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}5CBS 12522^{T}5NRRL Y-48784^T; allotype strain EBDC2Y25CBS 125215NRRL 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 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

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

The GenBank/EMBL/DDBJ 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.

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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 ascosporic yeast species were obtained from floral nectar of the beetle-pollinated common sugarbush [Protea caffra (Proteaceae)], three species of cetoniine beetles (Atrichelaphinis tigrina, Cyrtothyrea marginalis and Trichostetha fascicularis), one species of hopliinine 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 dehisced 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 (29u 16' 58.24" S 30u 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 ml 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 uC).

A representative colony of each different yeast morphotype was purified and preserved at 280 uC 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 uC) 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: T5type; AT5allotype.

Strain	Isolation source	Mating type	GenBank no.
Metschnikowia proteae			
EBDCdVSA 34_1	Nectar*	AT	JN935043
EBDCdVSA 35_1	Nectar*	T	JN935035
EBDCdVSA 36_1	Nectar*	T T	JN935044
EBDCdVSA 37_1	Nectar*	AT	JN935036
EBDCdVSA 39_1	Nectar*	T	JN935028
EBDCdVSA 46_1	Nectar*	T	JN935049
EBDSA45_2	Nectar*	AT	JN935026
EBDA7Y1	Beetle sp1D	AT	JN935034
EBDA10Y1	Beetle sp1D	AT	JN935042
EBDC1Y3	Beetle sp2d		JN935048
EBDC2Y2^{AT} (5CBS 125215NRRL Y-48785)	Beetle sp2d	AT	JN935027
EBDC3Y1	Beetle sp2d	AT	JN935050
EBDC4Y1	Beetle sp2d	AT	JN935029
EBDC6Y1	Beetle sp2d	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} (5CBS 12522^{T}5NRRL Y-48784^{T})	Beetle sp4¶	T	JN935033
EBDT2Y1	Beetle sp4¶	AT	JN935041
Candida sp. 1			
EBDM2Y3	Beetle sp3‖	–	JN935046
Candida sp. 2			
EBDM8Y1	Beetle sp3‖	–	JN935047

*Floral nectar of Protea caffra.
DAtrichelaphinis tigrina (Coleoptera: Scarabaeidae: Cetoniinae).
dCyrtothyrea 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

