Metschnikowia vanudenii sp. nov. and *Metschnikowia lachancei* sp. nov., from flowers and associated insects in North America

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Two new species of the ascospore yeast genus *Metschnikowia* were isolated from nectaries and associated muscoid flies of flowers from the common milkweed (*Asclepias syriaca*) in North America, and are described as *Metschnikowia vanudenii* [type strain = PYCC 4650^T = CBS 9134^T = NRRL Y-27243^T = UWO(PS) 86A4.1^T] and *Metschnikowia lachancei* [type strain = PYCC 4605^T = CBS 9131^T = NRRL Y-27242^T = UWO(PS) 7ASB2.3^T]. As with the previously described *Metschnikowia gruessii*, *M. vanudenii* has vegetative cells with an 'aeroplane' or cross-like configuration, produces ovoid chlamydospores and forms ellipsoidopedunculate asci with two acicular ascospores. *Metschnikowia lachancei* is distinguished from other *Metschnikowia* species by formation of club-shaped asci with 1–2 thick clavate ascospores. The phylogenetic positions of the proposed new species within *Metschnikowia* were determined from sequence analysis of the D1/D2 domain of 26S rDNA. The new species show low nuclear DNA relatedness with neighbouring taxa.

INTRODUCTION

Metschnikowia species often thrive in insect–flower ecosystems where they proliferate owing to the high sugar concentration of the nectar. The sugar composition of floral nectar varies among plant species, but shows a predominance of hexoses (glucose and fructose) and the disaccharide sucrose. Several recently described *Metschnikowia* species (Hong *et al.*, 2001; Kurtzman & Droby, 2001; Lachance *et al.*, 2001a, b; Lachance & Bowles, 2002) add to the abundance of flower-associated species over those of aquatic origin, suggesting that the proliferation of terrestrial flowering plants and associated insects has markedly impacted genetic diversification in *Metschnikowia*. The large number of nucleotide substitutions among *Metschnikowia* species in the D1/D2 domain of large subunit rDNA, especially among the large-spored species, further supports the idea that the genus is highly diverged and that there are numerous undetected species.

In the present study, two novel species of *Metschnikowia* are proposed. These species, to be named *Metschnikowia vanudenii* and *Metschnikowia lachancei*, were isolated in North America from the common milkweed *Asclepias syriaca* and associated insects. *M. vanudenii* shares with *Metschnikowia gruessii* the formation of 'aeroplane' cells, i.e. superimposed pairs of cells that form a cross-like configuration. The second species, *M. lachancei*, is characterized by clavate ascospores, which differentiates it from all other recognized species of *Metschnikowia*.

METHODS

Yeast strains. The five known strains of *M. vanudenii* were obtained from North America. The type strain of *M. vanudenii* (PYCC 4650^T) was isolated by M. A. Lachance, in 1986, from a 'walk plate' by allowing a muscoid fly collected from the common milkweed *Asclepias syriaca* in Guelph, Ontario, Canada, to walk over the solidified medium (Lachance *et al.*, 2001a). The origin of the remaining four strains (PYCC 4603, PYCC 4606, PYCC 4648 and PYCC 4649) was nectar from flowers of *A. syriaca* sampled in the province of Québec, Canada. The type strain of *M. lachancei* (PYCC 4605^T) was isolated in 1987 from nectar of the flower *A. syriaca* in South Bombay, NY, USA. W. T. Starmer recovered a second strain of this species (PYCC 5767) from a flower of *Calystegia sepium* (hedge bindweed), in Newfound Gap, Smoky Mountains National Park, TN, USA. All strains examined in this work have been deposited

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Abbreviation: nDNA, nuclear DNA.

The GenBank accession numbers for the D1/D2 26S rDNA sequences of strain PYCC 4650^T and PYCC 4605^T are AF017404 and AY080995, respectively.

(living and dried) in the Portuguese Yeast Culture Collection (PYCC), Monte de Caparica, Portugal, as well as in other collections, and accession numbers are given in Table 1.

