IJSEM Papers in Press. Published August 8, 2014 as doi:10.1099/ijs.0.068445-0

Metschnikowia drakensbergensis sp. nov. and *Metschnikowia caudata* sp. nov., two endemic yeasts associated with *Protea* flowers in South Africa

Clara de Vega[1*], Beatriz Guzmán[2], Sandy–Lynn Steenhuisen[3], Steven D. Johnson[4], Carlos M. Herrera[1], and Marc–André Lachance[5]

[1]Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Avenida de Américo Vespucio s/n, 41092 Sevilla, Spain

[2]Real Jardín Botánico, CSIC, Plaza de Murillo 2, 28014 Madrid, Spain

[3]Department of Biological Sciences, University of Cape Town, P/Bag, Rondebosch, 7701, South Africa

[4]School of Life Sciences, University of KwaZulu–Natal, P/ Bag X01, Scottsville, Pietermaritzburg 3209, South Africa

[5]Department of Biology, University of Western Ontario, N6A 5B7, London, Ontario, Canada

* Author for correspondence: Clara de Vega. Tel.: +34 954466700. Fax: +34 954621125. e-mail: cvega@ebd.csic.es

Running title: Two South African *Metschnikowia* species

The subject category: New taxa, Unicellular Eukaryotes

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are listed in Table 1 and Table S1.

Abbreviations: NJ, Neighbor–Joining; *ACT1*, actin gene; *RPB2*, RNA polymerase II gene; *EF2*, elongation factor 2 gene.

Abstract

In a taxonomic study of yeasts recovered from nectar of flowers and associated insects in South Africa, eleven strains were found to represent two novel species. Morphological and physiological characteristics and sequence analyses of the D1/D2 large subunit rRNA gene, as well as the actin, RNA polymerase II, and elongation factor 2 genes showed that the two novel species belonged to the genus *Metschnikowia*. *Metschnikowia drakensbergensis* sp. nov. was recovered from nectar of *Protea roupelliae* and the beetle *Heterochelus* sp. This species belongs to the large-spored *Metschnikowia* clade and is closely related to *M. proteae*, with which mating reactions and single-spored asci were observed. *Metschnikowia caudata* sp. nov. was isolated from nectar of *P. dracomontana, P. roupelliae, P. subvestita* and a honey bee, and is a sister species to *Candida hainanensis* and *M. lopburiensis*. Analyses of the four genes demonstrated the existence of three separate phylotypes. Intraspecies matings lead to the production of mature asci of unprecedented morphology, with a long, flexuous tail. A single ascospore was produced in all compatible crosses, regardless of sequence phylotype.

The two species appear to be endemic to South Africa. The ecology and habitat specificity of these novel species is discussed in terms of host plant and insect host species. The type cultures are: *Metschnikowia drakensbergensis* (type strain EBD-CdVSA09-2^{T}=CBS 13649^{T}=NRRL Y-63721^{T}, MycoBank no. MB809688; allotype EBD-CdVSA10-2^{A}=CBS13650^{A}=NRRL Y-63720^{A}); and *Metschnikowia caudata* (type strain EBD-CdVSA08-1^{T}=CBS 13651^{T}=NRRL Y-63722^{T}, MycoBank no. MB809689; allotype EBD-CdVSA57-2^{A}=CBS 13729^{A}=NRRL Y-63723^{A}).

Introduction

Flowers offer different food rewards to pollinators in exchange for pollination service. The primary floral reward is floral nectar, a complex fluid mainly containing sugars and amino acids that play a decisive role in the establishment of most plant-pollinator mutualisms (Simpson & Neff, 1983; Dupont *et al*., 2004; Nicolson, 2007). However, floral nectar is not exclusively used by pollinators. Its composition makes it a favourable environment for the growth of microorganisms, and it is exploited by floricolous yeasts that are vectored from flower to flower by floral visitors (Brysch-Herzberg, 2004; Herrera *et al*., 2008; Belisle *et al*., 2012; de Vega & Herrera, 2013).

A large number of novel yeast species have been isolated from flowers and pollinators, reflecting the high microbial diversity associated to them. The genus *Metschnikowia* (and anamorphs in the genus *Candida*) is one of the dominant taxa found in these substrates (Lachance *et al*., 2001; Lachance, 2011). For example, the cosmopolitan *M. reukaufii*, *M. gruessii* and *M. koreensis* have been repeatedly isolated from a wide diversity of flowers and associated bee, butterfly and bird pollinators in both the Old World and the New World (Hong *et al*., 2001; Pozo *et al*., 2011; Belisle *et al*., 2012). Interestingly, flowers visited by a distinct pollinator guild, the beetles, harbour different, highly specific yeast communities that are not found in plant species pollinated by other animals (Marinoni & Lachance, 2004; Lachance *et al*., 2005; Guzmán *et al*., 2013). Particularly well-studied is the yeast biota recovered from nitidulid beetles and associated flowers, mostly including large-spored haplontic *Metschnikowia* species (e.g. Marinoni & Lachance, 2004; Lachance *et al*., 2005).

Large-spored *Metschnikowia* species associated with beetles have distinct biogeographies, and their association with particular beetles and plants with restricted

distributions may have favoured speciation by allopatry or peripatry (Lachance *et al*., 2001, 2003a,b, 2005, 2006a; Lachance & Fedor, 2014). The most striking example is the *M. hawaiiensis* subclade, composed of six described and undescribed species associated with endemic nitidulid beetles of the genus *Prosopeus* and endemic plants of Hawaii (Lachance *et al*., 2005; Guzmán *et al*., 2013). Another interesting case is a subclade typified by *M. arizonensis* (Lachance & Fedor, 2014), represented by six described and undescribed species restricted to specific locations, sometimes to a single locality in the USA, Costa Rica, Brazil, or Belize, mainly in association with species of the nitidulids *Carpophilus* and *Conotelus*. Other *Metschnikowia* species associated with flowers and beetles, but not included in the large-spored clade, also have distinct ecologies and restricted geographical distributions, for example *M. corniflorae*, associated with chrysomelid beetles and flowers in the USA (Nguyen *et al*., 2006), *M. orientalis*, isolated from nitidulid beetles in the Cook Islands and Malaysia (Lachance *et al*., 2006b), and *Candida chrysomelidarum*, found in Panama in chrysomelid beetles (Nguyen *et al*., 2006).

The diversity of beetle-associated yeasts of flowers has been explored mostly in North, Central and South America, Hawaii, and to a lesser extent in Asia. Yeasts living in association with African plants and their beetles are only beginning to receive attention, even though their diversity may plausibly be as high as or even higher than that observed in other continents. Three beetle-associated *Metschnikowia* species have been described so far in Africa (Lachance *et al*., 2006a, 2008; de Vega *et al*., 2012). In an effort to gain further insight into the yeast biota associated with plants visited by beetles in poorly studied areas, we conducted a survey in the KwaZulu-Natal Region of South Africa.

