

Metschnikowia proteae sp. nov., a nectarivorous insect-associated yeast species from Africa

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A collection of yeasts isolated from nectar of flowers of *Protea caffra* (Proteaceae) and associated scarab beetles (*Atrichelaphinis tigrina*, *Cyrtothyrea marginalis*, *Trichostetha fascicularis* and *Heterochelus* sp.) and drosophilid flies in South Africa, contained 28 isolates that could not be assigned to known species. Comparisons of the D1/D2 domains of the large subunit rRNA gene demonstrated the existence of three separate phylotypes with an affinity to the genus *Metschnikowia* and more specifically to the beetle-associated large-spored *Metschnikowia* clade. Twenty-six strains that had similar D1/D2 sequences were mixed in all pairwise combinations. They were found to mate and give rise to large asci typical of those in the clade. The name *Metschnikowia proteae* sp. nov. (type strain EBDT1Y1^T=CBS 12522^T=NRRL Y-48784^T; allotype strain EBDC2Y2=CBS 12521=NRRL Y-48785) is proposed to accommodate this novel species. The ecology of this novel yeast species is discussed in relation to its potential plant and insect host species. The additional two single strains isolated from *Heterochelus* sp. represent two novel undescribed species (*Candida* sp. 1 EBDM2Y3 and *Candida* sp. 2 EBDM8Y1). As these single strains are probably haploid mating types of *Metschnikowia* species, their description is deferred until the species are sufficiently well sampled to permit meaningful descriptions.

INTRODUCTION

The wealth of yeast biodiversity in natural habitats still remains largely unexplored, as the diversity of yeasts associated with plants and their insect visitors is still grossly under-reported (Lachance, 2006; Ganter, 2006). The need to discover and describe as many species as possible is pressing and, as recommended by several authors, taxonomic surveys should be integrated within the biological context in which the yeast populations live in order to understand better their distribution, ecology and function within ecosystems (Spencer & Spencer, 1997; Lachance & Starmer, 1998).

Resuming some early attempts undertaken nearly one century ago (e.g. Boutroux, 1884; Schuster & Úlehla, 1913), an upsurge of interest in the yeasts associated with flowers

Abbreviations: BI, Bayesian Inference; NJ, neighbour-joining; NJ-BS, neighbour-joining bootstrap value.

The GenBank/EMBL/DBJ accession numbers for the sequences determined in this study are listed in Table 1.

Supplementary methodologies and a supplementary figure are available with the online version of this paper.

and their insect visitors has taken place recently. One outcome of this effort has been an explosion in the number of novel species in the *Metschnikowia* clade that are strongly associated with flower–insect systems (e.g. Giménez-Jurado *et al.*, 2003; Hong *et al.*, 2003; Lachance *et al.*, 2006; Rosa *et al.*, 2007). The number of *Candida* and *Metschnikowia* species in the *Metschnikowia* clade has almost quadrupled in the last ten years with 39 *Metschnikowia* species and 26 *Candida* species currently recognized (Lachance, 2011; Lachance *et al.*, 2011). Several of these yeast species have been specifically found in close association with floral nectar and insects that forage on flowers, and their ecology is becoming better understood as the knowledge on their genetic diversity and their role in plant–pollinator mutualism increases (Brysch-Herzberg, 2004; Herrera *et al.*, 2008, 2009, 2010, 2011; Herrera & Pozo, 2010; de Vega & Herrera, 2012). Species in the *Metschnikowia* clade have been described from five continents. Many are endemic and their distribution is determined by the restricted distribution of plants and insects (Lachance *et al.*, 2005). However, collecting efforts have not been uniform and surprisingly, given that the

African continent is a likely hot-spot of biodiversity for yeasts (Robert *et al.*, 2006) and has regions that are reservoirs of plant and entomofauna biodiversity (Myers *et al.*, 2000; Scholtz & Mansell, 2009), very few investigations of yeasts associated with angiosperms and their insect pollinators have been conducted in Africa.

