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

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In a taxonomic study of yeasts recovered from nectar of flowers and associated insects in South Africa, 11 strains were found to represent two novel species. Morphological and physiological characteristics and sequence analyses of the large-subunit rRNA gene D1/D2 region, 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. (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}) 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 *Metschnikowia proteae*, with which mating reactions and single-spored asci were observed. *Metschnikowia caudata* sp. nov. (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}) was isolated from nectar of *Protea dracomontana*, *P. roupelliae* and *P. subvestita* and a honeybee, and is a sister species to *Candida hainanensis* and *Metschnikowia lopburiensis*. Analyses of the four sequences demonstrated the existence of three separate phylotypes. Intraspecies matings led 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 are discussed in terms of host plant and insect host species.

INTRODUCTION

Flowers offer different food rewards to pollinators in exchange for pollination services. The primary floral reward is floral nectar, a complex fluid containing mainly 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 used exclusively by pollinators. Its composition makes it a favourable environment for the growth of micro-organisms, 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 with them. The genus *Metschnikowia* (and anamorphs in the genus *Candida*) is one of the dominant taxa found in these substrates (Lachance *et al.*,

Abbreviations: BI, Bayesian inference; ML, maximum-likelihood; NJ, neighbour-joining.

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

Two supplementary tables and two supplementary figures are available with the online version of this paper.

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2001; Lachance, 2011). For example, the cosmopolitan *Metschnikowia reukaufii*, *M. gruessii* and *M. koreensis* have been isolated repeatedly 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 *Metschnikowia 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 *Metschnikowia 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 nitidulids of the genera *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 *Metschnikowia corniflorae*, associated with chrysomelid beetles and flowers in the USA (Nguyen *et al.*, 2006), *Metschnikowia 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, 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 on 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 *Metschnikowia proteae*. The other has moderately sized ascospores with a novel morphology. Its closest described relatives are *Metschnikowia lopburiensis* and *Candida* (iter. nom. *Metschnikowia*) *hainanensis*, neither of which forms asci. We now describe the novel 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), *P. roupelliae* (n=19), *P. subvestita* (n=16) and *P. 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 the Mount Gilboa Estate. Sixteen samples from *P. welwitschii*, sampled in Winston Park (29° 49′ S 30° 47′ E, 530 m above sea level), did not yield any strains of *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 laboratory, where nectar sampling was done within a few hours of 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 novel species were isolated from a hopliinid beetle (*Heterochelus* sp.; Scarabaeidae) and a honeybee (*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 microlitres nectar was collected from each flower with a sterile microcapillary pipette. Nectar was diluted in 500 μl sterile MilliQ water, and 25 μl 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 (22–25 °C) for 3–8 days. A representative colony of each different morphotype was purified by repeated streaking on solid medium and preserved at −80 °C 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.

Evaluation of mating compatibility was performed by mixing pairs of active cultures on yeast carbon base supplemented with 0.01 % ammonium sulfate (YCBAS) or with 0.01 % yeast extract (YCBY) and dilute (1 : 10 and 1 : 20) V8. Cultures were incubated at both 16 and

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. Localities: GC, Garden Castle in uKhahlamba-Drakensberg Park, 29° 44′ S 29 °12′ E, 1820 m above sea level; MG, Mount Gilboa in the Karkloof Range, 29° 17′ S 30° 17′ E, 1520 m above sea level; SP, Sani Pass below the South African border post, 29° 35′ S 29° 17′ E, 2800 m above sea level.

Strain	26S rRNA gene sequence GenBank accession no.	Mating type	Isolation source	Locality
***M. drakensbergensis* sp. nov.**				
EBD-CdVSA09-2[T]	JN935056	(T)	*P. dracomontana*	GC
EBD-CdVSA10-2[A]	JN935054	(AT)	*P. dracomontana*	GC
EBD-CdVSA12-1	JN935055	AT	*P. dracomontana*	GC
EBD-M8Y1*	JN935047	T	*Heterochelus* sp.	MG
***M. caudata* sp. nov.**				
EBD-CdVSA08-1[T]	KJ736788	(T)	*P. dracomontana*	GC
EBD-CdVSA57-2[A]	KJ736790	(AT)	*P. subvestita*	SP
EBD-B8Y1	KJ736785	AT	*Apis mellifera*	MG
EBD-CdVSA21-2	KJ736786	T	*P. roupelliae*	MG
EBD-CdVSA23-1	KJ736787	T	*P. roupelliae*	MG
EBD-SA53	KJ736791	T	*P. roupelliae*	MG
EBD-SA54	KJ736789	AT	*P. roupelliae*	MG

*Already suggested to represent a novel species by de Vega *et al.* (2012).

