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TRICHOSPORONOIDES MEGACHILIENSIS, A NEW HYPHOMYCETE ASSOCIATED WITH ALFALFA LEAFCUTTER BEES, WITH NOTES ON TRICHOSPORONOIDES AND MONILIELLA

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ABSTRACT

A black yeast-like fungus was found to be commonly associated with provisions, larval gut contents and frass of alfalfa leafcutter bees (*Megachile rotundata*). The formation of dark colonies composed of hyphae developing chains of blastoconidia and cylindrical to barrel-shaped arthroconidia suggested an affinity to *Trichosporonoides* or *Moniliella*. Although lacking the synchronously produced blastoconidial state demonstrated by the type species *Trichosporonoides oedocephalis*, the leafcutter bee fungus has been named *T. megachiliensis* because of its morphological and physiological similarities with *T. madida*, *T. nigrescens* and *T. spathulata*. These species also lack the synchronously produced blastoconidia and are redescribed here. The recently described *T. australiense* was found to differ substantially from existing species in both morphology and physiology and is considered to be synonymous with *Brettanomyces anomalus* (anamorph of *Dekkera anomala*). A key to the species of *Trichosporonoides* and *Moniliella* is provided.

Key Words: Hyphomycetes, *Megachile rotundata*, *Moniliella*, *Trichosporonoides megachiliensis*

During a study to evaluate microorganisms associated with alfalfa leafcutter bees (*Megachile rotundata* Fabricius) and their possible influence on chalkbrood disease (*Ascosphaera aggregata* Skou), a black yeast-like fungus, having dark brown to black cerebriform colonies, was isolated frequently from food provisions, larval gut contents and frass. In producing both blastoconidia and arthroconidia, our fungus demonstrated an affinity to both *Trichosporonoides* Haskins & Spencer and *Moniliella* Stolk & Dakin. Although lacking the synchronously produced blastoconidia found in the type species *Trichosporonoides oedocephalis* Haskins & Spencer (1967), the leafcutter bee fungus resembled three species of *Trichosporonoides* (de Hoog, 1979; Hocking and Pitt, 1981) which also lacked this synanamorph. This report describes and illustrates *T. megachiliensis* Inglis & Sigler *sp. nov.* and briefly redescribes the morphological and physiological

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features of *T. madida* de Hoog, *T. nigrescens* Hocking & Pitt, *T. oedocephalis* and *T. spathulata* de Hoog. The distinctions between *Trichosporonoides* and *Moniliella* are examined and a key to the accepted species is provided. The recently described *T. australiense* Ramirez (1989) is examined and its placement in this genus revaluated.

MATERIALS AND METHODS

Collection and isolation of T. megachiliensis. — An isolate of a black yeast-like fungus was obtained from a sporulating chalkbrood cadaver collected near Tilley, Alberta in 1989. Three hundred and forty two morphologically similar isolates were obtained in 1990 from alfalfa leafcutter bee provisions, larval gut contents and frass. Bee cells were collected from a hive situated in an irrigated field of alfalfa near Lethbridge, Alberta. Provisions, predefecation larvae (instar III), postdefecation larvae (instar IV–V) and frass were removed from the cells and samples were macerated in 0.01 M phosphate buffer amended with 0.01% Tween 80 (pH 7) using a Potter-Elvehjem grinder. Prior to maceration, larvae were surface-sterilized with sodium hy-

pochlorite (1%), followed by two rinses in sterile dis-
tilled water. Homogenates from all treatments were
then spread onto the surface of sorbose yeast-extract
agar (SYE) (Bandoni, **1981**). After incubation for 5 da
at 25 C, individual colonies were transferred onto corn-
meal agar slants, incubated for an additional 10 da and
then stored at 4 C. The 1989 isolate and four other
strains were deposited in the University of Alberta
Microfungus Collection and Herbarium (UAMH) [cul-
ture collection designations follow Takishima et al.
(**1989**)] as: UAMH 6490 from sporulating cadaver,
UAMH 6820 from provisions, UAMH 6821 from frass,
UAMH 6822 (= CBS 191.92) from the gut contents
of a IV instar larva (postdefecation) and UAMH 6823
from the gut contents of a II–III instar larva (prede-
fecation). One isolate, UAMH 7060, was recovered
from the surface of an emerging bee. Twenty-five
emerging adult bees and 10 provisioning adults col-
lected from the field during the summer of 1991 at
Lethbridge were killed in CO_2, placed in phosphate
buffer and agitated at 250 rpm for 1 h on a rotary
shaker. The wash solutions were spread onto SYE and
the cultures incubated at 25 C for 5 da. The bees were
then surface-sterilized as previously described, mac-
erated and the homogenate spread onto SYE. Two ad-
ditional isolates were obtained from other sources:
UAMH 6969 was recovered from leafcutter bee pro-
visions collected in Saskatchewan by W. Goerzen, and
UAMH 6970 was isolated by G. D. Inglis from as-
cospores of *A. aggregata* obtained from chalkbrood
cadavers collected in Idaho. Although their morphol-
ogy was examined, the latter three isolates were not
included in the comparative growth studies.

Isolates studied. — Growth studies and all physiological
tests were conducted on five isolates of *T. megachi-
liensis* (UAMH 6490, 6820, 6821, 6822, 6823), the ex-
type cultures of *T. oedocephalis* (UAMH 3922), *T.
madida* (UAMH 4546 = CBS 240.79), *T. spathulata*
(UAMH 4547 = CBS 241.79) and *T. australiense*
(UAMH 6791 = TISTR 5242). Morphological obser-
vations and selected physiological tests also were con-
ducted on *T. nigrescens* (UAMH 7092 = CBS 269.81),
T. oedocephalis (UAMH 3923), *Moniliella acetoabu-
tens* Stolk & Dakin (UAMH 6850 = CBS 169.66;
UAMH 2498), *M. suaveolens* var. *suaveolens* (Lindner
ex Lindner) von Arx (UAMH 4580 = CBS 126.42) and
M. suaveolens var. *nigra* (Burri & Staub) de Hoog
(UAMH 4570 = CBS 314.31).

Growth studies. — Colonial features were recorded and
growth rates measured at 5 da intervals on Pablum
cereal agar (CER) (Padhye et al., **1973**) and on 50%
(w/v) glucose yeast-extract peptone agar (GYP50) (van
der Walt and Yarrow, **1984**) at 25 C, and on potato
dextrose agar (PDA, Difco) at 25 C and 37 C for 30
da. Color terminology follows Kornerup and Wanscher
(**1978**). Microscopic observations were made at 24 and
48 h of isolates grown in stationary culture in glucose
yeast-extract peptone broth (GYPB) (van der Walt and
Yarrow, **1984**) at 25 C. Morphological characteristics
also were observed on PDA in Dalmau culture (van
der Walt and Yarrow, **1984**) at 12, 24 and 48 h and at
5 d intervals for 30 da, and on CER in slide culture at
15 and 30 da.

Physiological tests. — Isolates were tested for their abil-
ity to grow at low water activities by growing them on
GYP50 and GYP66, and for their tolerance to cyclo-
heximide (400 μg ml^{-1}) and benomyl (2 μg ml^{-1}) by
recording growth rates on mycosel agar (MYC, BBL),
and on benomyl-amended modified Melin-Norkran's
medium (Sigler et al., **1990**), respectively. Growth
without and with vitamins was determined on vitamin-
free Casamino acid agar (CAS) (Georg and Camp, **1957**),
and on CAS amended with thiamine (0.02 μg ml^{-1});
growth was rated as positive only if aerial growth oc-
curred. Hydrolysis of urea to ammonia (urease activity)
was tested using Christensen's urea broth (Kane and
Fischer, **1971**) and the Diazonium Blue B (DBB) color
test was conducted as described by van der Walt and
Yarrow (**1984**). Ability to ferment glucose, galactose,
lactose, maltose, sucrose and trehalose was tested using
the Wickerham procedure (Rippon, **1982**). Tubes were
maintained at 25 C for 28 da. Assimilation profiles
were determined using API 20C (API Laboratory Prod-
ucts Ltd, St. Laurent, Quebec) and AutoMicrobic Sys-
tem (AMS)-Yeast Biochemical Cards (YBC) (Vitek
Systems, Inc., Hazelwood, Missouri). The manufac-
turer's instructions were followed for the API 20C sys-
tem except that strips were maintained at 25 C rather
than 30 C and examined daily for 5 da. Similarly, the
AMS-YBC cards were incubated at lower than rec-
ommended temperature (25 C) and read at 24 h in-
tervals for 4–5 da. All physiological tests were repeated.