During a study of yeasts associated with floral nectar in the KwaZulu-Natal region of South Africa, 26 isolates of a novel ascospore yeast species were obtained from floral nectar of the beetle-pollinated common sugarbush [*Protea caffra* (Proteaceae)], three species of cetonine beetles (*Atrichelaphinis tigrina*, *Cyrtothyrea marginalis* and *Trichostetha fascicularis*), one species of hopliine beetle (*Heterochelus* sp.) and drosophilid flies that were visiting *Protea* flowers. These strains are shown here to represent a novel species that belongs to the *Metschnikowia* clade, for which the name *Metschnikowia proteae* sp. nov. is proposed. The ecology and phylogenetic relationships of this novel yeast species will be discussed in relation to its potential host species. We also report the isolation of two additional strains associated with the beetle *Heterochelus* sp., each representing two novel undescribed *Candida* species (*Candida* sp. 1 EBDM2Y3 and *Candida* sp. 2 EBDM8Y1) related to the *Metschnikowia* clade. Description of these strains as members of novel species is deferred until a more representative sample is available.

METHODS

We examined 20 nectar samples of *Protea caffra* flowers, each corresponding to a fully dehiscent flower from a different plant, and 51 individual insects corresponding to the following species: *Atrichelaphinis tigrina* (Coleoptera: Scarabaeidae: Cetoniinae, $n=10$), *Cyrtothyrea marginalis* (Coleoptera: Scarabaeidae: Cetoniinae, $n=10$), drosophilid flies (Diptera: Drosophilidae, $n=10$), *Heterochelus* sp. (Scarabaeidae: Hopliinae, $n=10$), *Trichostetha fascicularis* (Coleoptera: Scarabaeidae: Cetoniinae, $n=2$) and *Apis mellifera scutellata* (Hymenoptera, Apidae, $n=9$). All insects were collected in the field while they were visiting *Protea caffra* flowers. Flowers and insects were collected on Mount Gilboa in the Karkloof Range, KwaZulu-Natal Province, South Africa (29° 16' 58.24" S 30° 17' 31.93" E; 1520 m above sea-level). Nectar samples were collected in 2008 ($n=4$) and 2010 ($n=16$). All insects were collected in 2008.

Yeast isolation from nectar and insects. Five microlitres of nectar was extracted from *Protea* flowers using sterile microcapillaries and then diluted in 500 μ l sterile MilliQ water. Twenty microlitres of each nectar dilution was streaked with a sterile loop onto YM agar plates (2.0% agar, 1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 0.01% chloramphenicol, pH 6.0). Insects were collected aseptically in the field, carried to the laboratory, placed into YM agar plates, and allowed to walk on the agar for 10 min after which they were removed. Plates were incubated at room temperature (22–25 °C).

A representative colony of each different yeast morphotype was purified and preserved at –80 °C on glycerol 10% and using the Microbank system (Pro-Lab diagnostics) for later identification.

Strain characterization. Cultures were characterized by the standard methods of Yarrow (1998). Formation of pseudohyphae was examined using a modified Dalmau plate (M. Sipiczki, personal communication).

Briefly, the agar was poured into a Petri dish containing a sterile microscope slide such that the slide itself was covered with a thin layer of medium. This prevented filamentous structures from growing in a deep submerged fashion as is often the case with the traditional Dalmau technique. Mating compatibility was investigated for all isolates by mixing pairs of cultures on yeast carbon base plus 0.01% ammonium sulfate and 1.5% agar (YCBAS), yeast carbon base plus 0.01% yeast extract (YCBY), and dilute (1:10 and 1:20) V8. Cultures were incubated at room temperature (22–25 °C) and examined periodically.

rDNA amplification and phylogenetic analysis. Yeasts were identified by sequencing the D1/D2 domain of the 26S rRNA gene following the methods of Kurtzman & Robnett (1998) and Lachance *et al.* (1999). Sequences were edited using Sequencher 4.9 (Gene Codes). The generated sequences were aligned with type strain sequences from related species (retrieved from GenBank) using M-Coffee (Wallace *et al.*, 2006). Ambiguously aligned regions were eliminated using the program Gblocks (Talavera & Castresana, 2007). DNA sequence variation was used to reconstruct phylogenetic relationships by neighbour-joining (NJ). The NJ (Saitou & Nei, 1987) analysis was performed in MEGA5 (Tamura *et al.*, 2011) using the Kimura two-parameter distance correction (Kimura, 1980). Bootstrap values (Felsenstein, 1985) were obtained from 10 000 random resamplings. See supplementary material available in IJSEM Online for additional phylogenetic analysis under Bayesian Inference (BI). *Candida ubatubensis* CBS 10003^T was used as outgroup.