25 °C and examined periodically by phase-contrast microscopy for the formation of zygotes, asci and ascospores. Strains of *M. drakensbergensis* sp. nov. were also mixed in all possible combinations with the type and allotype of its closest relative, *M. proteae*, as well as with strain *Metschnikowia* sp. EBDM2Y3. This last strain, also a member of the *Metschnikowia* clade, was obtained from a specimen of *Heterochelus* sp. in one of the study populations, on Mount Gilboa, in 2010 (de Vega *et al.*, 2012). It was considered premature to describe a novel species from this single strain.

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

PCR products were purified with Exo-SAP-IT enzyme mixture (USB) and sequenced on an ABI PRISM 3130xl DNA automatic sequencer. Sequences were assembled and edited using Sequencher 4.9 (Gene Codes). Alignment of generated sequences with related species from type strains was carried out using M-Coffee (Wallace *et al.*, 2006). D1/D2 sequences of type strains of related species were retrieved from the GenBank database. The alignment was used to reconstruct phylogenetic relationships using the neighbour-joining (NJ) method (Saitou & Nei, 1987). To avoid the presence of ambiguously aligned regions, an NJ analysis was performed separately for the two novel species. The analyses were performed in MEGA6 (Tamura *et al.*, 2013) using Kimura's two-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 *M. drakensbergensis* and shape parameter 0.55 for *M. caudata*). Bootstrap values (Felsenstein, 1985) were obtained from 10 000 random resamplings. *Candida hawaiiana* CBS 9146[T] and *Candida asparagi* CBS 9770[T] were used as outgroups for analyses of *M. drakensbergensis* sp. nov. and *M. caudata* sp. nov., respectively. Results of additional multilocus (*ACT1*, *EF2* and *RPB2*) phylogenetic analyses using Bayesian inference (BI) and maximum-likelihood (ML) analyses are given in Tables S1 and S2 and Figs S1 and S2, available in the online Supplementary Material.

RESULTS AND DISCUSSION

Species boundaries and phylogenetic position

The 83 nectar samples yielded 43 ascomycetous yeast isolates. Of these, three were assigned to the novel large-spored species *M. drakensbergensis* sp. nov. and six to the novel caudate ascus-forming species *M. caudata* sp. nov. Other yeast isolates from *Protea* nectar samples included strains of *Hanseniaspora thailandica*, *M. proteae*, *Candida corydalis* and *C. orthopsilosis* and 15 strains of two undescribed *Wickerhamiella* species.

M. 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 *M. drakensbergensis* sp. nov. into a sister clade to *M. proteae* (Figs 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 *M. proteae*, confirming the divergent status of the two species. *M. drakensbergensis* sp. nov. is polymorphic in the sequences examined. In particular, strain EBD-M8Y1 differed from the other three strains by four or five 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). NJ, BI 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 (Figs 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. *M. 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 honeybee 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 BI 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, and 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 *Metschnikowia lachancei* (Giménez-Jurado *et al.*, 2003). As shown by Marinoni & Lachance (2004), the formation of only one ascospore in *Metschnikowia* species may indicate in some cases 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* sp. nov., 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 was 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 BI and ML protein-coding gene phylogenies placed *M. caudata* sp. nov. close to flower- and insect-associated *Metschnikowia* species external to the large-spored *Metschnikowia* clade (Figs S1 and S2). The phylogenetic tree based on the 26S rRNA gene D1/D2 domain sequences showed that the clade comprising *M. caudata* sp. nov. has a clear sister relationship (Fig. 1b) to *C. 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 species or in more distant congeners (*Metschnikowia saccharicola* and *Candida 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* sp. nov. are typical of those of most *Metschnikowia* species. Unusual was the lack of assimilation of L-sorbose and 2-ketogluconate 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 biogeographical 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 the Equatorial East African species *Metschnikowia aberdeeniae* and *M. shivogae*, albeit with a lower level of statistical support (Figs 1a, S1 and S2). *Metschnikowia* sp. strain EBDM2Y3, recovered in the same population as *M. drakensbergensis* sp. nov., 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 occur mainly in nitidulid beetles and, in many cases, yeast endemism parallels beetle endemism. In contrast, African species exhibit associations not only with nitiidulids (Lachance *et al.*, 2008), but also mainly with 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 scarabaeids 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 of plants and insects from more sites.