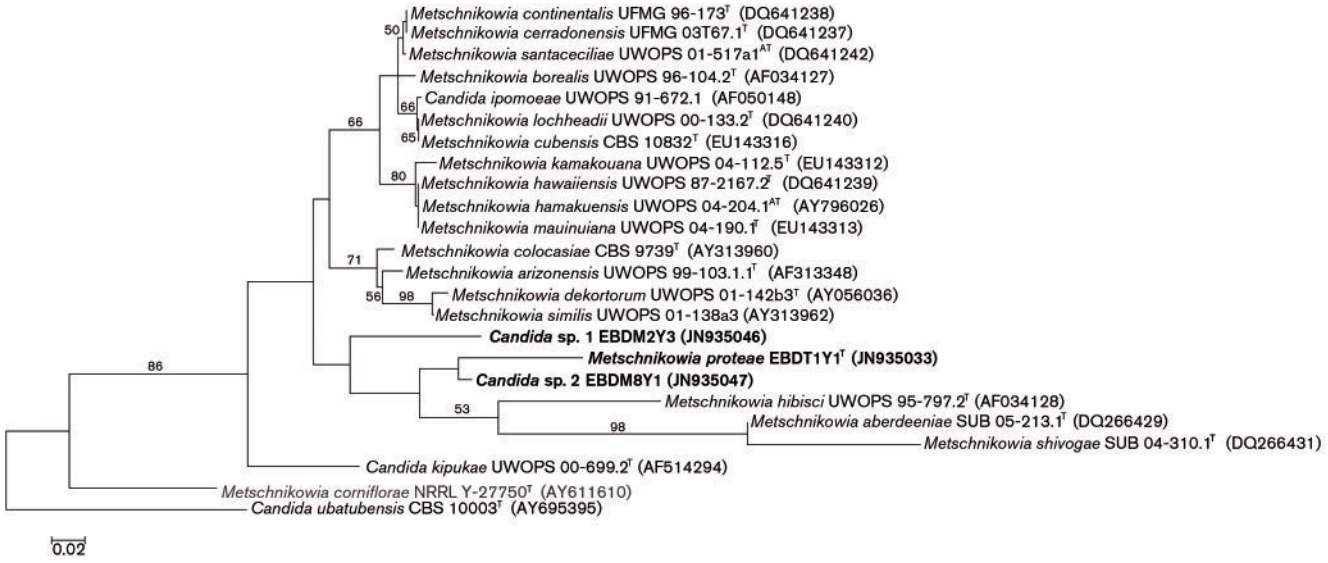

Fig. 1. Phylogeny of Metschnikowia proteae sp. nov. and related species based on NJ analysis of D1/D2 rDNA sequences. Numbers above branches show NJ bootstrap support. Candida ubatubensis CBS 10003^T was used as outgroup. Branch lengths are scaled to the expected number of nucleotide substitutions per site; bar, 0.02 nucleotide substitutions per site. Only bootstrap values ⩾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 monospecific 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