Eleven strains of two novel species were isolated from floral nectar of three species of *Protea* and associated insects. Sequence analyses of the D1/D2 regions of the large subunit rRNA gene as well as the actin (*ACT1*), RNA polymerase II (*RPB2*) and elongation factor 2 (*EF2*) genes showed that the two novel species belonged to the genus *Metschnikowia* and were phylogenetically distinct from any currently recognized species. One is part of the large-spored *Metschnikowia* clade and is closely related to the South African species *M. proteae*. The other has moderately-sized ascospores with a novel morphology. Its closest described relatives are *M. lopburiensis* and *Candida (iter. nom. Metschnikowia*) *hainanensis*, neither of which forms asci. We now describe the new species as *Metschnikowia drakensbergensis* sp. nov and *Metschnikowia caudata* sp. nov.

Methods

Collections

The origins of the strains considered in this study are described in Table 1. We examined 83 nectar samples from the following species: *Protea dracomontana* (*N* = 16), *Protea roupelliae* (*N* = 19), *Protea subvestita* (*N* = 16), and *Protea simplex* (*N* = 16). Flowers of *P. dracomontana* were collected from the Garden Castle area and *P. subvestita* from the Sani Pass area of the uKhahlamba-Drakensberg Park, and *P. roupelliae* and *P. simplex* from Mount Gilboa Estate. Sixteen samples from *P. welwitschii*, sampled in Winston Park (29º49′S 30º47′E, 530 m asl), did not yield any *Metschnikowia* species. All sites were located in KwaZulu-Natal Province, South Africa. The distances between sites ranged from 20 to 150 km. Flowers of all the plants were host to beetles and in addition, those of *P. roupelliae* and *P. subvestita* were

frequently visited by birds and those of *P. welwitschii* by bees. Samples were collected in 2011.

Flowers were cut and carried aseptically in a cooler to the lab where nectar sampling was done within a few hours after collection. Each nectar sample corresponded to a flower with fully dehisced anthers, each taken from a different plant, exposed to natural pollinator visitation. Additionally, two samples that yielded isolates of the new species were isolated from a hopliinid beetle (*Heterochelus* sp.; Scarabaeidae) and a honey bee (*Apis mellifera scutellata*) in previous sampling carried out in 2008 from insects visiting *Protea* flowers. Collection details for the insect isolates were given by de Vega *et al*. (2012).

Strain isolation and characterization

Five microliters of nectar were collected from each flower with a sterile microcapillary pipette. Nectar was diluted in 500 µl sterile MilliQ water, and 25 microliters 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). Yeasts from insects were isolated by allowing specimens to walk for 10 min on YM agar plates supplemented with 0.01% chloramphenicol. Plates with isolates from flowers and insects were incubated at room temperature (22 – 25ºC) for 3-8 days. A representative colony of each different morphotype was purified by repeated streaking on solid medium and preserved at -80 ºC in 10% glycerol and using the Microbank system (Pro-Lab diagnostics). Cultures were characterized by the standard methods of Kurtzman *et al*. (2011). Dalmau plates were prepared using Yeast Carbon Base agar supplemented with 0.01% yeast extract (YCBY) and 1.5% agar.

148 Evaluation of mating compatibility was performed by mixing pairs of active cultures on
149 Yeast Carbon Base supplemented with 0.01% ammonium sulfate (YCBAS), with 0.01%
150 yeast extract (YCBY), and dilute (1:10 and 1:20) V8. Cultures were incubated both at
151 16ºC and 25ºC and examined periodically by phase contrast microscopy for the
152 formation of zygotes, asci, and ascospores. Strains of *Metschnikowia drakensbergensis*
153 sp. nov. were also mixed in all possible combinations with the type and allotype of its
154 closest relative, *M. proteae*, as well as with strain *Metschnikowia* sp. EBDM2Y3. This
155 last strain, also a member of the *Metschnikowia* clade, was obtained from a specimen of
156 *Heterochelus* sp. in one of the study populations, on Mount Gilboa, in 2010 (de Vega *et*
157 *al*., 2012). It was considered premature to describe a new species from this single
158 strain.

159 DNA sequencing and phylogenetic analysis

160 Strains were identified by sequencing the D1/D2 domain of the 26S rRNA gene
161 following the methods of Kurtzman & Robnett (1998) and Lachance *et al*. (1999). The
162 D1/D2 domain was amplified by PCR using the primer combination NL1 and NL4. In
163 addition, three protein-coding genes, *ACT1*, *EF2*, *RPB2*, were amplified and sequenced.
164 Methods for DNA extraction, PCR amplifications and sequencing were described in
165 Guzmán *et al*. (2013).

166 PCR products were purified with Exo-SAP-IT enzyme mix (USB, Cleveland, OH) and
167 sequenced on an ABI PRISM 3130xl DNA automatic sequencer. Sequences were
168 assembled and edited using Sequencher 4.9 (Gene Codes, Ann Arbor, MI). Alignment
169 of generated sequences with related species from type strains was carried out using M–
170 Coffee (Wallace *et al*., 2006). D1/D2 sequences of type strains of related species were
171 retrieved from the GenBank database. The alignment was used to reconstruct

phylogenetic relationships using the Neighbor–Joining (NJ) method (Saitou & Nei, 1987). To avoid the presence of ambiguously aligned regions a NJ analysis was performed separately for the two new species. The analyses were performed in MEGA6 (Tamura *et al.*, 2013) using the Kimura 2–parameter distance correction (Kimura, 1980). The rate variation among sites was modelled with a gamma distribution determined using jModeltest (Posada, 2008; shape parameter = 0.39 for *Metschnikowia drakensbergensis* and shape parameter = 0.55 for *Metschnikowia caudata*). Bootstrap values (Felsenstein, 1985) were obtained from 10,000 random resamplings. *Candida hawaiiana* CBS 9146 and *Candida asparagi* CBS 9770 were used as outgroups for *Metschnikowia drakensbergensis* sp. nov. and *Metschnikowia caudata* sp. nov. analyses, respectively. See supplementary material for additional multi-locus (*ACT1*, *EF2*, *RPB2*) phylogenetic analyses using Bayesian Inference (BI) and Maximum Likelihood (ML) (Table S1, S2, Fig. S1, S2).

Results and Discussion

Species boundaries and phylogenetic position

The 83 nectar samples yielded 43 ascomycetous yeast isolates. Of these, three were assigned to the new large-spored species *Metschnikowia drakensbergensis* sp. nov. and six to the new caudate ascus-forming species *Metschnikowia caudata* sp. nov. Other yeast isolates from *Protea* nectar samples included *Hanseniaspora thailandica*, *Metschnikowia proteae*, *Candida corydalis*, *C. orthopsilosis*, and fifteen strains of two undescribed *Wickerhamiella* species.