DESCRIPTION OF NEW SPECIES

Trichosporonoides megachiliensis Inglis *et* Sig-
ler, *sp. nov.*   FIGS. 1–7

Coloniae moderatim bene crescentes, cerebriformes,
fusco-brunneae ad atrae cum partibus incrementi albi
floccosique pigmentum diffusile rubro-brunneum ef-
ficientibus. Hyphae primum hyaline, 1–2 μm diam,
deinde pigmentatae et latiores (2.5–3 μm) cum septis
prominentibus, deinde tumentes et rapide in arthro-
conidia cylindrica ad cupaeformae dilabentes, 6.5–20
(–25) × 3–6.5 μm. Blastoconidia nova hyalina, lacri-
maeformia ad ellipsoidea, 3.5–5.5 × 2.5–4.5 μm, mox
pigmentata, globosa, 4–7.5 × 4–7 μm, in catenis ac-
ropetalibus ramosis vel non-ramosis, congestim se-
cundum hyphas accumulantia.

TYPUS: Colonia exsiccata in herbario UAMH
(UAMH 6490, HOLOTYPUS), et in herbario DAOM
(DAOM 213330, ISOTYPUS). Cultus e cadavere lar-
vae *Megachiles rotundatae* ab *Ascosphaera aggregata*
contaminatae e Tilley, Alberta a M. Goettel, 1989.

On PDA (FIG. 2) and CER at 25 C, colonies
olive-brown (4D7) by 5 da, becoming brown
(4F7) to black, 24–30 mm diam by 15 da (TABLE
I); flat, finely furrowed or cerebriform, texture
butyraceous with velvet-like nap, small central
cerebriform umbo commonly covered with wisps
of white aerial hyphae; margins irregular, pin-
nate. Colonies on both media commonly sector-
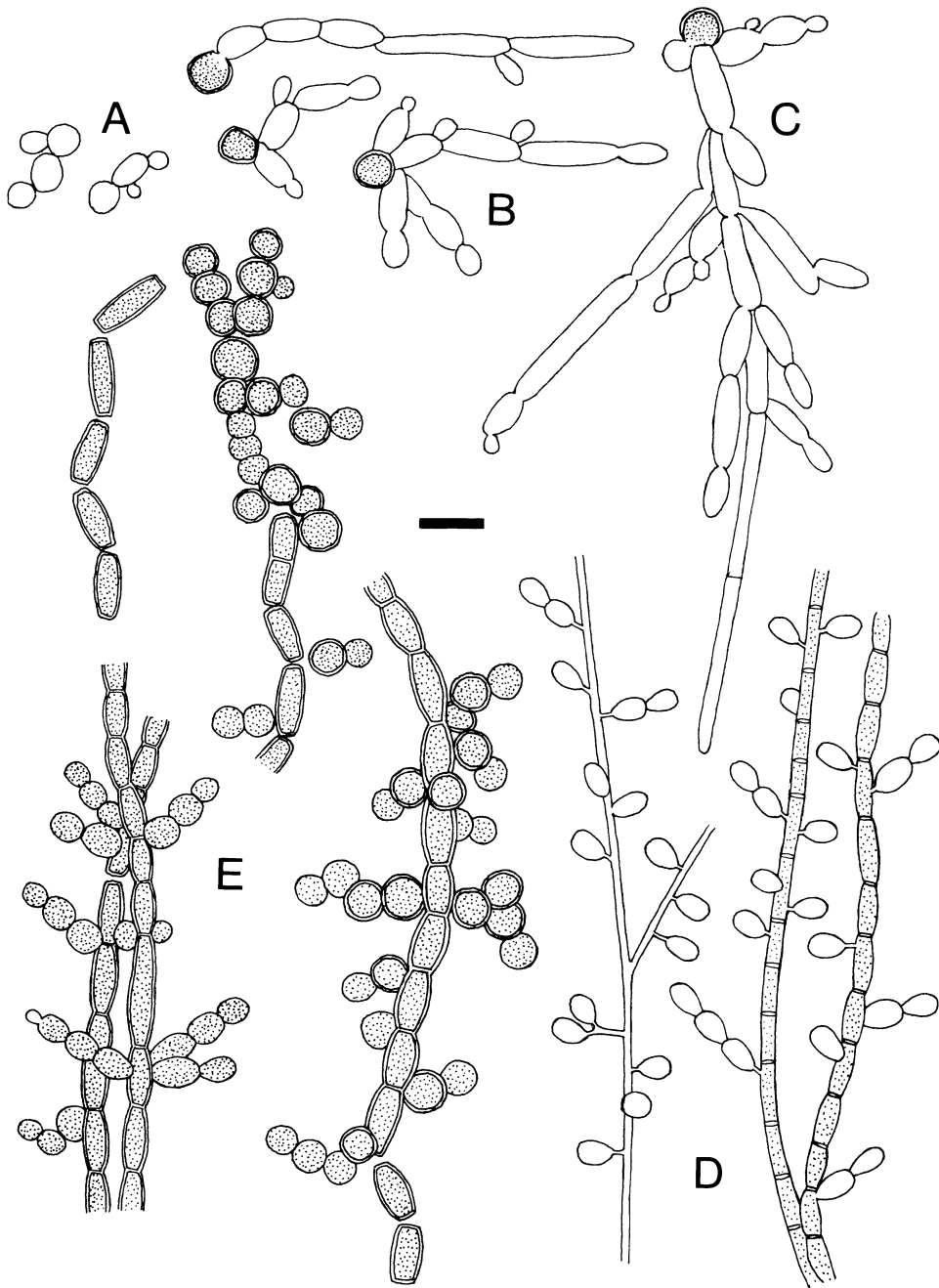ing to form areas of enhanced floccose, white