RESULTS AND DISCUSSION

Species delineation

Comparisons of the D1/D2 domains of the large subunit rRNA gene and physiological profiles demonstrated that the 28 isolates are representative of three undescribed species in the *Metschnikowia* clade. Twenty-six isolates assigned to the novel species *Metschnikowia proteae* sp. nov. differed in the D1/D2 complete sequence by 22 substitutions and 8 gaps from the closest relative, strain EBDM8Y1 (*Candida* sp. 2), and by 98 substitutions and 17 gaps from strain EBDM2Y3 (*Candida* sp. 1). When compared with their closest described relatives, *M. proteae* differed by 89 substitutions and 10 gaps from *Metschnikowia hibisci*, by 112 substitutions and 12 gaps from *Metschnikowia aberdeeniae*, and by 106 substitutions and 18 gaps from *Metschnikowia shivogae*. The strains of *M. proteae* were recovered from seven nectar samples of *P. caffra* collected in 2008 and 2010 and from 19 specimens of all sampled insect species except *Apis mellifera scutellata* (Table 1). Two D1/D2 sequence variants differing by one nucleotide substitution were identified in 18 and 8 strains, respectively. The strains are haploid mating types that formed asci typical of *Metschnikowia* when mixed in compatible pairs. The absence of any mating reaction with strains of other species, including strains EBDM8Y1 and EBDM2Y3 and the mating types of *Metschnikowia continentalis*, demonstrated that *M. proteae* is reproductively isolated (biological species) and further required that mating types be designated arbitrarily as h^+ for the type strain and h^- for the allotype. This contrasts with the rest of the large-spored *Metschnikowia* clade, where some mating response

Table 1. Origin of strains used in this study

Strain accession numbers are those in the yeast culture collection of the Evolutionary Ecology Department, Doñana Biological Station, Spanish National Research Council, CSIC (EBD), the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS), and the USDA ARS Culture Collection Culture Collection, Peoria, IL, USA (NRRL). Mating type: T=type; AT=allotype.

Strain	Isolation source	Mating type	GenBank no.
<i>Metschnikowia proteae</i>			
EBDCdVSA 34_1	Nectar*	AT	JN935043
EBDCdVSA 35_1	Nectar*	T	JN935035
EBDCdVSA 36_1	Nectar*	T	JN935044
EBDCdVSA 37_1	Nectar*	T	JN935036
EBDCdVSA 39_1	Nectar*	AT	JN935028
EBDCdVSA 46_1	Nectar*	T	JN935049
EBDSA45_2	Nectar*	T	JN935026
EBDA7Y1	Beetle sp1†	AT	JN935034
EBDA10Y1	Beetle sp1†	AT	JN935042
EBDC1Y3	Beetle sp2‡	AT	JN935048
EBDC2Y2 ^{AT} (=CBS 12521=NRRL Y-48785)	Beetle sp2‡	AT	JN935027
EBDC3Y1	Beetle sp2‡	AT	JN935050
EBDC4Y1	Beetle sp2‡	AT	JN935029
EBDC6Y1	Beetle sp2‡	AT	JN935037
EBDF1Y1	Fly§	AT	JN935045
EBDF2Y1	Fly§	AT	JN935051
EBDM1Y1	Beetle sp3	AT	JN935030
EBDM2Y1	Beetle sp3	AT	JN935038
EBDM3Y1	Beetle sp3	AT	JN935052
EBDM6Y1	Beetle sp3	AT	JN935031
EBDM7Y1	Beetle sp3	AT	JN935039
EBDM8Y3	Beetle sp3	AT	JN935053
EBDM9Y1	Beetle sp3	AT	JN935032
EBDM10Y1	Beetle sp3	AT	JN935040
EBDT1Y1 ^T (=CBS 12522 ^T =NRRL Y-48784 ^T)	Beetle sp4¶	T	JN935033
EBDT2Y1	Beetle sp4¶	AT	JN935041
<i>Candida</i> sp. 1			
EBDM2Y3	Beetle sp3	–	JN935046
<i>Candida</i> sp. 2			
EBDM8Y1	Beetle sp3	–	JN935047

*Floral nectar of *Protea caffra*.