Metschnikowia drakensbergensis sp. nov. was isolated exclusively from flowers of *P. dracomontana* and from a hopliinid beetle (Table 1). Phylogenetic analyses of both the

large subunit rRNA gene D1/D2 domain and the three protein-coding genes consistently placed isolates of *Metschnikowia drakensbergensis* sp. nov. into a sister clade to *Metschnikowia proteae* (Fig. 1a, S1 and S2). The D1/D2 sequence differed by 22-25 substitutions (4.6-5.2%) and five indels (1-4 bp) from that of the *M. proteae*, confirming the divergent status of the two species. *Metschnikowia drakensbergensis* sp. nov. is polymorphic in the sequences examined. In particular, strain EBD-M8Y1 differed from the other three strains by 4-5 substitutions, although the formation of mature asci with two ascospores in all mating pairs demonstrated their conspecificity (Fig. 2d). This is in contrast to crosses with *M. proteae*, which gave rise to mixtures of single-spored and empty asci (Fig. 2e). Neighbor–Joining, Bayesian and ML phylogenetic analyses suggested an affinity of the clade that comprises *M. proteae* and *M. drakensbergensis* sp. nov. with the large-spored *Metschnikowia* clade (Fig. 1a, S1 and S2), which is consistent with the striking similarity of their ascus morphologies. In addition, the growth characteristics of *M. drakensbergensis* sp. nov. (Table 2) are typical of those of most *Metschnikowia* species in the large-spored clade. *Metschnikowia drakensbergensis* sp. nov. differed by 97 substitutions and 20 indels in the D1/D2 sequence from strain *Metschnikowia* sp. EBDM2Y3, isolated from the same locality, and showed no signs of conjugation with this isolate.

Seven strains of *M. caudata* sp. nov. were recovered from three plant species (*P. dracomontana*, *P. subvestita*, and *P. roupelliae*) and a single honey bee in three different populations (Table 1). Three D1/D2 phylotypes were found. Strains EBD-CdVSA08-1 and EBD-CdVSA57-2 (type A) were isolated from nectar of *P. dracomontana* and *P. subvestita*, respectively (Table 1). Strains EBD-CdVSA21-2, EBD-CdVSA23-1, EBD-SA53, and EBD-SA54 (type B) were recovered from the nectar of *P. roupelliae* in a single population (Table 1). They differed from type A by

four substitutions. Strain EBD-B8Y1 (type C), isolated from a honeybee in Mount Gilboa, differed by three substitutions from type A and by seven substitutions from type B. The phylogenetic relationships elicited by analysis of D1/D2 sequences (Fig. 1b) were corroborated by both Bayesian and ML analyses of concatenated protein-coding genes (Figs. S1 and S2), indicating that a case might be made for considering strains of types A, B, and C to represent three species. The similarity among patterns arising from all four genes might even be seen as an example of genealogical concordance. However, the sample size for each phylotype is small and the four loci used are not particularly polymorphic (maximum total divergence of 39 substitutions, no indels, in the four concatenated gene sequences). Moreover, the different sequence types do not signify sufficient genetic differentiation to inhibit cross-breeding. When strains were mixed in every possible combination, compatible pairs conjugated and gave rise to asci with a long, flexuous tail and one fusiform spore with a tapered protuberance. A single ascospore (Fig. 2f) was produced in all compatible crosses, regardless of sequence type The ascus morphology is unprecedented, although the ascospore shape is vaguely reminiscent of that seen in *M. lachancei* (Giménez-Jurado *et al*., 2003). As shown by Marinoni & Lachance (2004), the formation of only one ascospore in *Metschnikowia* species may in some cases indicate that the spore is not viable and therefore that the conjugating strains are not members of the same biological species. In the absence of a clear pattern of mating success in *M. caudata*, we cannot rely on the biological species concept as a criterion for species delineation in the present case. The strains were physiologically homogeneous (Table 2), but the few polymorphic growth tests (cardinal growth temperatures, utilization of trehalose, maltose, melezitose, glucitol or glucosamine) varied in a manner that is somewhat consistent with the structure suggested by the sequences, indicating the possibility of varietal differentiation. We

favour prudence and assign all strains to a single species. This will avoid creating superfluous names that would later become confusing synonyms as more data become available.

Both Bayesian and Maximum Likelihood protein-coding genes phylogenies placed *M. caudata* sp. nov. close to flower- and insect-associated *Metschnikowia* species external to the large-spored *Metschnikowia* clade (Fig. S1 and S2). The phylogenetic tree based on the D1/D2 rDNA sequences showed that the clade comprising *M. caudata* sp. nov. has a clear sister relationship (Fig. 1b) to *Candida hainanensis* and *M. lopburiensis*, isolated from plants in China and Thailand, respectively (Wang *et al*., 2008; Kaewwichian *et al*., 2012). Ascus formation has not been observed in either of these two species or in more distant congeners (*M. saccharicola* and *C. robnettiae*), all of which were described on the basis of their asexual state. The eventual discovery of sexual states for *M. lopburiensis* and *C. hainanensis* may shed light on the significance of the unusual morphology seen in *M. caudata* sp. nov. and whether it represents a synapomorphy for the clade. The physiological characteristics of *M. caudata* are typical of those of most *Metschnikowia* species. Unusual was the lack of L-sorbose and 2-ketogluconate assimilation and the lack of fermentation seen in *M. caudata* sp. nov. These are normally positive in the clade.

Ecology and habitat specificity

Many members of the *Metschnikowia* clade have strong biogeographic patterns, while others are of a more cosmopolitan nature (Lachance 2011, Guzmán *et al*., 2013). The South African species *M. drakensbergensis* sp. nov. and *M. proteae* appear to provide yet another example of allopatric speciation as they seem to be moderately related to Equatorial East African species *M. aberdeeniae* and *M. shivogae*, albeit with a lesser degree of statistical support (Fig. 1a, Fig. S1, and Fig. S2). *Metschnikowia* sp. strain

EBDM2Y3 recovered in the same population as *M. drakensbergensis* does not seem to follow this pattern. Of considerable relevance here may be the group of beetles involved. Large-spored *Metschnikowia* species isolated in the New World and Hawaii mainly occur in nitidulid beetles and in many cases, yeast endemism parallels beetle endemism. In contrast, African species exhibit associations not only to nitiidulids (Lachance *et al*., 2008), but also mainly to other beetle families, such as the Meloidae, the Buprestidae (Tanzania and Kenya, Lachance *et al*., 2006a; 2008) and the Scarabaeidae (subfamily Cetoniinae and tribe Hopliini) in South Africa. Many groups of South African Scarabaeidae have undergone a spectacular adaptive radiation resulting in the evolution of hundreds of species, many of which are effective pollinators (Picker & Midgley 1996; Goldblatt *et al*. 1998; Steiner 1998). The potential importance of beetle diversification for speciation of *Metschnikowia* species in Africa could be resolved by further sampling plants and insects from more sites.