FIG. 1. *Trichosporonoides megachiliensis*. A. Growth at 6 h in GYPB. B. Pseudohyphal growth at 24 h in Dalmau culture. C. Formation of true hypha at 48 h in Dalmau culture. D. Hyaline, tear-shaped blastoconidia developing laterally on hyaline hyphae and older, swollen, pigmented hyphae in slide culture. E. Pigmented, globose blastoconidia and disarticulated arthroconidia in slide culture. Bar = 10 μm.
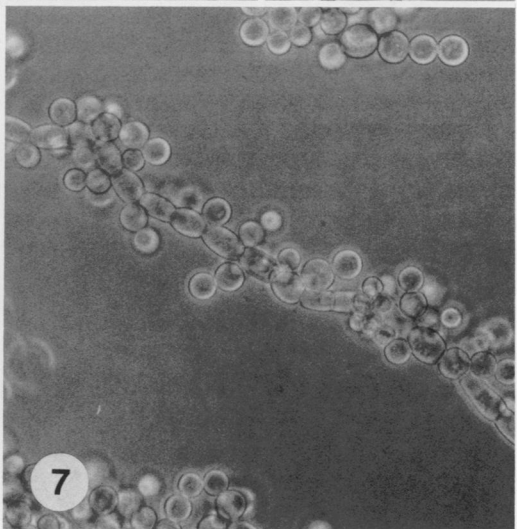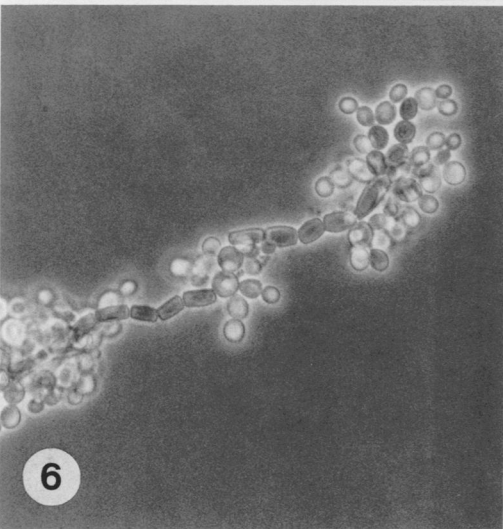
aerial mycelium; occasionally on PDA, forming sectors of butyraceous texture and cerebriform topography, but with a faster growth rate. Subcultures from more hyphal sectors produced colonies of similar type. Growth slightly inhibited on GYP50 (20–25 mm), simila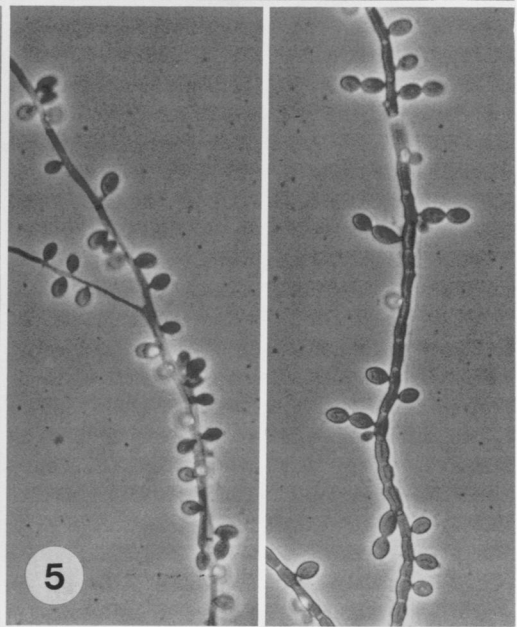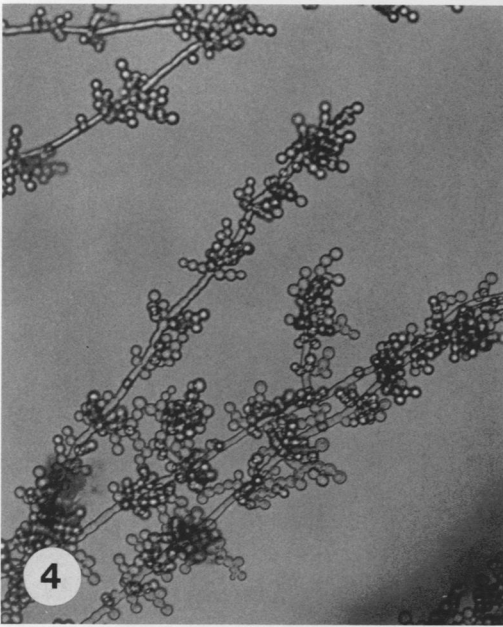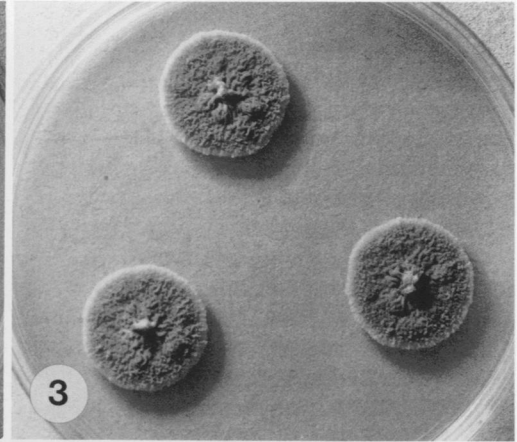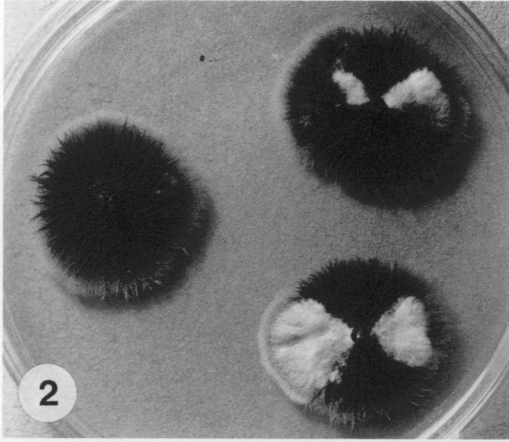r in appearance (FIG. 3) but without sectoring, and remaining yellowish-brown (5E8). At 37 C on PDA, growth 19–25 mm by 15 da. A reddish-brown diffusing pigment was observed on PDA at 20–30 da and a strong yeast-like smell was associated with all isolates.

Initial growth at 12 h in Dalmau culture on PDA consisted of pseudohyphae but by 24–48 h septate hyphae (3.5–4.5 μm diam) were formed (FIGS. 1A–C). In slide culture on CER, hyphae initially hyaline (1–2 μm diam), by 5–10 da hyphae becoming pigmented and broader (2.5–3 μm) with prominent septa at 7–12 μm intervals (FIGS. 1D, 5), then swelling and disarticulating into arthroconidia. Arthroconidia brown, unicellular, cylindrical to barrel-shaped, 6.5–20 (–25) × 3–6.5 μm (av 13.9 × 4.8 μm), l/w = 2.88 (FIGS. 1E, 6). Blastoconidia initially hyaline, ovoid or subglobose, forming apically or laterally, either sessile or on short unswollen stalks, solitary or in short chains, 3.5–5.5 × 2.5–4.5 μm, becoming swollen, brown, globose to subglobose, 4–7.5 × 4–7 μm (av 5.6 × 5.7), l/w = 1.0 (FIGS. 1D–E, 4–7), accumulating in masses along hyphae.

Isolates were slightly inhibited on GYP66 (11–14 mm diam at 15 da) compared with GYP50 (20–25 mm) (TABLE I). Growth was completely inhibited or restricted on the benomyl-amended medium but all isolates stained positively with DBB. There was no growth on vitamin-free CAS, CAS amended with thiamine or in the presence of cycloheximide. All isolates hydrolyzed urea. They fermented glucose and sucrose but only two isolates showed weak fermentation of galactose and maltose. All assimilated cellobiose, glucose, glycerol, maltose, palatinose and sucrose in the API 20C and/or AMS-YBC systems; only some assimilated galactose.

TYPIFICATION: Dried colony derived from a living culture obtained from chalkbrood (*Ascosphaera aggregata*) cadaver, 1989, by M. Goettel, HOLOTYPE (UAMH 6490), ISOTYPE (DAOM 213330). Ex-type cultures UAMH 6490 = CBS 190.92 = ATCC 76718.

HABITAT: Associated with alfalfa leafcutter bees, including provisions, larvae, frass, emerging adults and larval cadavers infected with *A. aggregata.*

ECOLOGY AND DISTRIBUTION: *Trichosporonoides megachiliensis* is known so far only from leafcutter bees and provisions; its geographic range appears to parallel that of the bee in western North America. Bissett (1988) and Goerzen (1991) have also recovered an unnamed *Trichosporonoides* associated with alfalfa leafcutter bees. Although representative living cultures are no longer available, Bissett (pers. comm.) has observed two different black yeast-like fungi: the most prevalent one, associated with provisions, adults and larval cadavers infected with *Ascosphaera* spp. including *A. aggregata,* demonstrated features similar to *T. megachiliensis.* The second, isolated on ten occasions from sporulating and nonsporulating chalkbrood cadavers, demonstrated synchronously produced conidia reminiscent of *T. oedocephalis.* Goerzen (1991) recovered *Trichosporonoides* sp. from cell leaf pieces, provisions, nest material, larval cadavers infected with *A. variegata* Bissett and adult bees in Saskatchewan. One of his isolates (UAMH 6969) was examined by us and found to be conspecific with *T. megachiliensis.*

Although populations of *T. megachiliensis* were found to be the most prevalent taxon of filamentous fungi recovered from larval guts (unpublished), reasons for its prevalence and mechanisms of entry are unclear. We were unable to isolate the fungus from pollen and nectar obtained from bees provisioning the cells, nor did we isolate it from any of 10 provisioning bees. Our isolation of *T. megachiliensis* from the surface of 1 of 25 emerging adult bees, together with its recovery at low levels from bee provisions (unpublished), suggests that emerging bees are

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FIG. 2–7. *Trichosporonoides megachiliensis.* (FIGS. 2–3 = UAMH 6490; FIGS. 4–6 = 6820; FIG. 7 = 6821) 2–3. Colonies at 15 da at 25 C, ×0.85. 2. On PDA. 3. On GYP50. 4. Globose blastoconidia in acropetal chains in Dalmau culture, ×460. 5. Hyaline, ovoid to subglobose blastoconidia from young hyaline hypha (left) and older, subhyaline, swollen hypha (right) in slide culture, ×585. 6. Globose blastoconidia and disarticulating arthroconidia in slide culture, ×585. 7. Globose pigmented blastoconidia and hypha constricted at septa in Dalmau culture, ×585.