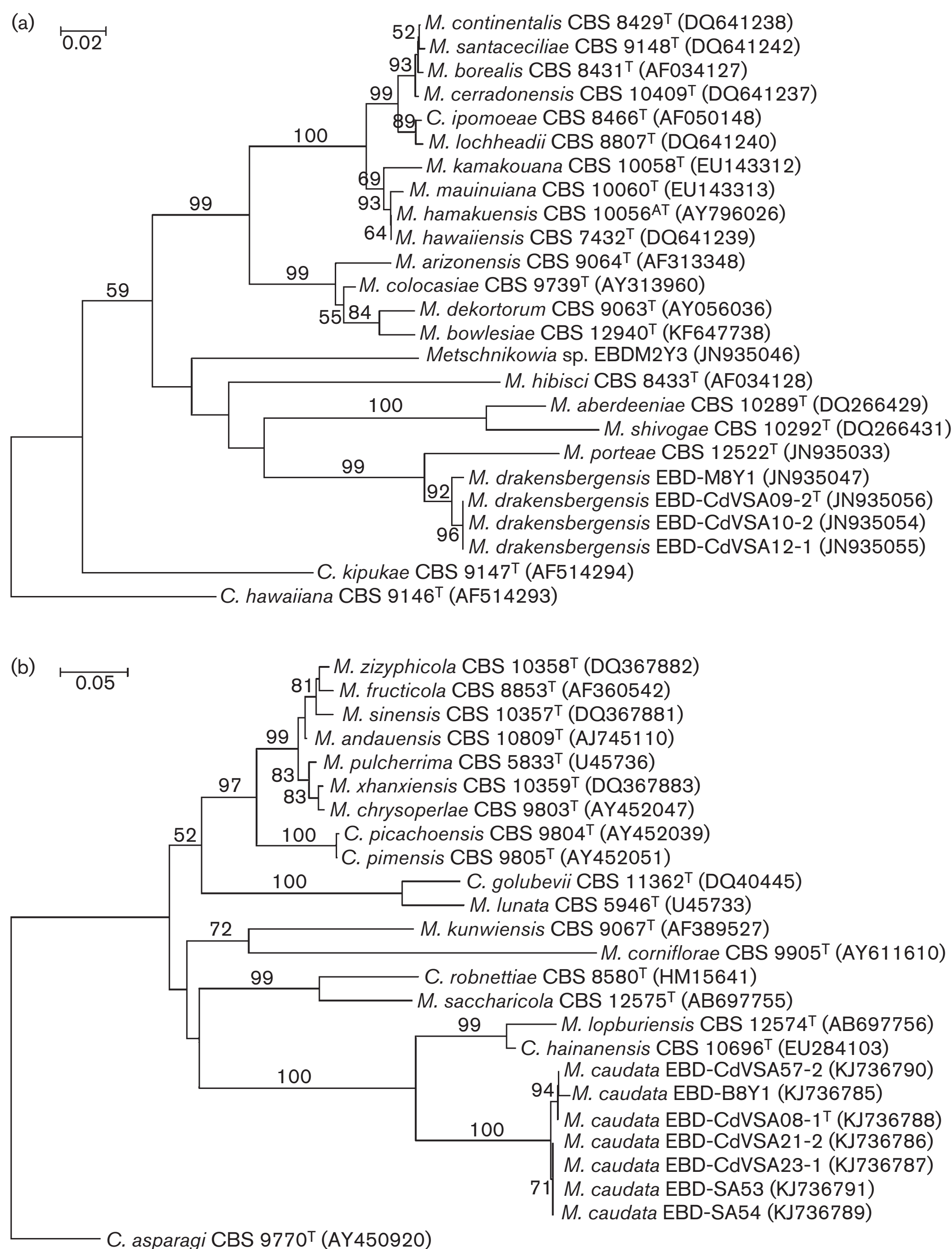

Fig. 1. Phylogeny of *M. drakensbergensis* sp. nov. and related species (a) and *M. caudata* sp. nov. and related species (b) based on NJ analyses of 26S rRNA gene D1/D2 sequences. Numbers above branches show NJ bootstrap support. *Candida hawaiiana* CBS 9146^T and *Candida asparagi* CBS 9770^T were used as outgroups in (a) and (b), respectively. Branch lengths are scaled to the expected number of nucleotide substitutions per site. Bars, 0.02 (a) and 0.05 (b) nucleotide substitutions per site. Only bootstrap values $\geqslant$50 % are shown. GenBank accession numbers are indicated after the strain name. CBS, Centraalbureau voor Schimmelcultures; EBD, Estación Biológica de Doñana.