Biogeographic subdivision or host specificity at a much finer scale was observed in *Metschnikowia caudata* sp. nov., where, for example, strains possessing sequence type B were exclusively isolated from a single locality (Mount Gilboa) and a single plant species (*Protea roupelliae*). However, as a relatively small number of *Protea* flowers (84 samples) were analysed, the ability of these species to live in nectar of other *Protea* plants cannot be ruled out.

A characteristic common to all recently described *Metschnikowia* species from South Africa, including the new species described here and the recently described *M. proteae*, and *Metschnikowia* sp. strain EBDM2Y3, is a strong association with *Protea* plants visited by beetles and other pollinators. The microbiota observed in *Protea* species differed markedly from that of *ca*. 300 nectar samples from *ca*. 40 plant species visited by bees, butterflies, and birds, taken across several localities in South Africa (de Vega *et*

295 *al*., unpublished research). The dominant yeasts recovered in those plant species were
296 the small-spored *Metschnikowia* clade species *Candida rancensis*, *Metschnikowia*
297 *reukaufii*, and *M. koreensis*. These three species appear to be cosmopolitan, being
298 commonly isolated worldwide from floral nectar in plants pollinated by a diverse array
299 of pollinators, primarily bees, butterflies, and birds (Brysch-Herzberg, 2004; Pozo *et al*.,
300 2011; Belisle *et al*., 2012; de Vega & Herrera, 2012). As nectar yeasts are thought to be
301 vectored by the main animal visitors, and the newly described species appear associated
302 with a small set of plant species visited by beetles, our findings suggests that the new
303 species are highly selective in terms of host and the habitat requirements.

304 Description of *Metschnikowia drakensbergensis* sp. nov. de Vega, Guzmán & Lachance

305 *Metschnikowia drakensbergensis* (dra.kens.berg.en'sis. N.L. fem. adj. drakensbergensis
306 referring to to the South African mountains where the species was isolated).

307 After 3 days at 25°C on YM agar, the cells are ovoid to ellipsoid, 2-3 × 4-5 μm, and
308 occur singly, in mother-bud pairs, or in chains (Fig. 2a, d). After one week the colonies
309 are low-convex and slightly umbonate with entire margins. In slide cultures on YCBY
310 agar after two weeks at 25°C, short chains of undifferentiated cells are formed.

311 Asci (Fig. 2d) arise from the conjugation of cells of complementary mating types,
312 reaching nearly full size 6-8 hours after mixing agar media. The asci are fusiform (4-5
313 × 100-120 μm) and typically contain two aciculate spores (1-2 × 80-90 μm). Vestiges
314 of the original conjugated cells are usually present. Ascospore maturity is reached after
315 2-3 days at 25°C. Single spored asci are formed in crosses with *M. proteae* (Fig. 2e).
316 Ascus formation occurs on a large variety of media but is generally easier to observe
317 under conditions of nitrogen limitation (*e.g*., YCBAS agar).

Growth responses are given in Table 2.

The type is strain EBD-CdVSA09-2^{T}, recovered from nectar of *Protea dracomontana* in Garden Castle in uKhahlamba-Drakensberg Park, KwaZulu-Natal, South Africa. It has been deposited in the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, under number CBS 13649^{T} (NRRL Y-63721^{T}). The MycoBank accession number is MB809688. It has the mating type T. The designated allotype, of mating type AT, is EBD-CdVSA10-2^{A} (CBS 13650^{A}, NRRL Y-63720^{A}) and has a similar origin.

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

Metschnikowia caudata (cau.da'ta, L. fem. adj. *caudata* with a tail, referring to the unusual appearance of the ascus of the species).

After 3 days at 25°C on YM agar, the cells are globose to ovoid, 2-3 × 3-4 µm, and occur singly or in mother-bud pairs (Fig. 2b). After one week the colonies are low-convex and slightly umbonate with entire margins. In slide cultures on YCBY agar after two weeks at 25°C, pseudohyphae or hyphae are absent.

Mixtures of complementary mating types give rise within 2 days at 16°C to zygotes (Fig. 2c) some of which feature a pointy protuberance. After 3-4 days, elongate asci with a flexuous, tapered extremity are formed (0.5-1.5 × 70-100 µm) typically containing a single fusiform ascospore (25 µm) with a tapering end (Fig. 2f). The spores range in width from ca. 1 µm in the swollen part to less than 0.2 µm at the fine end. Vestiges of the original conjugated cells are usually present. Ascus formation was observed on YCBY agar.

The physiological characteristics are presented in Table 2.

The type is strain EBD-CdVSA08-1^{T}, recovered from nectar of *Protea dracomontana* in Garden Castle in uKhahlamba-Drakensberg Park, KwaZulu-Natal, South Africa. It has been deposited in the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, under number CBS 13651^{T} (NRRL Y-63722^{T}). The MycoBank accession number is MB809689. It has the mating type T. The designated allotype, of mating type AT, is EBD-CdVSA57-2^{A}, (CBS 13729^{A}, NRRL Y-63723^{A}) and was recovered from nectar of *Protea subvestita* in Sani Pass below the South African border post, KwaZulu-Natal, South Africa.

Acknowledgements

We thank L. Cabral and E. López for technical assistance and Dr. R.G. Albaladejo for comments on the manuscript. Authors gratefully acknowledge financial support from the Natural Science and Engineering Research Council of Canada (M.A.L.), the Spanish Ministry of Economy and Competitiveness through the Severo Ochoa Programme for Centres of Excellence in R&D&I (SEV-2012-0262; Postdoctoral fellowship to C.d.V.), Junta de Andalucía (P09–RNM– 4517 grant to C.M.H.), and Spanish National Research Council (CSIC)-European Social funds (JAE–DOC Programme to B.G.).

References

Belisle, M., Peay, K. G. & Fukami T. (2012). Flowers as islands, spatial distribution of nectar–inhabiting microfungi among plants of *Mimulus aurantiacus*, a hummingbird–pollinated shrub. *Microb Ecol* **63**, 711–718.