TABLE I

PHYSIOLOGICAL CHARACTERISTICS OF *TRICHOSPORONOIDES OEDOCEPHALIS* (To), *T. MEGACHILIENSIS* (TME), *T. MADIDA* (TMA), *T. SPATHULATA* (Ts), *T. NIGRESCENS* (TN) AND *T. AUSTRALIENSE* (TA)

	To (3922)	Tme (pooled)	Tma (4546)	Ts (4547)	Tn (7092)	Ta (6791)
FERMENTATION						
Glucose	+	+	+	+	+	+
Galactose	+	v^a	−	+	+	+
Sucrose	+	+	−	+	−	v^a
Maltose	−	v^a	+	−	+	−
Lactose	−	−	−	−	−	+
Trehalose	−	−	−	−	−	−
API 20C AND AMS-YBC						
Glucose	+	+	+	+	+	+
Galactose	+	v^c	−	−	+	+
D-glucosamine	−	−	−	+	−	+^{c}
D-xylose	−	−	−	+^{c}	−	−
L-arabinose	+^{c}	−	−	−	−	−
Sucrose	+	+	+	+	−	+
Maltose	+	+	+	+^{c}	+	+
Trehalose	+	−	−	−	−	+
Methyl-D-glucoside	−	−	−	−	−	+
Cellobiose	+	+	+^{c}	+	−	+
Melibiose^b	−	−	−	−	−	−
Lactose	−	−	−	−	−	+
Raffinose	−	−	−	+^{c}	−	−
Melezitose	−	−	−	+^{c}	−	−
Glycerol	+	+	+	+	+	+
Erythritol^b	−	−	+^{d}	−	+	−
Ribitol (adonitol)	−	−	−	−	+^{e}	−
Xylitol	−	−	−	−	+^{e}	−
Galactitol (dulcitol)^b	−	−	−	−	−	−
D-glucitol (sorbitol)	−	−	−	+^{c}	+^{e}	−
Inositol	−	−	−	−	−	−
Palatinose^b	+	+	+^{d}	−	+	+
2-keto-D-gluconate	+	−	+	+	−	−
Nitrate^b	+	v^a	+	+	+	+
ADDITIONAL TESTS						
No vitamins	−	−	−	+	−	−
Thiamine	+	−	−	+	+	−
DBB	+	+	+	+	+^{f}	−
Urease	+	+	+	+	+	−
PDA^g	27–28	24–30	22–24	45–46	20–21	17–18
CER^g	27–29	26–29	20–21	35–38	10–11	16–19
PDA 37 C^g	21–25	19–25	19–20	37–41	0	8–9
GYP50^g	19–22	20–25	10–12	23–25	11–14	0
MYC (400 µg/ml cycloheximide)^g	0	0	0	0	0	5–6
MMN (2 µg/ml benomyl) 20 da^g	9–10	0–12	2–4	7–8	0–2	12–15

^{a} Variable response.
^{b} Tested in AMS-YBC but not API 20C.
^{c} Results positive with API 20C but not AMS-YBC.
^{d} Positive in one of two trials in AMS-YBC.
^{e} Results positive with AMS-YBC but not API 20C.
^{f} As reported by Hocking and Pitt (**1981**).
^{g} Colony diameters (mm) measured at 15 da at 25 C unless otherwise stated.

infested with the fungus and introduce propagules in the larval provisions at very low frequencies. However, since our isolation methods from provisions and larvae were bulked (10 samples), we have no measure of variation between individuals.

We previously showed (Inglis et al., 1992) that provisions were comprised of nectar and pollen with a total sugar content of 66% (w/provision dry weight) and that the water soluble component consisted of fructose (48.1%), glucose (43.6%), sucrose (1.4%), turanose and/or palatinose (1.8%). The present study demonstrated that *T. megachiliensis* was able to grow on media with reduced water activity, that it fermented glucose and sucrose, and that it assimilated all of the main sugars found in provisions (TABLE I), with the exception of turanose which was not tested. The high incidence of recovery and the potential ability of *T. megachiliensis* to assimilate and ferment sugars found in provisions and subsequently in guts of leafcutter bee larvae may influence the gut environment, in particular CO_2. Since high concentrations of CO_2 are necessary for the germination of ascospores of *A. aggregata* (Kish, 1980), large populations of *T. megachiliensis* in larval guts may contribute to the development of chalkbrood disease, a possibility presently being investigated in our laboratories.

COMPARISONS WITH OTHER SPECIES OF TRICHOSPORONOIDES

Since the species of *Trichosporonoides* are not readily distinguished from each other, we provide brief descriptions of the type and accepted species to enable others to make comparisons with the new species. In addition, the original descriptions of *T. madida* and *T. spathulata* did not provide photomicrographs. Colonial descriptions are based on growth at 15 da at 25 C unless otherwise stated. Physiological characters are presented in TABLE I.

Trichosporonoides spathulata de Hoog, Stud. Mycol. **19**: 23. 1979.   FIGS. 8–11

Colonies on PDA (FIG. 8) and CER pale, grey-brown to olivaceous, becoming dark brown, slightly floccose, cerebriform or with radial furrows, frequently sectoring, reverse olive-brown. Growth rate on PDA moderately rapid (45–46

mm); slower on CER (35–38 mm) and on GYP50 (23–25 mm) (FIG. 9), and on PDA at 37 C (37–41 mm) (TABLE I). Blastoconidia hyaline, broadly ellipsoidal, 5–10 × 3–4 μm formed apically and laterally in acropetal chains (FIGS. 10–11); in older (20–30 da) cultures, some globose, darkly pigmented blastoconidia present. Arthroconidia hyaline to subhyaline, cylindrical, 5–15 × 2–4 μm.

HABITAT. Ghee, a fat from fermented buffalo milk.

Trichosporonoides madida de Hoog, Stud. Mycol. **19**: 25. 1979.   FIGS. 12–15

Colonies on PDA (FIG. 12) and CER olive-brown, becoming dark brown to black, cerebriform with white aerial hyphae in sectors. Similar growth rates on PDA and CER at 25 C (20–24 mm) and on PDA at 37 C (19–20 mm) (TABLE I) but slower on GYP50 (10–12 mm) (FIG. 13). Blastoconidia hyaline to subhyaline, ellipsoidal to subglobose, 6–12 × 3–4 μm, formed apically and laterally in acropetal chains (FIGS. 14–15). Arthroconidia hyaline to subhyaline, cylindrical, 5–20 × 3–4 μm.

HABITAT. Margarine.

Trichosporonoides nigrescens Hocking & Pitt, Antonie van Leeuwenhoek Ned. Tijdschr. Hyg. **47**: 413. 1981.   FIGS. 16–20