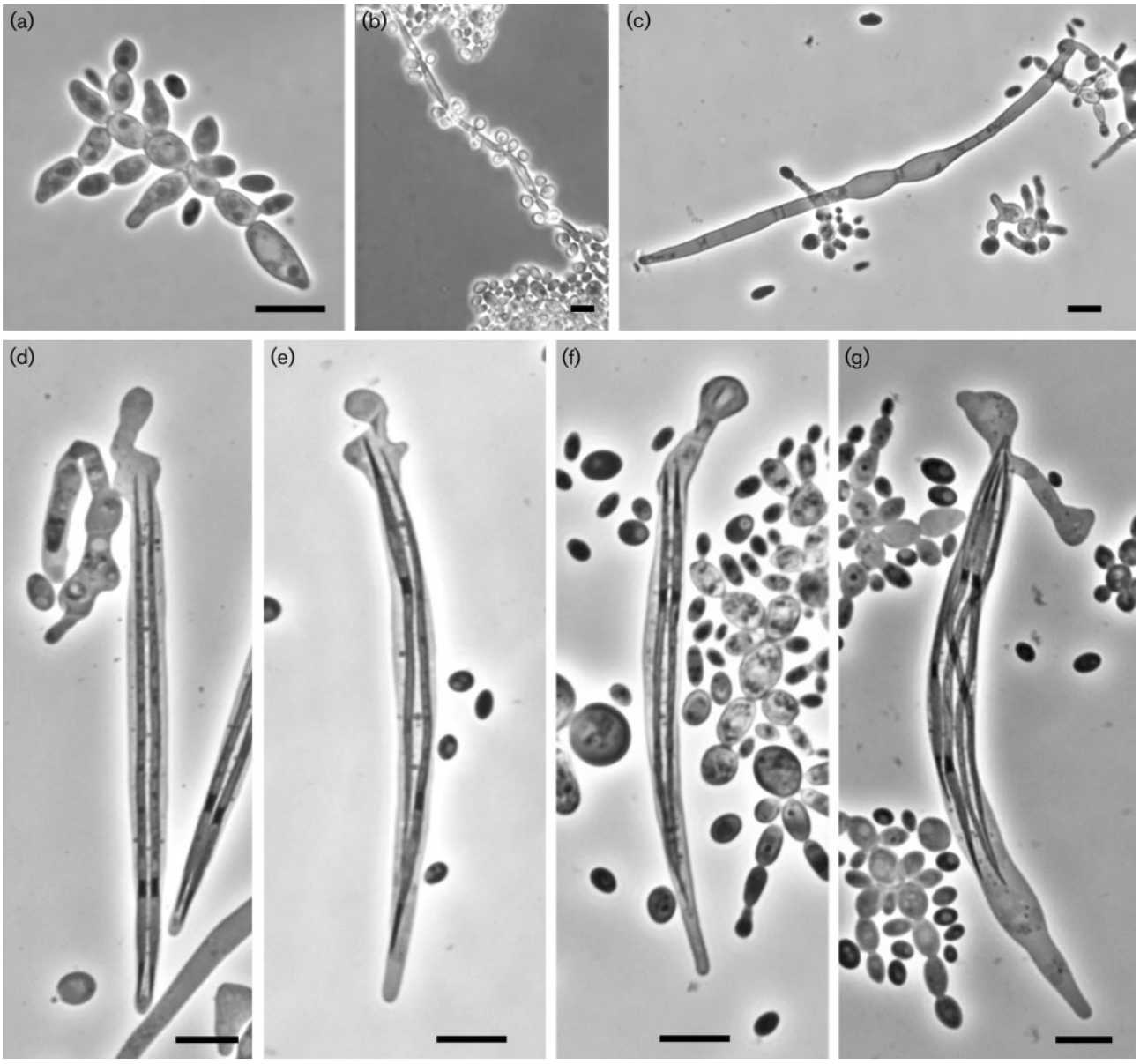

Fig. 2. Phase-contrast micrographs of Metschnikowia proteae. (a) Vegetative cells on YM agar. (b) Pseudohyphae on YCBY agar. (c) Mixed culture of strains EBDSA45_2 and EBDF2Y1 showing mating reaction. (d–g) Asci obtained from mixing strains EBDT1Y1^{T} and EBDC2Y2^{AT} (d) and EBDSA45_2 and EBDT2Y1 (e–g) 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 μm.

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 uC, growth at 31 uC, 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^{21}. The following tests gave negative results: assimilation of inulin, raffinose, melibiose, lactose, methyl a-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 uC, growth at 33 uC, hydrolysis of gelatin or casein, utilization of sodium nitrate or nitrite, growth in the presence of 10 mg cycloheximide l^{21}, 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; 2, negative; s, slow; w, weak.

Characteristic	1	2	3
Galactose	+ (sometimes s)	w	+
Trehalose	2 (sometimes w or s)	2	2
Cellobiose	2 (rarely w or s)	2	+
Salicin	w (rarely 2 or s)	2	+
Xylose	s (rarely w or 2)	2	2
Ribose	2 (rarely w)	2	w
Ethanol	s (rarely +)	+	s
Ribitol	2 (rarely w)	2	s
Xylitol	2, w or s	s	s
Mannitol	+ (sometimes s or w)	+	+
Glucitol	+ (rarely w or 2)	w	+
Succinic acid	w (rarely 2)	w	w
Citric acid	2 (rarely w)	s	s
Gluconic acid	w (rarely – or +)	2	+
Glucono-d-lactone	w (sometimes 2)	w	s
D-Glucosamine	w or 2	s	+
Ethyl acetate	2 (rarely w or +)	2	w
Hexadecane	2 (rarely w)	w	w
Hydrolysis of Tween 80	2	2	+
Lysine	+ (sometimes s or w)	w	+
NaCl (10%)	w	+	w
Glucose (50%)	2	2	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 brevis; cellulae ellipsoidae (2–3×5–8 mm). 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 (exigue, rare non), acidum D-gluconicum (exigue et variabile) et N-acetylglucosaminum (exigue) assimilantur at non inulinum, raffinosum, melibiosum, trehalosum (variabile), methyl a-D-glucosidum, lactosum, cellobiosum (variable), amylum solubile, L-rhamnosum, L-arabinosum, D-arabinosum, D-ribosum (exigue), methanolum, 1-propanol, 2-propanolum, 1-butanolum, erythritolum, ribitolum (rare exigue), xylitolum (variabile), galactitolum, meso-inositolum, acidum D-glucoronicum, acidum lacticum, acidum citricum (rare exigue), acidum malicum, D-gluconolactonum, 2-keto-D-gluconatum, D-glucosaminum, hexadecanum (rare exigue), acetonum nec ethyl acetas (variabile). Ethylaminum, lysinum et cadaverinum assimilantur at non natrium nitricum et natrium nitrosum. Ad crescentiam vitaminae externae necessaria sunt. Augmentum in 31 uC at non 33 uC. Habitat nectar nectarum Protea caffra. Typus EBDT1Y1^T (5NRRL 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 (pro9te.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 uC, the cells are ellipsoid, 2–3×5–8 mm, 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 uC on YCBY agar, the slide culture shows mostly clusters of individual cells interconnected by pseudohyphae consisting of elongate cells of up to 30 mm 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 mm) 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.

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