Brysch–Herzberg, M. (2004). Ecology of yeasts in plant–bumblebee mutualism in Central Europe. *FEMS Microbiol Ecol* **50**, 87–100.

de Vega, C. & Herrera, C. M. (2013). Relationships among nectar-dwelling yeasts, flowers and ants: patterns and incidence on nectar traits. *Oikos* **121,** 1878–1888.

de Vega, C. & Herrera, C. M. (2013). Microorganisms transported by ants induce changes in floral nectar composition of an ant–pollinated plant. *Am J Bot* **100**, 792–800.

de Vega, C., Guzmán, B., Lachance, M. A., Steenhuisen, S. L., Johnson, S. D. & Herrera, C. M. (2012). *Metschnikowia proteae* sp. nov., a nectarivorous insect-associated yeast species from Africa. *Int J Syst Evol Microbiol* **62**, 2538-2545.

Dupont, Y. L., Hansen, D. M., Rasmussen, J. T. & Olesen, J. M. (2004). Evolutionary changes in nectar sugar composition associated with switches between bird and insect pollination, the Canarian bird–flower element revisited. *Funct Ecol* **18**, 670–676.

Felsenstein, J. (1985). Confidence limits on phylogenies, an approach using the bootstrap. *Evolution* **39**, 783–791.

Giménez–Jurado, G., Kurtzman, C. P., Starmer, W. T. & Spencer–Martins, I. (2003). *Metschnikowia vanudenii* sp. nov. and *Metschnikowia lachancei* sp. nov., from flowers and associated insects in North America. *Int J Syst Evol Microbiol* **53**, 1665–1670.

Goldblatt, P., Bernhardt, P. & Manning, J. C. (1998). Pollination of petaloid geophytes by monkey beetles (Scarabaeidae, Rutelinae, Hopliini) in Southern Africa. *Ann Mo Bot Gard* **85**, 215–230.

Guzmán, B., Lachance, M. A. & Herrera C. M. (2013). Phylogenetic analysis of the angiosperm–floricolous insect–yeast association: have yeast and angiosperm lineages co–diversified? *Mol Phylogenet Evol* **68**, 161–175.

Herrera, C. M., García, I. M. & Pérez, R. (2008). Invisible floral larcenies, microbial communities degrade floral nectar of bumble bee–pollinated plants. *Ecology* **89**, 2369–2376.

Hong, S. G., Chun, J., Oh, H. W. & Bae, K. S. (2001). *Metschnikowia koreensis* sp. nov., a novel yeast species isolated from flowers in Korea. *Int J Syst Evol Microbiol* **51**, 1927–1931.

Kaewwichian, R., Yongmanitchai, W., Kawasaki, H. & Limtong, S. (2012). *Metschnikowia saccharicola* sp. nov. and *Metschnikowia lopburiensis* sp. nov., two novel yeast species isolated from phylloplane in Thailand. *Antonie Van Leeuwenhoek* **102**, 743–751.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kurtzman, C. P. & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* **73**, 331–371.

Kurtzman, C. P., Fell, J. W., Boekhout, T. & Robert, V. (2011). Methods for the isolation, phenotypic characterization and maintenance of yeasts. In *The Yeasts, a Taxonomic Study*, vol 1, pp 87–110. Edited by Kurtzman C. P., Fell J. W. & Boekhout ,T. Amsterdam: Elsevier.

Lachance, M. A. (2011). *Metschnikowia* Kamienski. In, *The Yeasts, a Taxonomic Study*, vol 1, pp 575-620. Edited by Kurtzman C. P., Fell J. W. & Boekhout, T. Amsterdam: Elsevier.

Lachance, M. A. & Fedor, A. N. (2014). Catching speciation in the act: *Metschnikowia bowlesiae* sp. nov., a yeast species found in nitidulid beetles of Hawaii and Belize. *Antonie van Leeuwenhoek* **105**, 541–550.

Lachance, M. A., Bowles, J. M., Starmer, W. T. & Barker, J. S. F. (1999). *Kodamaea kakaduensis* and *Candida tolerans*, two new ascomycetous yeast species from Australian *Hibiscus* flowers. *Can J Microbiol* **45**, 172–177.

Lachance, M. A., Starmer, W. T., Rosa, C. A., Bowles, J. M., Barker, J. S. F. & Janzen, D. H. (2001). Biogeography of the yeasts of ephemeral flowers and their insects. *FEMS Yeast Res* **1**, 1–8.

Lachance, M. A., Bowles, J. M. & Starmer, W. T. (2003a). Geography and niche occupancy as determinants of yeast biodiversity, the yeast–insect–morning glory ecosystem of Kípuka Puaulu, Hawai'i. *FEMS Yeast Res* **4**, 105–111.

Lachance, M. A., Bowles, J. M. & Starmer, W. T. (2003b). *Metschnikowia santaceciliae*, *Candida hawaiiana*, and *Candida kipukae*, three new yeast species associated with insects of tropical morning glory. *FEMS Yeast Res* **3**, 97–103.

Lachance, M. A., Ewing C. P., Bowles J. M. & Starmer, W. T. (2005). *Metschnikowia hamakuensis* sp. nov., *Metschnikowia kamakouna* sp. nov. and *Metschnikowia mauinuiana* sp. nov., three endemic yeasts from Hawaiian nitidulid beetles. *Int J Syst Evol Microbiol* **55**, 1369–1377.

Lachance, M. A., Anderson, T. M. & Starmer, W. T. (2006a). A new subclade of haplontic *Metschnikowia* species associated with insects of morning glory flowers in Africa and description of the yeast *Metschnikowia aberdeeniae* sp. nov. *Int J Syst Evol Microbiol* **56**, 1141–1145.

Lachance, M. A., Bowles, J. M., Wiens, F., Dobson, J. & Ewing C. P. (2006b). *Metschnikowia orientalis* sp. nov., an Australasian yeast from nitidulid beetles. *Int J Syst Evol Microbiol* **56**, 2489–2493.

Lachance, M. A., Bowles, J. M., Anderson, T. M. & Starmer, W. T. (2008). *Metschnikowia shivogae* sp. nov., a yeast species associated with insects of morning glory flowers in East Africa. *Int J Syst Evol Microbiol* **58**, 2241–2244.

Marinoni, G. & Lachance, M. A. (2004). Speciation in the large–spored *Metschnikowia* clade and establishment of a new species, *Metschnikowia borealis* comb. nov. *FEMS Yeast Res* **4**, 587–596.

Nguyen, N. H., Suh, S. O., Erbil, C. K. & Blackwell, M. (2006). *Metschnikowia noctiluminum* sp. nov., *Metschnikowia corniflorae* sp. nov., and *Candida chrysomelidarum* sp. nov., isolated from green lacewings and beetles. *Mycol Res* **110**, 346–356.

Nicolson, S. W. (2007). Nectar consumers. In, *Nectaries and Nectar*, pp. 289–342. Edited by Nicolson, S.W., Nepi, M., Pacini, W. Dordrecht: Springer–Verlag.