Colonies on PDA (FIG. 16) initially greyish-yellow with radiating furrows, becoming olive-brown, reverse blond with patches of brown, butyraceous, no aerial hyphae or sectors, conspicuously cerebriform with irregular margins, raised 3–4 mm, 20–21 mm in diam (TABLE I), commonly cracking medium. On CER and GYP50 (FIG. 17) growth slower, colonies 10–11 mm and 11–14 mm, respectively, raised 2–3 mm, golden-brown to yellowish-brown with darker olive-brown central umbo on CER; topography and texture similar to colonies on PDA, but colonies on GYP50 developing a superficial nap of white velvet-like hyphae at margins. No growth on PDA at 37 C. Initial growth pseudohyphal (FIG. 19). Blastoconidia predominantly subhyaline, becoming brown in older cultures, subglobose to ellipsoidal, 3–7 × 2–4 μm, formed apically or laterally in acropetal chains (FIG. 18). Arthroconidia not produced in slide culture on CER or in Dalmau culture on PDA at 15 da but
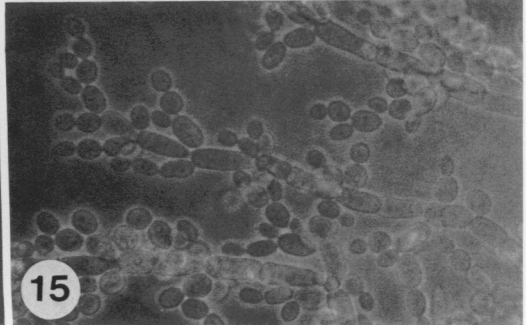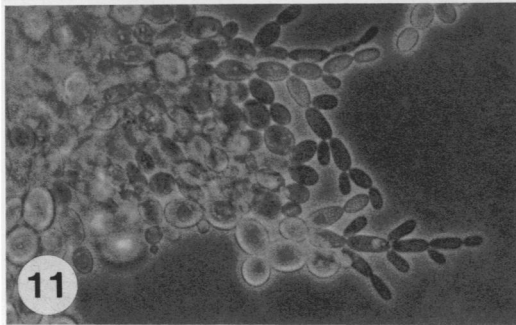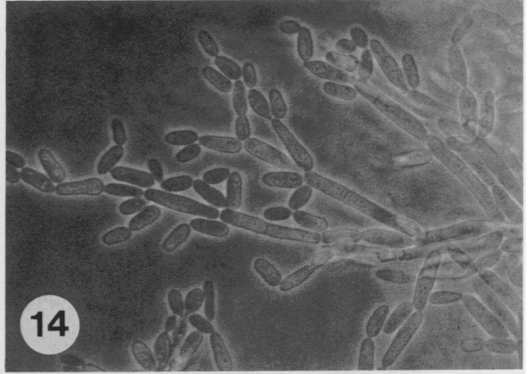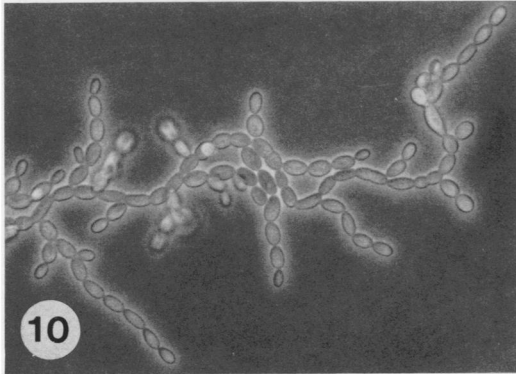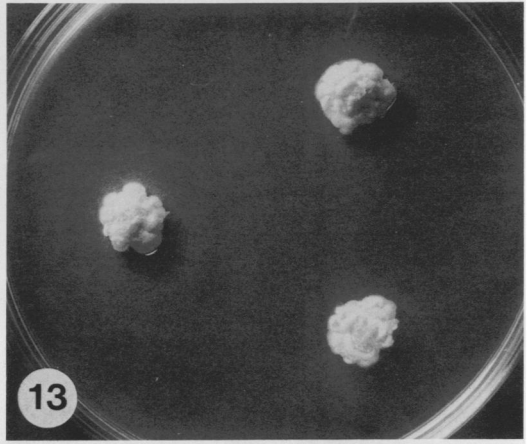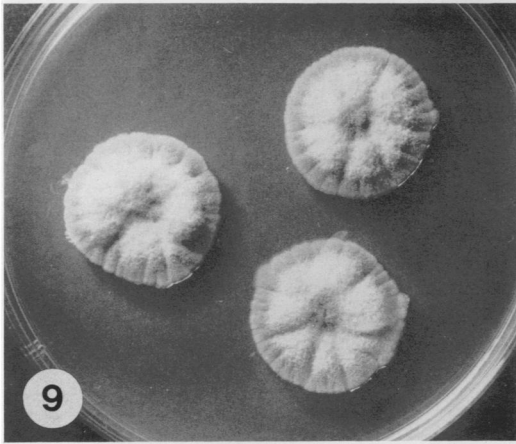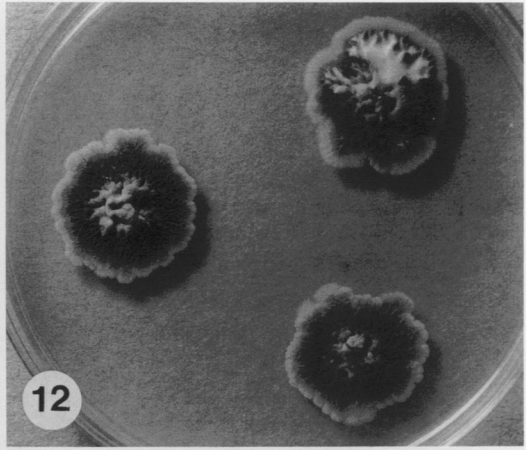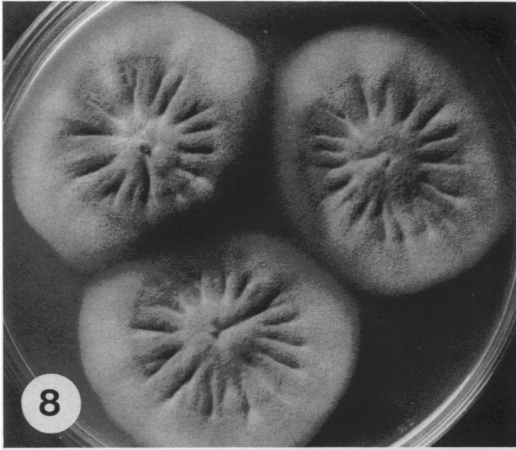
occurring in the hyphal nap at the colony margins on GYP50, hyaline to subhyaline, cylindrical, 6–10 (–15) × 3–4 μm (FIG. 20), hyphae also fragmenting into longer lengths of up to 80 μm.

HABITAT. Jam.

Trichosporonoides oedocephalis Haskins & Spencer, Canad. J. Bot. **45**: 519. 1967.

FIGS. 24–27

Colonies on PDA and CER initially creamy-white and butyraceous, developing patches of brown to grey-brown, 27–29 mm diam, becoming uniformly dark brown by 30 da, reverse pale turning brown. Colonies sometimes becoming hyphal, velvet-like or floccose. Growth on GYP50, 19–22 mm, and on PDA at 37 C, 21–25 mm (TABLE I). Initial growth by formation of pseudohyphae (elongated blastoconidia), true hyphae forming lateral or terminal chains of blastoconidia and dividing by schizolytic dehiscence to form arthroconidia. Blastoconidia ellipsoidal to subglobose, hyaline to subhyaline, 3–6 × 3–4 μm (FIG. 27); arthroconidia hyaline to subhyaline, cylindrical, 5–20 × 3–4 μm. By 7 da, and corresponding to the darkening of the colony, yellowish-brown conidia were produced synchronously on vesicles, 7–12 μm diam (FIGS. 24–27), borne at the ends of non-septate conidiophores or in intercalary positions. They measured 4.5–9 × 4.5–6.5 μm and were cylindrical, subglobose and then broader than long, or globose, often without a noticeable dehiscence scar except for a narrow thin-walled area. The synchronous conidia did not detach as readily as the blastic conidia and often remained connected in sets of 2–3.

HABITAT. Brood cell of a domestic honeybee comb.

Except for *T. australiense,* all species of *Trichosporonoides* formed dark butyraceous colonies which were slightly to markedly cerebriform, grew initially as pseudohyphae, produced nontruncate hyaline to pigmented blastoconidia and cylindrical to barrel-shaped arthroconidia, were able to grow on low water activity media, and had positive reactions with DBB. In contrast to the other species, *T. megachiliensis* formed globose blastoconidia, which like the arthroconidia, were conspicuously darkly pigmented (FIG. 7), and produced a diffusing reddish-brown pigment on PDA. *Trichosporonoides madida,* the most similar species, differed from *T. megachiliensis* by its subhyaline arthroconidia and blastoconidia, which were predominantly ellipsoidal, by its slower growth rate on GYP50 and CER at 25 C (TABLE I), by its inability to ferment sucrose and galactose, and by its ability to assimilate erythritol and 2-keto-D-gluconate. *Trichosporonoides spathulata,* in comparison to *T. megachiliensis,* grew faster on PDA and CER at 25 and on PDA at 37 C (TABLE I), grew on a vitamin-free medium, assimilated N-acetyl-D-glucosamine, xylose, raffinose, melizitose, glucitol, 2-keto-D-gluconate, and failed to assimilate palatinose. Although *T. spathulata* produced some dark, globose blastoconidia of similar size to those of *T. megachiliensis* in aged cultures (20–30 da), most blastoconidia were hyaline and broadly ellipsoidal, and arthroconidia remained hyaline to subhyaline. The morphological and physiological differences between these three species are further supported by their ecological differences: *T. madida* and *T. spathulata* from high fat substrates, margarine and ghee (a fat from fermented buffalo milk), respectively and *T. megachiliensis* from high sugar content alfalfa leafcutter bee provisions. In addition to *T. megachiliensis,* two accepted species of *Trichosporonoides* are known from high sugar substrates: *T. oedocephalis,* from honeybee comb and *T. nigrescens* from jam. *Trichosporonoides oedocephalis,* the only other species known from bees, differed from all species in forming darkly pigmented conidia synchronously on a slightly ampulliform conidiogenous cell (FIGS. 24–27). These conidia were observed in both strains of *T. oedocephalis* examined by us on media such as CER and PDA. The type species further differed from *T. megachiliensis* in being more hyphal, in forming