Phenotypic characterization. The morphological and physiological characteristics of the strains were determined by conventional techniques described by Yarrow (1998). Sporulation was induced using the conditions recommended by Pitt & Miller (1968).

DNA base composition and DNA–DNA reassociations. For thermal denaturation and reassociation experiments, DNA was purified by chromatography on hydroxylapatite columns following the protocol of Britten *et al.* (1970). The mean nuclear DNA (nDNA) G+C content from *Metschnikowia* strains was determined by thermal denaturation (T_m) (Marmur & Doty, 1962) using a Gilford Response II Spectrophotometer and its thermal programming software. The G+C content was determined at least twice for each strain in 0.1 × SSC (Owen *et al.*, 1969). The extent of DNA relatedness was determined spectrophotometrically as described by Seidler & Mandel (1971) and modified by Kurtzman *et al.* (1980), using a Gilford Response II Spectrophotometer and its thermal kinetics program.

rDNA sequencing and sequence analysis. Methods for nDNA isolation, amplification by PCR of the 26S rDNA domain D1/D2 and sequencing with the ABI TaqDyeDeoxy Terminator Cycle Sequencing kit and the ABI model 377 automated DNA sequencer (PE Biosystems) were previously described (Kurtzman & Robnett, 1998). Sequence data were visually aligned with QEDIT 2.15 (SemWare, Marietta, GA, USA). Phylogenetic relationships were calculated with the maximum-parsimony program of PAUP*4.0b3a (Swofford, 1998), as well as with the neighbour-joining program using the Kimura two-parameter distance measure. *Schizosaccharomyces pombe* was the designated outgroup species in all analyses.

Confidence limits for phylogenetic trees were estimated from bootstrap analysis (1000 replications). The nucleotide sequences for the new species reported here, as well as for reference species, have been deposited with GenBank under the accession numbers given in Fig. 3.

RESULTS AND DISCUSSION

Latin diagnosis of *Metschnikowia vanudenii* Giménez-Jurado, Kurtzman & Spencer-Martins sp. nov.

In medio liquido cum dextroso et peptono et extracto levedinis post dies 3 ad 25 °C cellulae sunt ovoideae aut cylindrateae (3–5 × 7–9 μm), binae vel in catenis brevis. Chlamydo sporae praesentes plerumque, globosus (6–8 × 8–10 μm) refractivae. Reproductio vegetativae per gemmatae multipolarem et cellulae cruciformis formatur. Post dies 7 ad 25 °C neque pseudohyphae nec hyphae formatur, sedimentum abundus. Cultura in medio agaro cum dextroso et peptono et extracto levedinis post dies 7 ad 25 °C albida vel crenea butyrosa cum margine glabro. In agaro malti post dies 7 ad temperatura ambeunte, chlamydo sporae globosus refractivae abundus et cellulae conjugationem tubi junctioniz formatur. In agaro farinae Zea mays post dies 7 ad 25 °C pseudohyphae rudimentari formatur. Asci in agaro V8 dilutus post dies 12 ad 17 °C ellipsoidopedunculati (6–8 × 25–45 μm), pedunculi cylindrati, 2-spori. Ascosporae aciculares vel filiformes. Fermentatio glucosum. Assimilat glucosum, sucrosum, maltosum,