Picker, M. D. & Midgley, J. J. (1996). Pollination by monkey beetles (Coleoptera, Scarabaeidae, Hopliini), flower and colour preferences. *Afr Entomol* **4**, 7–14.

Posada, D. (2008). jModeltest: phylogenetic model averaging. *Mol Biol Evol* **25**, 1253-1256.

Pozo, M. I., Herrera, C. M. & Bazaga, P. (2011). Species richness of yeast communities in floral nectar of southern Spanish plants. *Microb Ecol* **6**, 82–91.

Saitou, N. & Nei, M. (1987). The Neighbor–joining method, a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Simpson, B. B. & Neff, J. L. (1983). Evolution and diversity of floral rewards. In *Handbook of Experimental Pollination Biology*, pp. 142–159. Edited by Jones, C. E. & Little, R. J. New York: Van Nostrand Reinhold.

Steiner, K. E. (1998). Beetle pollination of peacock moraeas (Iridaceae) in South Africa. *Plant Syst Evol* **209**, 47–65.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6, Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* **30**, 2725–2729.

Wallace, I. M., O'Sullivan, O., Higgins, D. G. & Notredame, C. (2006). M-Coffee: combining multiple sequence alignment methods with TCoffee. *Nucleic Acids Res* **34**, 1692–1699.

Wang, S. A., Jia, J. H. & Bai, F. Y. (2008). *Candida alocasiicola* sp. nov., *Candida hainanensis* sp. nov., *Candida heveicola* sp. nov. and *Candida musiphila* sp. nov., novel anamorphic, ascomycetous yeast species isolated from plants. *Antonie Van Leeuwenhoek* **94**, 257–265.

Captions

Figure 1. Phylogeny of *Metschnikowia drakensbergensis* sp. nov. and related species (a) and *Metschnikowia caudata* sp. nov. and related species (b) based on NJ analyses of D1/D2 rDNA sequences. Numbers above branches show NJ bootstrap support. *Candida hawaiiana* CBS 9146 and *Candida asparagi* CBS 9770 were used as outgroups for *Metschnikowia drakensbergensis* sp. nov. and *Metschnikowia caudata* sp. nov. analyses. Branch lengths are scaled to the expected number of nucleotide substitutions per site; bar, 0.02 and 0.05 nucleotide substitutions per site. Only bootstrap values ≥ 50% are shown. GenBank accession numbers of all sequences are indicated after strain name. ^{T}Type strain; ^{A}Allotype. Culture collection prefixes: EBD, Estación Biológica de Doñana; CBS, Centraalbureau voor Schimmelcultures.

Figure 2. Phase contrast photomicrographs of *Metschnikowia drakensbergensis* sp. nov. and *Metschnikowia caudata* sp. nov. (a) Budding cells (strain EBD-CdVSA09-2^{T}) and (d) mature ascus of *M. drakensbergensis* sp. nov (asci obtained from mixing strains EBD-CdVSA09-2^{T} and EBD-CdVSA10-2^{A}). (e) Single-spored and sterile asci resulting from a cross between *M. drakensbergensis* sp. nov. (strain EBD-CdVSA09-2^{T}) and *M. proteae* (strain EBDC2Y2). (b) Budding cells (strain EBD-CdVSA08-1^{T}), (c) zygote and developing ascus (from mixing strains EBD-CdVSA08-1^{T} and EBD-CdVSA57-2^{A}), and (f) mature ascus (from mixing strains EBD-CdVSA21-2 and EBD-CdVSA57-2^{A}) of *M. caudata*. Scale bar=10 μm.

Table 1. Origin of strains of *Metschnikowia drakensbergensis* sp. nov., and *Metschnikowia caudata* sp. nov. Mating type: T = same as the type; AT = same as the allotype. Isolation source. Localities: GC (Garden Castle in uKhahlamba-Drakensberg Park, 29º44′S 29º12′E, 1820 m asl); MG (Mount Gilboa in the Karkloof Range, 29°17′ S 30°17′E, 1520 m asl); SP (Sani Pass below the South African border post, 29°35′S 29°17′E, 2800 m asl). *Already suggested as a new species in de Vega *et al*. (2012) IJSEM 62, 2538–2545. [T]Type strain; [A]Allotype.

Strains	GenBank no.	Mating type	Isolation source	Localities
***Metschnikowia drakensbergensis* sp. nov.**				
EBD-CdVSA09-2[T]	JN935056	T	*Protea dracomontana*	GC
EBD-CdVSA10-2[A]	JN935054	AT	*Protea dracomontana*	GC
EBD-CdVSA12-1	JN935055	AT	*Protea dracomontana*	GC
EBD-M8Y1*	JN935047	T	*Heterochelus sp*	MG
***Metschnikowia caudata* sp. nov.**				
EBD-CdVSA08-1[T]	KJ736788	T	*Protea dracomontana*	GC
EBD-CdVSA57-2[A]	KJ736790	AT	*Protea subvestita*	SP
EBD-B8Y1	KJ736785	AT	*Apis mellifera*	MG
EBD-CdVSA21-2	KJ736786	T	*Protea roupelliae*	MG
EBD-CdVSA23-1	KJ736787	T	*Protea roupelliae*	MG
EBD-SA53	KJ736791	T	*Protea roupelliae*	MG
EBD-SA54	KJ736789	AT	*Protea roupelliae*	MG