Biogeographical subdivision or host specificity at a much finer scale was observed in *M. caudata* sp. nov., where, for example, strains possessing sequence type B were isolated exclusively from a single locality (Mount Gilboa) and a single plant species (*P. roupelliae*). However, as a relatively small number of *Protea* flowers (83 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

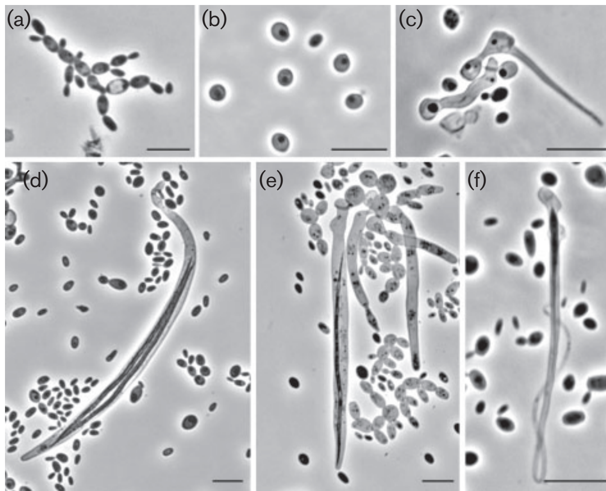

Fig. 2. Phase-contrast photomicrographs of *M. drakensbergensis* sp. nov. (a, d, e) and *M. caudata* sp. nov. (b, c, f). (a) Budding cells of *M. drakensbergensis* EBD-CdVSA09-2^T. (b) Budding cells of *M. caudata* EBD-CdVSA08-1^T. (c) Zygote and developing ascus from mixing *M. caudata* strains EBD-CdVSA08-1^T and EBD-CdVSA57-2^A. (d) Mature asci obtained from mixing *M. drakensbergensis* strains EBD-CdVSA09-2^T and EBD-CdVSA10-2^A. (e) Single-spored and sterile asci resulting from a cross between *M. drakensbergensis* EBD-CdVSA09-2^T and *M. proteae* EBDC2Y2. (f) Mature ascus from mixing *M. caudata* strains EBD-CdVSA21-2 and EBD-CdVSA57-2^A. Bars, 10 μm.

novel 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 about 300 nectar samples from about 40 plant species visited by bees, butterflies and birds, taken across several localities in South Africa (C de Vega, SD Johnson & CM Herrera, unpublished). The dominant yeasts recovered in those plant species were the small-spored *Metschnikowia* clade species *Candida rancensis*, *M. reukaufii* and *M. koreensis*. These three species appear to be cosmopolitan, being commonly isolated worldwide from floral nectar in plants pollinated by a diverse array of pollinators, primarily bees, butterflies and birds (Brysch-Herzberg, 2004; Pozo *et al.*, 2011; Belisle *et al.*, 2012; de Vega & Herrera, 2012). As nectar yeasts are thought to be vectored by the main animal visitors, and the newly described species appear associated with a small set of plant species visited by beetles, our findings suggests that the novel species are highly selective in terms of host and habitat requirements.

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

Metschnikowia drakensbergensis (dra.kens.ber.gen′sis. N.L. fem. adj. *drakensbergensis* referring to the South African Drakensberg mountains, where the species was first isolated).

After 3 days at 25 °C on YM agar, the cells are ovoid to ellipsoid, 2–3 × 4–5 μm, and occur singly, in mother–bud pairs or in chains (Fig. 2a, d). After 1 week, the colonies are low-convex and slightly umbonate with entire margins. In slide cultures on YCBY agar after 2 weeks at 25 °C, short chains of undifferentiated cells are formed. Asci (Fig. 2d) arise from the conjugation of cells of complementary mating types, reaching nearly full size 6–8 h after mixing agar media. The asci are fusiform (4–5 × 100–120 μm) and typically contain two aciculate spores (1–2 × 80–90 μm). Vestiges of the original conjugated cells are usually present. Ascospore maturity is reached after 2–3 days at 25 °C. Single-spored asci are formed in crosses with *M. proteae* (Fig. 2e). Ascus formation occurs on a large variety of media, but is generally easier to observe under conditions of nitrogen limitation (e.g. YCBAS agar). Growth responses are given in Table 2.