†*Atrichelaphinis tigrina* (Coleoptera: Scarabaeidae: Cetoniinae).

‡*Cyrtothyrea marginalis* (Coleoptera: Scarabaeidae: Cetoniinae).

§Drosophilidae species.

||*Heterochelus* sp. (Scarabaeidae: Hopliinae).

¶*Trichostetha fascicularis* (Coleoptera: Scarabaeidae: Cetoniinae).

can be observed when strains of opposite mating types are mixed (Lachance *et al.*, 2006), allowing a non-arbitrary mating type assignment. The two additional strains considered in this study represent two novel undescribed species provisionally assigned to the genus *Candida* (*Candida* sp. 1 EBDM2Y3 and *Candida* sp. 2 EBDM8Y1; Table 1), as a sexual cycle has not been observed for those strains. Both were isolated from specimens of *Heterochelus* sp. As it is highly probable that these strains are haploid mating types of two heterothallic *Metschnikowia* species, we feel that formal species descriptions based on incomplete biological information would be premature.

Comparison with other large-spored *Metschnikowia* species

M. proteae differs in a number of ways from other members of the large-spored *Metschnikowia* clade defined in its broadest sense to include *Metschnikowia hawaiiensis*, *Metschnikowia arizonensis*, *M. hibisci* and their respective relatives (Fig. 1). Among these species, proliferation is normally by way of budding cells that may form pseudohyphae under certain conditions (Lachance, 2011). In contrast, in *M. proteae*, the cells tend to remain attached and to form dense chains. A similar tendency is observed to a lesser