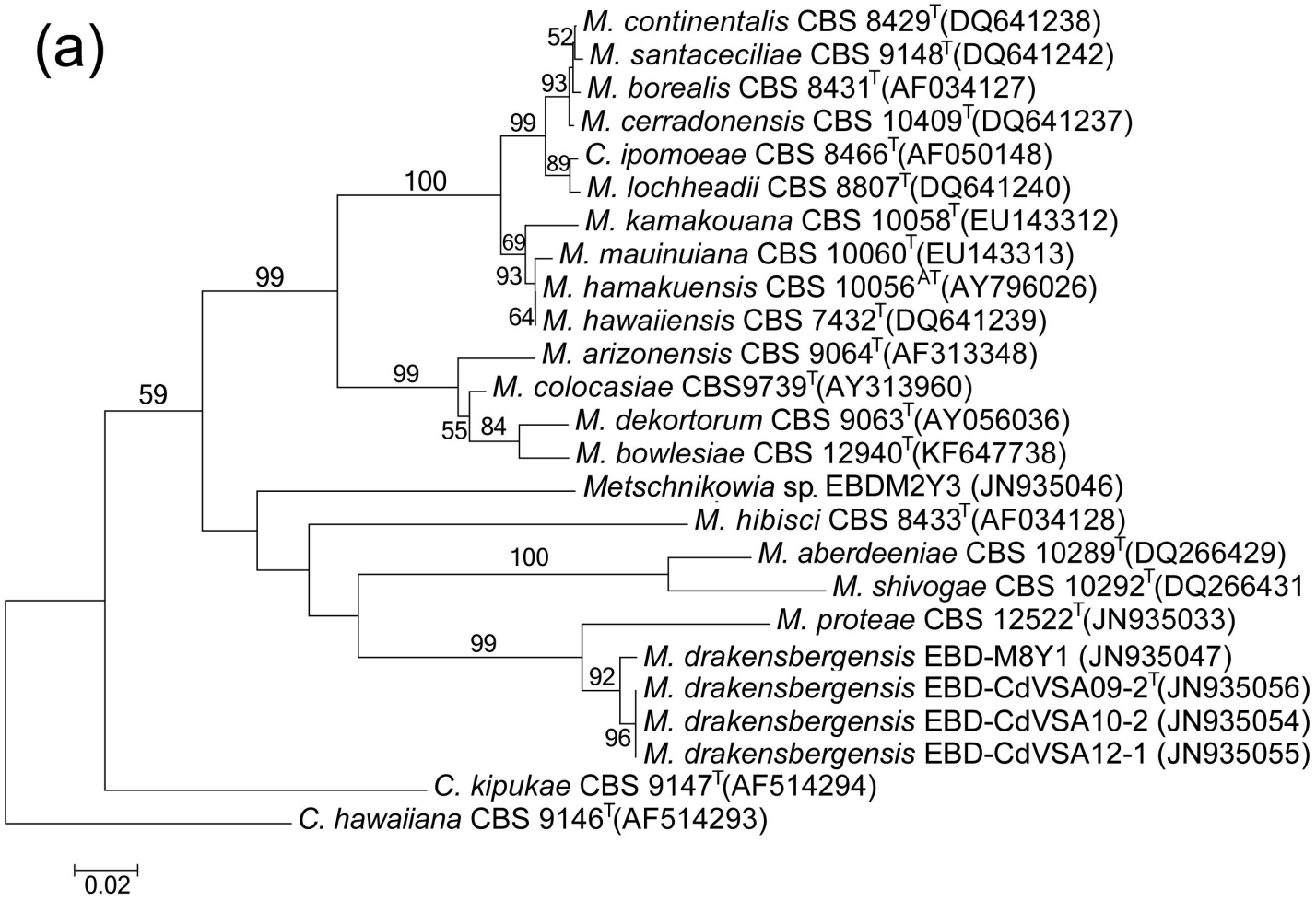
Table 2. Growth characteristics of *Metschnikowia drakensbergensis* sp. nov., *Metschnikowia caudata* sp. nov., and related species. +, Positive; -, negative; s, slow; w, weak. Invariant responses: assimilation of sucrose, melezitose, are positive; assimilation of inulin, raffinose, melibiose, lactose, starch, L-rhamnose, L-arabinose, methanol, 1–propanol, 2–propanol, 1–butanol, erythritol, galactitol, inositol, and lactate negative. Utilization of nitrate and nitrite negative; ethylamine, lysine, and cadaverine, positive. Growth in the presence of 10 ppm cycloheximide is negative. The data for *C. hainanensis* and *M. lopburiensis* are from the original descriptions (Wang *et al*., 2008; Kaewwichian *et al*., 2012).

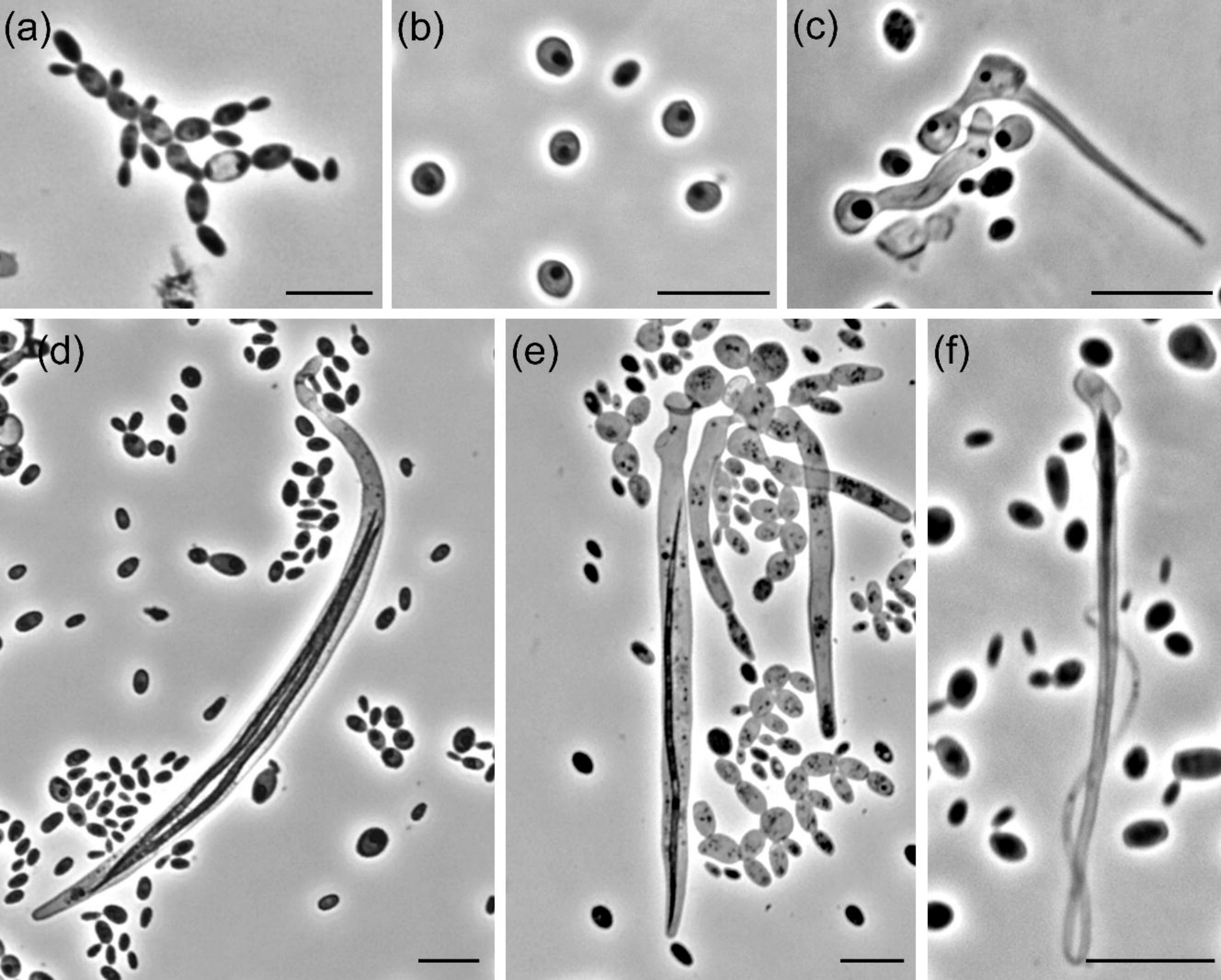
	M. proteae	EBD-M8Y1	EBD-CdVSA09-2	EBD-CdVSA10-2	EBD-CdVSA12-1	*C. hainanensis*	*M. lopburiensis*	EBD-B8Y1	EBD-CdVSA08-1	EBD-CdVSA57-2	EBD-CdVSA21-2	EBD-CdVSA23-1	EBD-SASA53	EBD-SASA54
Galactose	+	w	s	s	s	–	–	–	–	–	–	–	–	–
Trehalose	w	–	w	w	w	+	+	–	+	+	–	–	–	–
Maltose	+	+	+	+	+	+	+	–	–	–	w	–	w	w
Methyl glucoside	–	–	–	–	–	+	+	–	–	–	–	–	–	–
Cellobiose	–	–	+	w	s	+	+	w	+	w	w	w	–	–
Salicin	–	–	+	w	w	+	+	w	w	w	w	w	w	w
Sorbose	+	+	+	+	+	+	+	–	–	–	–	–	–	–
Xylose	w	–	–	–	–	+	+	–	–	–	–	–	–	–
D–arabinose	–	–	–	–	–	–	w	–	–	–	–	–	–	–
Ribose	–	s	w	–	w	–	–	w	w	w	w	w	s	s
Ethanol	+	+	s	s	s	+	+	s	s	s	s	s	w	w
Glycerol	–	–	–	–	–	+	S	s	w	s	w	w	s	s
Ribitol	–	–	–	–	–	+	+	–	–	–	–	–	–	–
Xylitol	w	s	–	–	–	–	+	–	–	–	–	–	–	–
Mannitol	s	+	w	w	w	+	+	+	+	+	+	+	+	+
Glucitol	+	w	w	w	w	+	+	+	+	+	w	w	w	w
Succinic	w	w	s	s	s	s	+	w	s	w	s	s	w	w
Citric	–	s	–	–	–	+	+	–	–	–	–	–	–	–
Gluconic	w	–	–	–	–	–	+	–	–	–	–	–	–	–
Gluconolactone	w	w	w	w	–	–	+	w	s	w	w	w	w	w
2–ketogluconate	+	+	+	+	+		+	–	–	–	–	–	–	–
Glucosamine	v	s	w	w	–	+	+	–	–	–	w	w	s	s
N–acetyl glucosamine	+	+	+	+	+		+	+	+	+	+	+	+	+
Hexadecane	–	w	–	–	–	–	–	s	–	s	–	–	–	–
Growth at 4 ˚C	–	–	–	–	–			+	+	+	+	+	+	+
Growth at 30 ˚C	+	+	w	w	w	+	+	+	+	+	w	+	+	+
Growth at 31 ˚C	+	+	–	s	s			+	+	+	–	+	+	+
Growth at 32 ˚C	s	–	–	–	–			+	+	+	–	+	+	+
Growth at 33 ˚C	–	–	–	–	–			+	+	+	–	–	w	w