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FIGS. 8–15. *Trichosporonoides spathulata* and *T. madida.* 8–11. *Trichosporonoides spathulata* (UAMH 4547). 8–9. Colonies at 15 da at 25 C, ×0.85. 8. On PDA. 9. On GYP50. 10–11. Subhyaline, ellipsoidal blastoconidia in acropetal chains in Dalmau culture, ×585. 12–15. *Trichosporonoides madida* (UAMH 4546). 12–13. Colonies at 15 da at 25 C, ×0.85. 12. On PDA. 13. On GYP50. 14–15. Subhyaline, ellipsoidal to subglobose blastoconidia in acropetal chains in Dalmau culture, ×585.
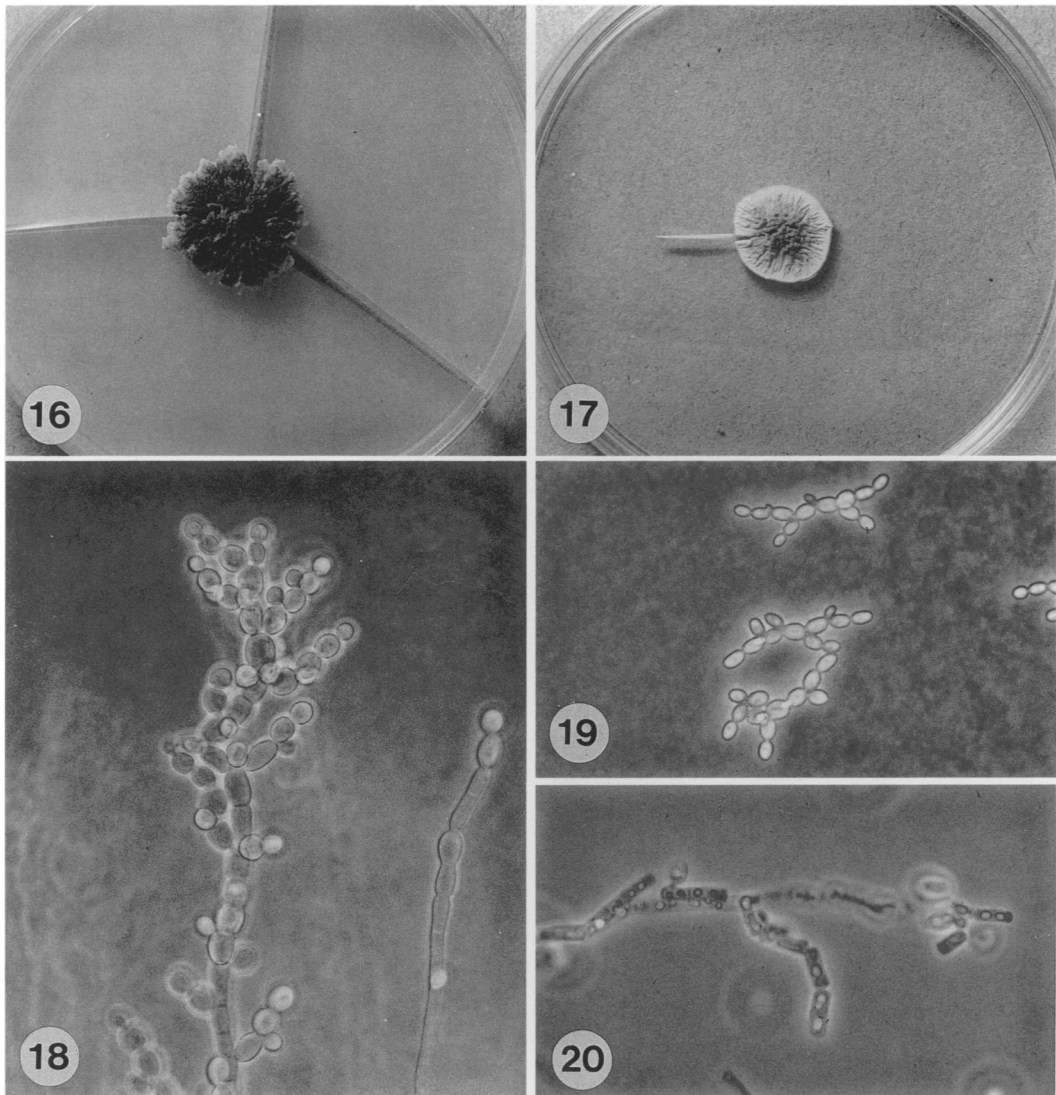
FIGS. 16–20. *Trichosporonoides nigrescens* (UAMH 7092). 16–17. Colonies at 15 da at 25 C, ×0.85. Note splitting of media. 16. On PDA. 17. On GYP50. 18. Subhyaline, subglobose to ellipsoidal blastoconidia in acropetal chains in Dalmau culture, ×585. 19. Pseudohyphal growth at 24 h in Dalmau culture, ×340. 20. Subhyaline arthroconidia from GYP50, ×585.

shorter blastoconidia which were ellipsoidal to subglobose and remained hyaline, and in assimilating L-arabinose, trehalose and 2-keto-D-gluconate. *Trichosporonoides nigrescens* differed from *T. megachiliensis* and all other species by its colonial and microscopic features. Colonies were raised 2–4 mm and lacked the sectoring so prominent in the new species. Growth was slow on CER and there was no growth at 37 C (TABLE I). In Dalmau culture the PDA medium commonly split. Hocking and Pitt (**1981**) reported an extremely slow growth rate on glucose yeast-extract peptone agar (1–4 mm after 3 wk). Although we did not culture the type strain on the same medium, we observed slower growth only on 2% malt extract agar (5–6 mm at 15 da). No arthroconidia formed under our Dalmau conditions or in slide culture. We observed them
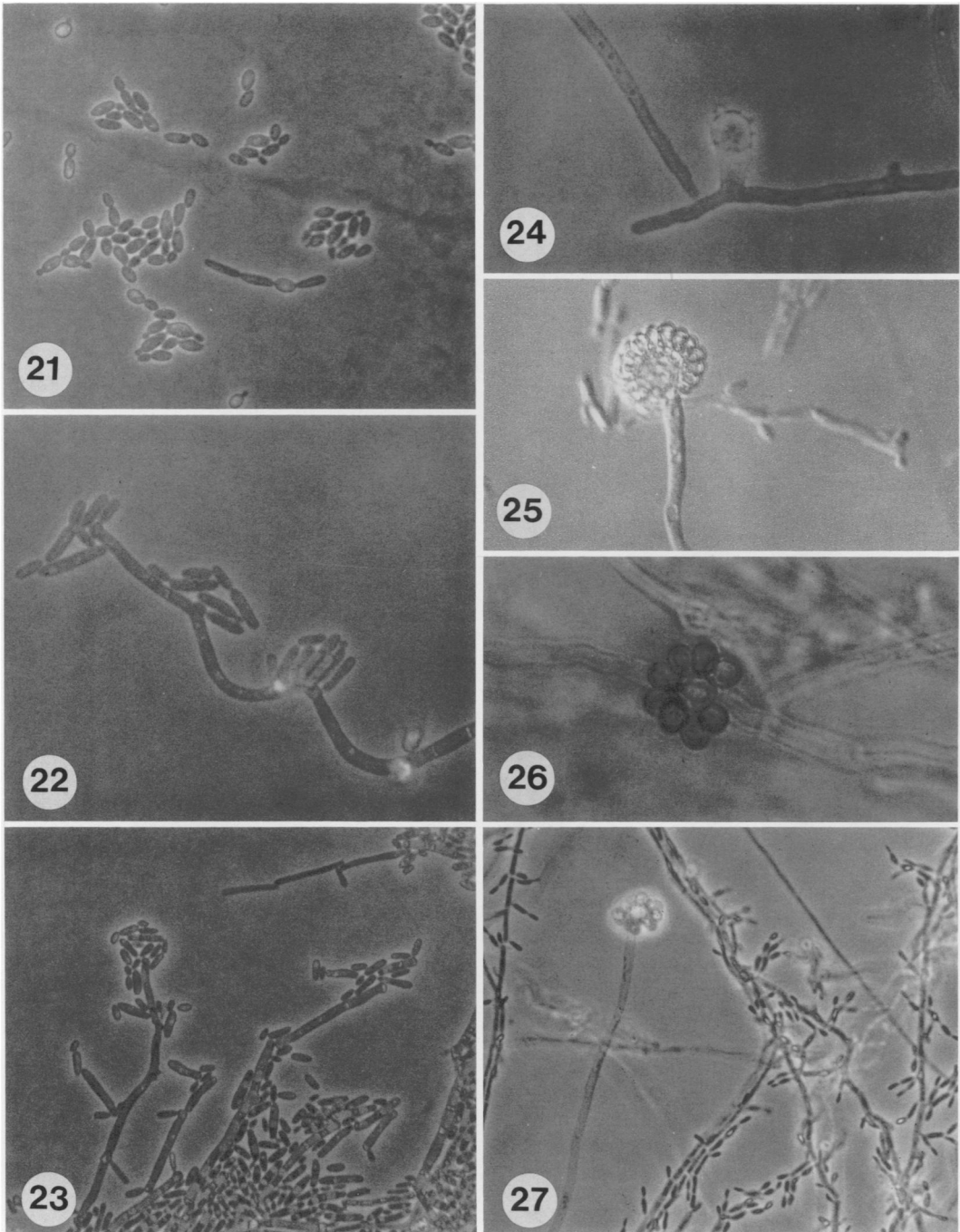
FIGS. 21–27. *Trichosporonoides australiense* and *T. oedocephalis*. 21–23. *Trichosporonoides australiense* (UAMH 6791). 21. Polar and bipolar growth at 24 h in Dalmau culture, ×950. 22. Ellipsoidal blastoconidia at 5 da from hyaline hypha in Dalmau culture, ×950. 23. Blastoconidia at 15 da, ×585. 24–27. *Trichosporonoides oedocephalis* (FIGS. 24–26 = UAMH 3922; FIG. 27 = 3923). 24. Hyphal branch with swollen vesicle producing conidial primordia at 20 da in Dalmau culture, ×950. 25–27. Synchronously produced blastoconidia. 25. In slide culture, ×770. 26. In Dalmau culture, ×950. 27. Blastoconidia produced laterally from hyphae in short acropetal chains and synchronously from a swollen vesicle at the apex of a nonseptate conidiophore, ×460.