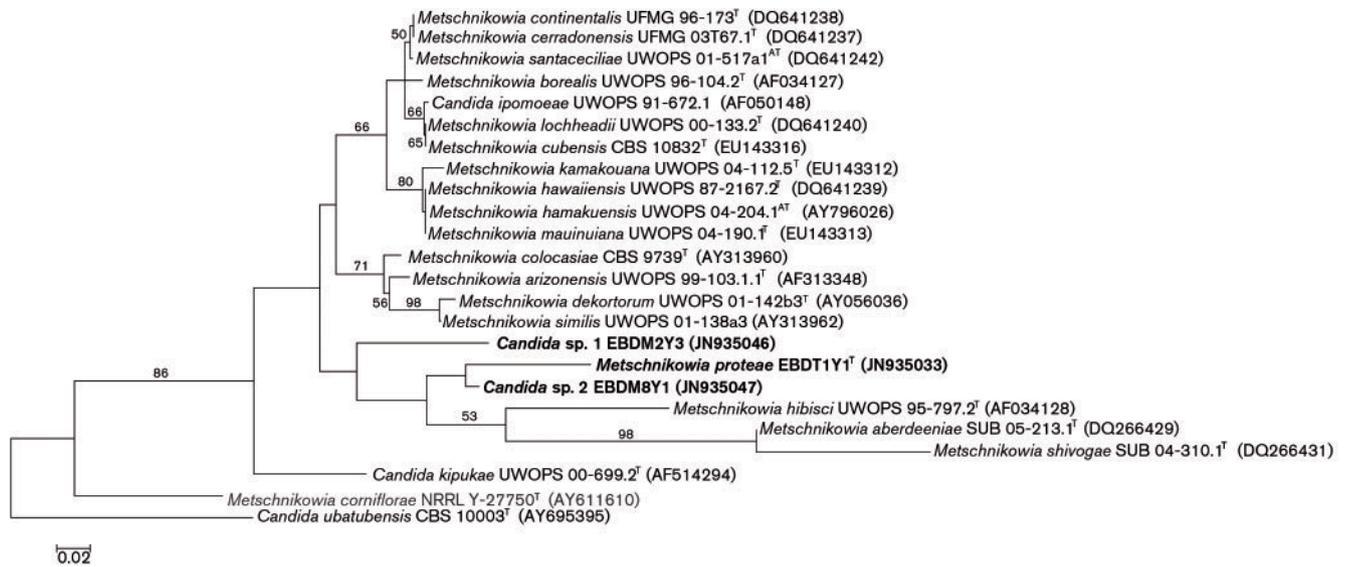


Fig. 1. Phylogeny of *Metschnikowia proteae* sp. nov. and related species based on NJ analysis of D1/D2 rDNA sequences. Numbers above branches show NJ bootstrap support. *Candida ubatubensis* CBS 10003^T was used as outgroup. Branch lengths are scaled to the expected number of nucleotide substitutions per site; bar, 0.02 nucleotide substitutions per site. Only bootstrap values $\geq 50\%$ are shown. GenBank accession numbers of all sequences are indicated after strain name. T, type strain; AT, allotype strain. Culture collection prefixes: NRRL Y, ARS Culture Collection; EBD, Estación Biológica de Doñana; CBS, Centraalbureau voor Schimmelcultures; SUB, Syracuse University; UFMG, Universidade Federal de Minas Gerais; UWOPS, University of Western Ontario.

extent in *Candida ipomoeae*. The small, high density area reported in the cytoplasm of the ascospores, near the distal end of the ascus (Fig. 2d), is also present in all members of the clade, but in *M. proteae*, the position of the dense area may vary as indicated below. Particularly remarkable was the unprecedented case of a four-spored ascus (Fig. 2g). To our knowledge, this has never been reported for species of the genus *Metschnikowia*. Also unique to *M. proteae* is the presence of a phase-dark area near the ascospore tip. The significance of these structures is not known.

Phylogenetic position

NJ analysis inferred a relationship between *Metschnikowia proteae* and the strain EBDM8Y1 (hereafter *Metschnikowia proteae* subclade) but with weak bootstrap support (BS) ($< 50\%$ NJ-BS) (Fig. 1) and retrieved an ill-defined position of the *M. proteae* subclade with respect to related species suggesting a relationship between the *M. proteae* subclade and the basal members of the large-spored *Metschnikowia* clade associated with floricolous insects from equatorial Africa (*M. aberdeeniae*, *M. shivogae*) and Australia (*M. hibisci*). The results from the BI analysis (see Fig. S1) were consistent with the NJ tree (Fig. 1), but with poorer resolution and similar or higher support values in most cases. The BI reconstruction retrieved the *M. proteae* subclade and its close relationship to large-spored equatorial African and Australian species but with no support (see supplementary material in IJSEM Online for additional information about NJ and BI results).

Ecology

The high number of yeast strains isolated from *Protea* floral nectar and insects allowed us to generate plausible generalizations about the natural habitat of *M. proteae*. The species was isolated from different individual plants and five insect species on two separate occasions (austral summers of 2008 and 2010), highlighting its prevalence in the sampling area. *M. proteae* and the two related strains were only a subset of the microbiota isolated from insects and nectar. The body surfaces of the insects harboured a diverse yeast biota comprising several species with affinities to the genera *Kluyveromyces*, *Lodderomyces*, *Metschnikowia*, *Meyerozyma* and *Wickerhamiella*, and the nectar yielded several species of *Hanseniaspora*, *Lodderomyces* and *Yarrowia* (C. de Vega and others, unpublished data).

All evidence available points towards *M. proteae* being engaged in a close, specific relationship with *Protea* plants and their associated insects. We have inspected nectar samples from 40 other taxonomically diverse South African plant species in the KwaZulu-Natal Province, and *M. proteae* was never recovered (C. de Vega and others, unpublished data). In many of the plant species examined the nectar contained nearly mono-specific populations of *Metschnikowia koreensis*, *Metschnikowia reukaufii* or *Candida rancensis*. The differences in the nectar microbiota between nectar samples of *P. caffra* and those of many other plant species may be attributable to the activity of different pollinators. Yeasts are actively vectored by insects (Ganter, 2011), and *P. caffra* flowers are visited mostly by