Growth at 34 ˚C	–	–	–	–	–			+	+	+	–	–	–	–
Growth at 35 ˚C	–	–	–	–	–			+	–	–	–	–	–	–
Growth at 37 ˚C	–	–	–	–	–	+	+	–	–	–	–	–	–	–
NaCl 10%	w	+	w	w	w		w	+	s	+	s	s	+	+
NaCl 15%	–	–	–	–	–		–	s	–	w	–	–	–	–
Glucose 50%	–	–	–	–	–		+	s	–	–	–	–	–	–
Cycloheximide 10 ppm	–	–	–	–	–	–		–	–	–	–	–	–	–
Glucose fermentation	w	+	+	+	+	+	+	–	w	–	–	–	–	–

510

511


(a)
M. continentalis CBS 8429T(DQ641238)
M. santaceciliae CBS 9148T(DQ641242)
M. borealis CBS 8431T(AF034127)
M. cerradonensis CBS 10409T(DQ641237)
C. ipomoeae CBS 8466T(AF050148)
M. lochheadii CBS 8807T(DQ641240)
M. kamakouana CBS 10058T(EU143312)
M. mauinuiana CBS 10060T(EU143313)
M. hamakuensis CBS 10056AT(AY796026)
M. hawaiiensis CBS 7432T(DQ641239)
M. arizonensis CBS 9064T(AF313348)
M. colocasiae CBS9739T(AY313960)
M. dekortorum CBS 9063T(AY056036)
M. bowlesiae CBS 12940T(KF647738)
Metschnikowia sp. EBDM2Y3 (JN935046)
M. hibisci CBS 8433T(AF034128)
M. aberdeeniae CBS 10289T(DQ266429)
M. shivogae CBS 10292T(DQ266431
M. proteae CBS 12522T(JN935033)
M. drakensbergensis EBD-M8Y1 (JN935047)
M. drakensbergensis EBD-CdVSA09-2T(JN935056)
M. drakensbergensis EBD-CdVSA10-2 (JN935054)
M. drakensbergensis EBD-CdVSA12-1 (JN935055)
C. kipukae CBS 9147T(AF514294)
C. hawaiiana CBS 9146T(AF514293)
0.02
(b)
M. zizyphicola CBS 10358T(DQ367882)
M. fructicola CBS 8853T(AF360542)
M. sinensis CBS 10357T(DQ367881)
M. andauensis CBS 10809T(AJ745110)
M. pulcherrima CBS 5833T(U45736)
M. xhanxiensis CBS 10359T(DQ367883)
M. chrysoperlae CBS 9803T(AY452047)
C. picachoensis CBS9804T(AY452039)
C. pimensis CBS 9805T(AY452051)
C. golubevii CBS 11362T(DQ40445)
M. lunata CBS 5946T(U45733)
M. kunwiensis CBS 9067T(AF389527)
M. corniflorae CBS 9905T(AY611610)
C. robnettiae CBS 8580T(HM15641)
M. saccharicola CBS 12575T(AB697755)
M. lopburiensis CBS 12574T(AB697756)
C. hainanensis CBS 10696T(EU284103)
M. caudata EBD-CdVSA57-2 (KJ736790)
M. caudata EBD-B8Y1 (KJ736785)
M. caudata EBD-CdVSA08-1T(KJ736788)
M. caudata EBD-CdVSA21-2 (KJ736786)
M. caudata EBD-CdVSA23-1 (KJ736787)
M. caudata EBD-SA53 (KJ736791)
M. caudata EBD-SA54 (KJ736789)
C. asparagi CBS 9770T(AY450920)
0.05


(a)
(b)
(c)
(d)
(e)
(f)