The type strain 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, as 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), which 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 1 week, the colonies are low-convex and slightly umbonate with entire margins. In slide cultures on YCBY agar after 2 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 about 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 is 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, as CBS 13651^T (=NRRL Y-63722^T). The

Table 2. Growth characteristics of strains of *M. drakensbergensis* sp. nov. and *M. caudata* sp. nov. and related species

Strains/species: 1, *M. proteae*; 2, EBD-M8Y1; 3, EBD-CdVSA09-2^{T}; 4, EBD-CdVSA10-2^{A}; 5, EBD-CdVSA12-1; 6, *C. hainanensis*; 7, *M. lopburiensis*; 8, EBD-B8Y1; 9, EBD-CdVSA08-1^{T}; 10, EBD-CdVSA57-2^{A}; 11, EBD-CdVSA21-2; 12, EBD-CdVSA23-1; 13, EBD-SA53; 14, EBD-SA54. +, Positive; −, negative; S, slow; V, variable; W, weak; ND, no data available. Invariant responses: positive for assimilation of sucrose and melezitose; negative or assimilation of inulin, raffinose, melibiose, lactose, starch, L-rhamnose, L-arabinose, methanol, 1-propanol, 2-propanol, 1-butanol, erythritol, galactitol, inositol and lactate; negative for utilization of nitrate and nitrite; positive for utilization of ethylamine, lysine and cadaverine. Data for *C. hainanensis* and *M. lopburiensis* are from the original descriptions (Wang *et al.*, 2008; Kaewwichian *et al.*, 2012).

Characteristic	1	*M. drakensbergensis*				6	7	*M. caudata*						
		2	3	4	5			8	9	10	11	12	13	14
Assimilation of:														
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 acid	W	W	S	S	S	S	+	W	S	W	S	S	W	W
Citric acid	−	S	−	−	−	+	+	−	−	−	−	−	−	−
Gluconic acid	W	−	−	−	−	−	+	−	−	−	−	−	−	−
Gluconolactone	W	W	W	W	−	−	+	W	S	W	W	W	W	W
2-Ketogluconate	+	+	+	+	+	ND	+	−	−	−	−	−	−	−
Glucosamine	V	S	W	W	−	+	+	−	−	−	W	W	S	S
N-Acetylglucosamine	+	+	+	+	+	ND	+	+	+	+	+	+	+	+
Hexadecane	−	W	−	−	−	−	−	S	−	S	−	−	−	−
Growth at/in:														
4 °C	−	−	−	−	−	ND	ND	+	+	+	+	+	+	+
30 °C	+	+	W	W	W	+	+	+	+	+	W	+	+	+
31 °C	+	+	−	S	S	ND	ND	+	+	+	−	+	+	+
32 °C	S	−	−	−	−	ND	ND	+	+	+	−	+	+	+
33 °C	−	−	−	−	−	ND	ND	+	+	+	−	−	W	W
34 °C	−	−	−	−	−	ND	ND	+	+	+	−	−	−	−
35 °C	−	−	−	−	−	ND	ND	+	−	−	−	−	−	−
37 °C	−	−	−	−	−	+	+	−	−	−	−	−	−	−
10 % NaCl	W	+	W	W	W	ND	W	+	S	+	S	S	+	+
15 % NaCl	−	−	−	−	−	ND	−	S	−	W	−	−	−	−
50 % Glucose	−	−	−	−	−	ND	+	S	−	−	−	−	−	−
10 p.p.m. Cycloheximide	−	−	−	−	−	−	ND	−	−	−	−	−	−	−
Glucose fermentation	W	+	+	+	+	+	+	−	W	−	−	−	−	−

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}), which was recovered from nectar of *Protea subvestita* in Sani Pass below the South African border post, KwaZulu-Natal, South Africa.

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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. (2012). 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 rates of base substitutions 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*, 5th edn, vol. 1, pp. 87–110. Edited by C. P. Kurtzman, J. W. Fell & T. Boekhout. Amsterdam: Elsevier.

Lachance, M.-A. (2011). *Metschnikowia* Kamienski. In *The Yeasts: a Taxonomic Study*, 5th edn, vol. 1, pp. 575–620. Edited by C. P. Kurtzman, J. W. Fell & T. Boekhout. 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). *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., Bowles, J. M. & Starmer, W. T. (2003b). Geography and niche occupancy as determinants of yeast biodiversity: the yeast–insect–morning glory ecosystem of Kīpuka Puaulu, Hawai'i. *FEMS Yeast Res* **4**, 105–111.

Lachance, M.-A., Ewing, C. P., Bowles, J. M. & Starmer, W. T. (2005). *Metschnikowia hamakuensis* sp. nov., *Metschnikowia kamakouana* 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 *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 S. W. Nicolson, M. Nepi & W. Pacini. Dordrecht: Springer.

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* **61**, 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 C. E. Jones & R. J. Little. 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 T-Coffee. *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.