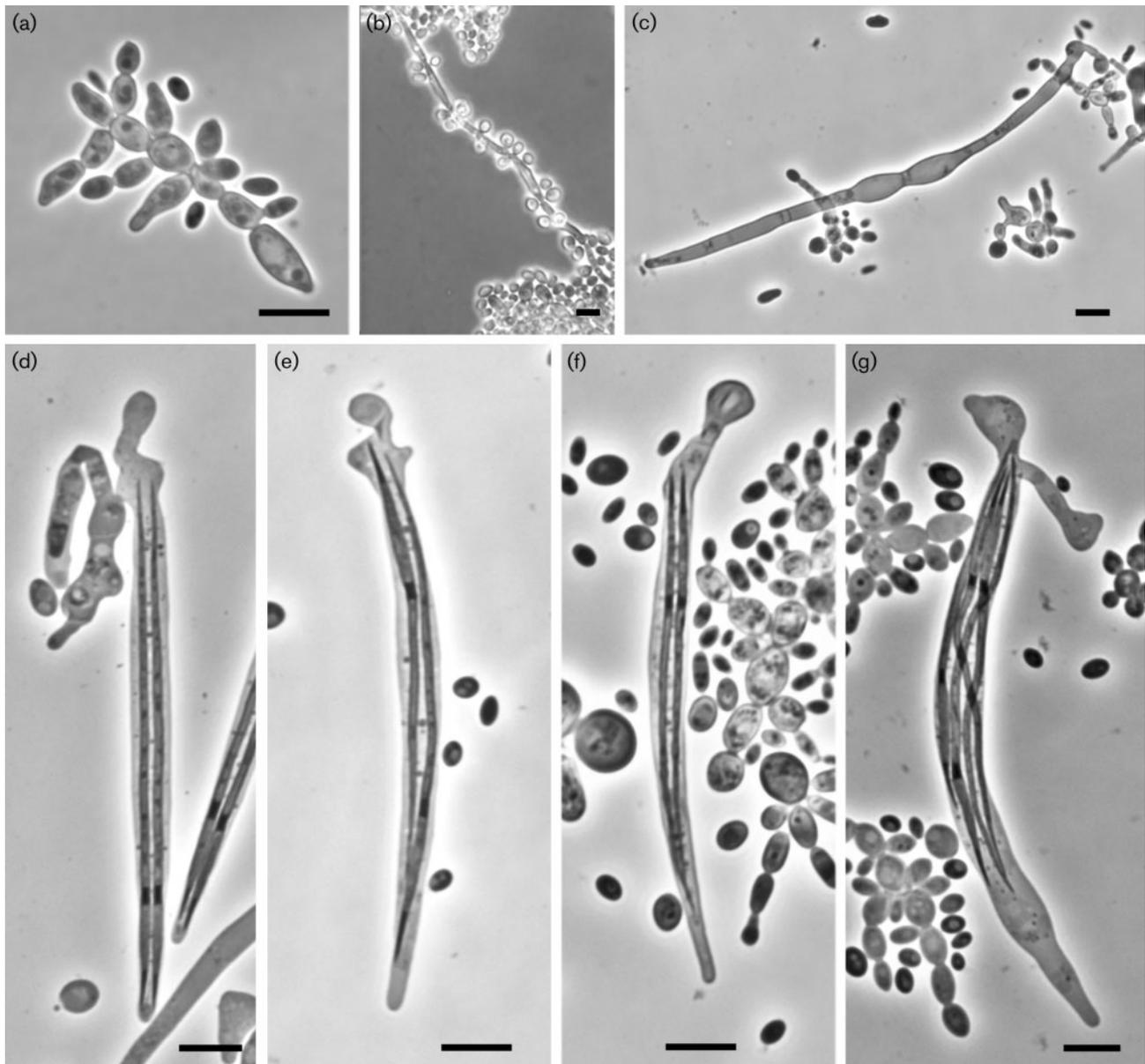


Fig. 2. Phase-contrast micrographs of *Metschnikowia proteae*. (a) Vegetative cells on YM agar. (b) Pseudohyphae on YCBY agar. (c) Mixed culture of strains EBDSA45_2 and EBDF2Y1 showing mating reaction. (d–g) Asci obtained from mixing strains EBDT1Y1^T and EBDC2Y2^{AT} (d) and EBDSA45_2 and EBDT2Y1 (e–g) after 2 days' incubation on YCBAS agar supplemented with 0.01% ammonium sulfate and 1.5% agar. Asci were either two- (d–f) or four-spored (g). Note the position of the two ascospores with a short phase-dark zone about 1/4 from the distal end of the ascus, and a dark zone in the tip (d), one ascospore with a dark zone on the proximal and one on the distal end (e), or two ascospores with the dark zone at the proximal end (f). Bars, 10 μ m.

