification in our laboratory, revealed that RAPD analysis is superior to the other two methods. The PCR fingerprinting analysis revealed no differences among the four *Mrakia* species. When amplified 26S rDNA from a *Mrakia* species was digested with *Hinf*I and *Hae*III, we obtained 10 and 8 fragments, respectively (Fig. 6). The more variable region, which included both internal transcribed spacers, also did not reveal any differences among the *Mrakia* species, although five different enzymes were used. Digestion with *Hinf*I, *Hae*III, *Taq*I, *Alu*I, and *Msp*I resulted in three, four, four, three, and three fragments, respectively (data not shown). These results contrast with the results obtained by the RAPD analyses, which completely differentiated all species except the *M. frigida*-*M. nivalis* and *M. stokesii*-*M. gelida* pairs.

Although analysis of yeast cell wall proteins was superior to whole-cell protein electrophoresis for the identification of *Taphrina* and *Exobasidium* species (4), this method often has the disadvantage that interpretation and assignment of thin or weak bands in a high-band-density background are ambiguous (Fig. 7). In Fig. 7 we indicate bands which may further differentiate the *M. frigida*-*M. nivalis* and *M. gelida*-*M. stokesii* pairs. However, these differences are less convincing compared with the results obtained in the RAPD analysis.

In conclusion, we tried to show that morphological characteristics have to be checked very carefully for their usefulness for generic assignment. Stalked conidia occur in members of at least four different genera of basidiomycetes that are not closely related (the genera *Fellomyces*, *Kurtzmanomyces*, *Sterigmatomyces*, and *Tilletiopsis*). In addition, RAPD analysis is a new, simple, and highly sensitive method which differentiates organisms at the nDNA level. This method may replace

nDNA-nDNA hybridization experiments for species identification, characterization, and delimitation.

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