only on GYP50 (FIG. 20). In addition, *T. nigrescens* failed to ferment sucrose or to assimilate cellobiose, but did assimilate erythritol, ribitol, xylitol and D-glucitol. The latter three carbohydrates were positive in the AMS-YBC test but not in API 20C. In contrast to our findings, Hocking and Pitt (**1981**) reported fermentation of sucrose, and assimilation of sucrose and cellobiose at standard concentrations.

In a few other instances, the physiological data that we obtained varied from that previously reported. This was probably due to our use of the rapid yeast identification methods, the API 20C and automated AMS-YBC systems. Previous studies (Martinez et al., **1979**; Hocking and Pitt, **1981**) reported results of traditional liquid assimilation methods read at 28 da. Although our results were obtained at 4–5 da, both AMS-YBC and the API 20C systems are well known to provide fast (within 48–72 h) and accurate identification of medically important yeasts (Warren and Shadomy, **1991**). Their efficacy for measuring carbohydrate assimilation by yeast-like or filamentous fungi is not yet known since few reports are available (Espinel-Ingroff et al., **1989**; Sigler, **1990**). Only one possible false positive result appeared to occur with our methods: *T. oedocephalis* assimilated trehalose whereas Martinez et al. (**1979**) reported a negative result. Moreover, erythritol, lactose, and D-glucitol were usually negative by our methods but positive in the liquid assimilation methods. Discrepancies also were encountered in the ability of isolates to grow without vitamins. In contrast to Martinez et al. (**1979**) and Hocking and Pitt (**1981**) who found that all species of *Trichosporonoides,* except *T. oedocephalis,* were capable of growth without vitamins, we judged only *T. spathulata* to be positive. The results were difficult to interpret since species other than *T. spathulata* produced some degree of submerged growth, but we limited a positive response to the presence of aerial mycelium.

EXCLUDED SPECIES

Trichosporonoides australiense Ramirez, Mycopathologia **108**: 25. 1989.   FIGS. 21–23

Colonies on PDA and CER, 16–19 mm (TABLE I), white to cream, smooth, slightly raised, pasty, developing a fringe of submerged mycelium, but remaining creamy-white by 30 da. No growth was observed on GYP50 and growth was inhibited at 37 C. Initial growth was by polar and bipolar budding (FIG. 21). In Dalmau culture, blastoconidia germinated to form true branched hyphae which were initially nonseptate, eventually becoming septate; clusters of elongated, ellipsoidal, hyaline blastoconidia, 6–8 × 2–3 μm accumulated along sides of hyphae (FIGS. 22–23). Physiological characteristics are presented in TABLE I.

HABITAT. Sweetened fruit drink.

Ramirez (**1989**) placed this fungus in *Trichosporonoides* because of the production of variously shaped bipolar budding cells, the presence of pseudomycelia, and smooth-walled hyaline hyphae, which, when mature, disarticulated into long cylindrical arthroconidia. However, our examination of the ex-type strain revealed several characteristics not typical of *Trichosporonoides* including initial growth by budding cells, rudimentary hyphae, elongated ellipsoidal cells dissimilar to the arthroconidia of *Trichosporonoides,* colonies which remained creamy-white and pasty, and fermentation of lactose. Some physiological characteristics varied from those reported in the published description. Sucrose was fermented and maltose assimilated. The ex-type strain failed to grow on high sugar medium or to hydrolyze urea and showed a negative reaction with DBB. A negative reaction with Diazonium Blue B, and to a lesser extent the inability to hydrolyze urea suggested an ascomycetous affinity. However, the fungus appeared to be more tolerant to benomyl at 2 ppm, than were other species of *Trichosporonoides* (TABLE I). Although many fungi with ascomycetous teleomorphic affinities are highly susceptible to benomyl (Edgington et al., **1971**; Summerbell, **1988**), variations in susceptibility exist, limiting tolerance to benomyl as a definitive taxonomic tool. The morphological and physiological characteristics suggest that *T. australiense* is conspecific with *Brettanomyces anomalus* Custers (Barnett et al., **1990**); moreover, it was isolated from a sweetened drink, a substrate in which *B. anomalus* is commonly found (Jong et al., **1985**). Although we did not induce ascospore formation on PDA or on cornmeal agar after 30 da, Smith and van Grinsven (**1984**) observed ascospores in two strains of *B. anomalus* and they named the teleomorph *Dekkera anomala* Smith & van Grinsven. We propose the following synonymy:

Brettanomyces anomalus Custers, Ph.D. Thesis, Delft University of Technology, Delft, Netherlands. p. 79. 1940.

= *Trichosporonoides australiense* Ramirez, Mycopathologia **106**: 25. 1989.

TELEOMORPH: *Dekkera anomala* Smith & van Grinsven, Antonie van Leeuwenhoek Ned. Tijdschr. Hyg. **50**: 142. 1984.

GENERIC RELATIONSHIPS

The fungus associated with alfalfa leafcutter bees produced dark brown to black colonies, pigmented blastoconidia in acropetal chains, pigmented arthroconidia and a positive DBB reaction thereby suggesting an affinity to *Trichosporonoides* and/or *Moniliella*. In deciding on the most appropriate genus for its placement, we found difficulty with the current concepts of these genera. The type species of *Trichosporonoides* and *Moniliella* demonstrate morphological features which allow them to be easily distinguished. *Trichosporonoides oedocephalis* forms a distinctive accessory state characterized by synchronous formation of darkly pigmented conidia in a single layer on ampulliform vesicles borne at the apex of non-septate conidiophores (FIGS. 25, 27) or in intercalary positions (FIG. 26). Furthermore, the blastoconidia are more rounded and lack distinctive truncate scars, 0.5–1 μm long and wide *fide* Stolk and Dakin (**1966**) typical of the blastoconidia and ramoconidia of *M. acetoabutens,* the type species. The latter is further distinguished by the formation of thick-walled, brown globose chlamydospores. These may be sparse or abundant, depending upon the growth medium, and occur in the submerged mycelium or in sparsely septate aerial hyphae which may reach 12 μm in width. Stolk and Dakin commented on the tendency of the hyphae to fragment into truncate arthroconidia of regular size and shape, or into "long thinwalled hyphal fragments up to 150 μ long." Colonies of both species tend to be soft, butyraceous and yeast-like, similar to colonies of yeast-like fungi, such as *Geotrichum* or *Trichosporon,* but with gradations in color from light to dark brown. They also frequently sector to form areas of enhanced aerial mycelium. Subcultures from hyphal sectors usually produce more hyphal colonies.