beetles. The other plant species examined are pollinated mainly by bees, butterflies and birds (de Vega *et al.*, 2009) that are likely to have their own associated microbiota. In this respect, it is worth noting that *M. proteae* was not found on *Apis mellifera scutellata* individuals collected as they were visiting *P. caffra* flowers, suggesting that this bee is not a suitable vector for *M. proteae*. This fits well within the general observation that the entire large-spored *Metschnikowia* clade is beetle-associated

and conspicuously absent from bees that visit the same flowers, although they may be present in sympatric drosophilids. Interestingly, members of the *M. hawaiiensis* and *M. arizonensis* subclades also appear to be restricted to beetles of the family Nitidulidae (Lachance, 2011), whereas those of the remaining subclade have been found in several families, including Nitidulidae, Meloidae and Buprestidae (Lachance *et al.*, 2006, 2008). We now add to these two subfamilies in the

Table 2. Growth characteristics of *Metschnikowia proteae* and related strains

Taxa: 1, *Metschnikowia proteae*; 2, strain EBDM8Y1; 3, strain EBDM2Y3. The following tests gave positive responses for all taxa: assimilation of glucose, sucrose, maltose, melezitose, sorbose, 2-ketogluconic acid and *N*-acetylglucosamine, growth in the absence of amino acids, growth at 10 °C, growth at 31 °C, utilization of ethylamine and cadaverine, growth in the presence of 5% sodium chloride, fermentation of glucose, and growth in the presence of 50 mg CTAB l⁻¹. The following tests gave negative results: assimilation of inulin, raffinose, melibiose, lactose, methyl α -D-glucoside, soluble starch, L-rhamnose, L-arabinose, D-arabinose, methanol, 1-propanol, 2-propanol, 1-butanol, glycerol, erythritol, galactitol, inositol, D-glucuronic acid, lactic acid, malic acid, and acetone, growth in vitamin-free medium, growth at 8 °C, growth at 33 °C, hydrolysis of gelatin or casein, utilization of sodium nitrate or nitrite, growth in the presence of 10 mg cycloheximide l⁻¹, growth in the presence of 1% acetic acid, and growth in the presence of 6% ethanol. Strains EBDM2Y3 and EBDM8Y1 represent the two novel undescribed species *Candida* sp. 1 and *Candida* sp. 2. +, Positive; -, negative; s, slow; w, weak.

Characteristic	1	2	3
Galactose	+ (sometimes s)	w	+
Trehalose	- (sometimes w or s)	-	-
Cellobiose	- (rarely w or s)	-	+
Salicin	w (rarely - or s)	-	+
Xylose	s (rarely w or -)	-	-
Ribose	- (rarely w)	-	w
Ethanol	s (rarely +)	+	s
Ribitol	- (rarely w)	-	s
Xylitol	-, w or s	s	s
Mannitol	+ (sometimes s or w)	+	+
Glucitol	+ (rarely w or -)	w	+
Succinic acid	w (rarely -)	w	w
Citric acid	- (rarely w)	s	s
Gluconic acid	w (rarely - or +)	-	+
Glucono- δ -lactone	w (sometimes -)	w	s
D-Glucosamine	w or -	s	+
Ethyl acetate	- (rarely w or +)	-	w
Hexadecane	- (rarely w)	w	w
Hydrolysis of Tween 80	-	-	+
Lysine	+ (sometimes s or w)	w	+
NaCl (10%)	w	+	w
Glucose (50%)	-	-	w

Scarabaeidae, Cetoniinae and Hopliinae, and confirm transfer of the yeasts to drosophilid flies in South African flowers. It has been suggested that flower-visiting insects may themselves be the hosts of certain *Metschnikowia* species or alternatively they may act as vectors between flowers, which may be the actual hosts (Lachance *et al.*, 1990; Brysch-Herzberg, 2004). The

answer to this question will require determination of where proliferation of the yeasts takes place. Our study adds credibility to the hypothesis that the geographical distribution of yeast is largely determined by the distribution of their host species.

Latin diagnosis of *Metschnikowia proteae* de Vega, Guzmán, Lachance et Herrera sp. nov.

In medio agaro YM post dies tres cellulae in catenatis brevibus; cellulae ellipsoideae (2–3 × 5–8 µm). Post unum mensem velum non formatur. Cultura candida, humilis-convexa et rugosa. In agaro YCBY post dies 14 pseudohyphae formatur. Glucosum fermentatur. Glucosum, saccharosum, galactosum (aliquando lente), maltosum, melezitosum, salicinum (variabile), L-sorbosum, D-xylosum (variabile), ethanolum, glycerolum, D-mannitolum, glucitolum (rare exigue aut non), acidum succinicum (exiguum, rare non), acidum D-gluconicum (exiguum et variabile) et N-acetylglucosaminum (exiguum) assimilantur at non inulinum, raffinolum, melibiosum, trehalosum (variabile), methyl α -D-glucosidum, lactosum, cellobiosum (variabile), amyllum solubile, L-rhamnosum, L-arabinosum, D-arabinosum, D-ribosum (exiguum), methanolum, 1-propanol, 2-propanolum, 1-butanolum, erythritolum, ribitolum (rare exiguum), xylitolum (variabile), galactitolum, meso-inositolum, acidum D-gluconicum, acidum lacticum, acidum citricum (rare exiguum), acidum malicum, D-gluconolactonum, 2-keto-D-gluconatum, D-glucosaminum, hexadecanum (rare exiguum), acetonum nec ethyl acetatum (variabile). Ethylaminum, lysinum et cadaverinum assimilantur at non natrium nitricum et natrium nitrosolum. Ad crescentiam vitaminarum externarum necessariae sunt. Augmentum in 31 °C at non 33 °C. Habitat nectar nectarum Protea caffra. Typus EBDT1Y1^T (=NRRL Y-48784^T). In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 12522^T typus stirps deposita est.

Description of *Metschnikowia proteae* de Vega, Guzmán, Lachance & Herrera sp. nov.

Metschnikowia proteae (pro'te.ae. L. gen. fem. n. *proteae* of *Protea*, in reference to the plant *Protea caffra*, from which the isolates were recovered).

After 3 days on YM agar at 25 °C, the cells are ellipsoid, 2–3 × 5–8 µm, and occur in small chains. Budding is multilateral (Fig. 2a). After 5 days, the colony is white, low-convex, umbonate, verrucose with a smooth centre and a lacy edge, and with a clumpy texture. Pellicles are not formed on liquid media. After 2 weeks at 25 °C on YCBY agar, the slide culture shows mostly clusters of individual cells interconnected by pseudohyphae consisting of elongate cells of up to 30 µm long with small clusters of spheroid blastoconidia (Fig. 2b). A few undifferentiated pseudohyphae are also formed at the edge of the growth. After 3–4 days, mixed cultures of complementary mating types produce a low to moderate number of conjugated cells, zygotes, and mature asci (7–10 × 70–120 µm) containing

two aciculate ascospores (0.8–1.2 µm wide) that fill three-quarters or more of the ascus length (Fig. 2c–f). The asci are persistent under the conditions used. Asci containing four ascospores may occur (Fig. 2g). Most ascospores have a short, phase-dark zone usually 1/4 from the distal end of the ascus, and another dark zone at the tip itself (Fig. 2d). Exceptionally, some asci contain ascospores with the dark zones near the proximal end of the ascus or even two ascospores with different orientations (Fig. 2e–g). Sporulation is best observed on YCBY and YCBAS agars. Glucose is fermented. Other growth responses are given in Table 2.

The type strain is EBDT1Y1^T (=CBS 12522^T=NRRL Y-48784^T), recovered from the beetle *Trichostetha fascicularis*, and the allotype is EBDC2Y2 (=CBS 12521=NRRL Y-48785), recovered from the beetle *Cyrtothyrea marginalis*, both from Mount Gilboa in the Karkloof Range, KwaZulu-Natal Province, South Africa.

ACKNOWLEDGEMENTS

We thank E. López, L. Cabral and P. Bazaga for technical assistance and Dr R. G. Albaladejo for comments on the manuscript. This work was supported by funds from Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía (Proyecto de Excelencia P09-RNM-4517 to C. M. H.), Ministerio de Ciencia e Innovación (grant CGL2010-15964 to C. M. H. and Juan de la Cierva Program to C. d. V.), Consejo Superior de Investigaciones Científicas and Fondo Social Europeo (JAE-DOC Program to B. G.) and the Natural Science and Engineering Research of Canada (M. A. L.).

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