The addition of several species to these genera has made the distinctions between them less clearcut. None of the newly added *Trichosporonoides*

species, including the one described here, produces the accessory synchronous conidia. On the basis of this character alone, it could be argued that all other species should be excluded. However, de Hoog (**1979**) disregarded their development as a key character. Instead, he placed emphasis on other features including: 1) blastoconidial size where conidia of *Trichosporonoides* were less than 10 μm in length and up to 4.5 μm in width, whereas those of *Moniliella* were greater than 10 μm in length and usually wider than 4–4.5 μm; 2) blastoconidia with truncate abscission scars in *Moniliella* compared to nontruncate blastoconidia in *Trichosporonoides*; and 3) presence of hyphal fascicles on high sugar substrates in *Trichosporonoides*. Although we did not find the presence or absence of fascicles on GYP50 to be a useful key character, we concur with the use of the first two characters in differentiating between the genera. We would also add an additional distinguishing feature: germination of all *Trichosporonoides* species occurs by formation of pseudohyphae whereas *M. acetoabutens* and *M. suaveolens* var. *suaveolens* germinate by formation of hyphae. *Moniliella suaveolens* var. *nigra* seems to differ in this respect, although both varieties are highly variable in morphological characters and may represent different taxa (de Hoog, **1979**; de Hoog and Guého, **1984**).

De Hoog and cooperators examined species within the genera on the basis of morphology (de Hoog, **1979**), physiology (Martinez et al., **1979**), production of volatile organic compounds (de Hoog and Roeymans, **1979**) and analysis of cell wall carbohydrates (Weijman, **1979**) but they could not resolve relationships within this complex. Although differences in physiological characteristics could be observed, the small number of genotypes of *Trichosporonoides* species precluded an adequate measure of intraspecies variation, and thus limited the usefulness of a key based on these characters (Martinez et al., **1979**). A relatively high percent of guanine and cytosine (de Hoog and Guého, **1984**) combined with positive DBB, production of urease and reduced dolipores without pore caps (Martinez, **1979**) suggested a basidiomycetous affinity for members of both genera. Quantification of nuclear cytosine and guanine showed considerable divergence between the type species of each genus, with *T. oedocephalis* at 45.8 mol% G + C compared with 61.1–61.3 mol% G + C in *M. acetoabutens* (de

Hoog and Guého, **1984**). The two species closest to *T. oedocephalis* were *T. madida* at 50.2% and *M. pollinis* (Hennebert & Verachtert) de Hoog & Guého at 50.4%; all other species had mol% G + C greater than 57%. *Trichosporonoides madida* is morphologically most similar to *T. megachiliensis*. Assessment of cell wall hydrolysates demonstrated the presence of glucose, galactose and mannose, but no xylose in any species of *Trichosporonoides* or *Moniliella* (Weijman, **1979**). His study also demonstrated the presence of erythritol in most taxa, but this polyol notably was lacking in the ex-type culture of *M. acetoabutens*. Haskins and Spencer (**1967**) had also commented on the formation of erythritol in *T. oedocephalis*, and de Hoog made reference to its formation in *M. pollinis* (de Hoog, **1979**, as *M. tomentosa* var. *pollinis*), isolated from pollen in a honey comb.

Although some genetic analysis has been done, the relationships between members of the genera remain unclear. Sequencing of 5S ribosomal RNA has demonstrated identical signature nucleotides between *T. oedocephalis* and *M. acetoabutens* (Walker, **1984**) and suggested a closer relationship to some heterobasidiomycetes, especially some species of smuts occurring on monocotyledonous plants (Blanz and Unseld, **1987**) than to other basidiomycetes. Reduced dolipores without pore caps occur in the Filobasidiaceae, Ustilaginaceae and Tilletiaceae (Khan and Kimbrough, **1982**; Roberson and Luttrell, **1989**). The rather low %G + C and presence of septa with both reduced dolipores and simple pores (Haskins, **1975**) appear to set *T. oedocephalis* apart from all other taxa. The synchronously produced spores on ampulliform sporophores have been considered to be mitotic structures but evidence is lacking on the nuclear events occurring therein.

Since they bear some resemblance to the basidia of some members of the Filobasidiaceae, it is tempting to speculate whether they may be meiosporangia of a homothallic heterobasidiomycete rather than conidiophores. Members of the Filobasidiaceae have dolipore-like septa without parenthosomes, form nonseptate sporophores and holobasidia, produce sessile terminal basidiospores singly or in chains, and have yeast anamorphs (Kwon-Chung, **1987**). However, additional physiological markers emphasized by Kwon-Chung including limited fermentative ability, assimilation of inositol, urea hydrolysis and production of soluble starch are not compatible with the physiological features of *Trichosporonoides*. Hocking and Pitt (**1981**) questioned the basidiomycetous affinity of *Trichosporonoides* as some species showed ascomycetous characteristics including xerophily and *T. nigrescens* demonstrated an ascomycete-like mitotic cycle.

While we found difficulty with the current generic concepts, we found no convincing reason to combine the genera. Similarly to de Hoog, we have placed emphasis on the acropetally produced blastoconidial state, rather than on the synchronously produced conidial state, in assessing the generic limits. In *Trichosporonoides*, the longest blastoconidia were usually less than 10 μm in length, usually rounded, rarely truncate and ramoconidia with denticles were not conspicuous. The key to the species presented below is based primarily on conidial morphology and colonial appearance with a minor emphasis on physiology. Descriptions are based on growth at 15 da at 25 C; colonies on PDA, microscopic details on CER and PDA, except where noted.

KEY TO THE SPECIES OF *TRICHOSPORONOIDES* AND *MONILIELLA*

1. Germination of conidia predominantly hyphal, blastoconidia usually borne on ramoconidia, blastoconidia and ramoconidia with truncate denticles (0.5–1 μm long and wide), blastoconidia usually exceeding 10 μm in length .... *Moniliella* 2
1. Germination of conidia by pseudohyphae, blastoconidia broadly ellipsoidal or globose, usually less than 10 μm in length, generally not truncate, sometimes borne synchronously .... *Trichosporonoides* 3
    2. Colonies off-white turning dark brown with development of chlamydospores, grows at 37 C; terminal or intercalary pigmented, thick-walled chlamydospores present; long, nonseptate hyphal fragments of variable width (up to 12 μm wide) common .... *Moniliella acetoabutans*
    2. Colonies tan or pale grey, no growth at 37 C; pigmented chlamydospores and nonseptate hyphal fragments absent .... *M. suaveolens*
3. Colonies greater than 35 mm at 25 and 37 C .... *T. spathulata*
3. Colonies less than 35 mm diam at 25 and 37 C 4
    4. Darkly pigmented blastoconidia borne synchronously on swollen vesicles present ... *T. oedocephalis*
    4. Synchronous conidia absent 5

5. Colonies olive-brown, conspicuously raised (3–4 mm), membranous (lacking aerial hyphae), not sectoring; arthroconidia absent except on GYP50; no growth at 37 C; associated with jam .... *T. nigrescens*
5. Colonies dark brown to black, flat but with small central umbo, commonly with sectors of white aerial hyphae; arthroconidia present; grows at 37 C; associated with other substrates 6
    6. Colonies with pinnate or feathery margins except in hyphal sectors, diffusing reddish-brown pigment present (20–30 da); blastoconidia and arthroconidia darkly pigmented, blastoconidia globose; good growth on high sugar medium (>20 mm diam on GYP50); associated with alfalfa leafcutter bees .
    .... *T. megachiliensis*
    6. Margins paler, broad, entire, diffusing pigment absent; blastoconidia and arthroconidia hyaline to subhyaline, blastoconidia ellipsoidal to subglobose; poor growth on high sugar medium (<15 mm diam on GYP50); associated with fatty substrates .... *T. madida*

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