Table 1. Origin and DNA base composition of the *Metschnikowia* strains examined

Strain*	Other designation(s)*	Isolation source	G + C (mol%)†	GenBank no.
<i>M. lachancei</i>				
PYCC 4605 ^T	CBS 9131 ^T , UWO(PS) 7ASB2.3 ^T , NRRL Y-27242 ^T	Flower of <i>Asclepias syriaca</i> , South Bombay, NY, USA	46.9 ± 0.4	AY080995
PYCC 5767	SU(B) 99.220.4	Flower of <i>Calystegia sepium</i> , Newfound Gap, Smoky Mountains National Park, TN, USA	ND	AF313346
<i>M. vanudenii</i>				
PYCC 4650 ^T	CBS 9134 ^T , UWO(PS) 86AW4.1 ^T , NRRL Y-17036 ^T	Muscoid fly plate of <i>Asclepias syriaca</i> , Ontario, Canada	44.4 ± 0.6	AF017404
PYCC 4603	UWO(PS) 87AD3.1, NRRL Y-27241	Nectar of <i>Asclepias syriaca</i> , Québec, Canada	43.3 ± 0.2	
PYCC 4606	UWO(PS) 86A1.1	Nectar of <i>Asclepias syriaca</i> , Québec, Canada	43.4 ± 0.2	
PYCC 4648	CBS 9132, UWO(PS) 86A2.1	Nectar of <i>Asclepias syriaca</i> , Québec, Canada	42.7 ± 0.5	
PYCC 4649	CBS 9133, UWO(PS) 86AW6.1	Nectar of <i>Asclepias syriaca</i> , Québec, Canada	ND	
<i>M. gruessii</i>				
PYCC 4382 ^T	CBS 7657 ^T , NRRL Y-17809 ^T	Flower of <i>Hebe salicifolia</i> , Oeiras, Portugal	39.2 ± 0.6	U45737
<i>M. reukaufii</i>				
PYCC 4446 ^T	CBS 5834 ^T , NRRL Y-7112 ^T	Flower of <i>Epilobium angustifolium</i> , Canada	42.8 ± 0.2	U44825

ND, Not determined.

*CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA; PYCC, Portuguese Yeast Culture Collection, Centro de Recursos Microbiológicos (CREM), Universidade Nova de Lisboa, Monte de Caparica, Portugal; UWO, University of Western Ontario, Canada; SU, Syracuse University, NY, USA.

†DNA base composition determined by melting temperature (T_m), based on three or more determinations.

L-sorbose, cellobiose (variable), α,α -trehalose (variable), melezitose, amylose soluble (variable), D-xylose (variable), ethanol (variable), glycerol (variable), ribitol (variable), D-mannitol (variable), D-sorbitol, salicin (variable), arbutin, D-glucosamine, gluconolactone (variable), D-glutamate (variable), N-acetylglucosamine (variable) et 2-ketogluconate ad non D-galactose, lactose, melibiose, raffinose, inulin, L-arabinose, D-arabinose, D-ribose, L-rhamnose, erythritol, D-glucitol, methyl- α -D-glucoside, acidum lacticum, acidum succinicum, acidum citricum, myo-inositol, methanol, acidum glucuronicum, acidum gluconicum, acidum tartaricum et acidum malicum. Assimilat L-lysini, ethylamini, cadaverini, D-glucosaminum (variable), betanini, D-glutaminum ad non kalli nitratis, sodii nitrosi, creatino nec creatinino. Vitaminae externae ad crescentiam necessariae sunt. Crescit in 30 °C sed non in 35 °C. Materia amyloidea non formatur. Ureum non finditur. Ubiquinonum majus Q-9. Proportio molaris guanini plus cytosini in acidi deoxyribonucleati 44.4 mol%.

Typus: PYCC 4650^T, ex flore, exsiccatus et vivus in collectione zymotica Centraalbureau voor Schimmelcultures (CBS 9134^T), Trajectum ad Rhenum, praeservatus.

Description of *Metschnikowia vanudenii* Giménez-Jurado, Kurtzman & Spencer-Martins sp. nov.

Growth in glucose (2%) yeast extract (0.5%) peptone (1%) broth. After 3 days at 25 °C, vegetative cells are ovoid to cylindrical (3–5 × 7–9 µm) and occur mostly in pairs, tetrads or in short chains. Some cells develop into characteristic ‘aeroplane’ or cross formations. Chlamydospores, when present, are globose to subglobose in shape (6–8 × 8–10 µm), highly refractile and contain several spherical lipid globules. Some strains produce few or no chlamydospores. No pseudohyphae or hyphae were observed in this medium. Cell division occurs by multilateral budding. After 1 week at 25 °C, abundant sediment is formed.

Growth on glucose (2%) yeast extract (0.5%) peptone (1%) agar. After 1 week at room temperature, strains form white to cream, smooth to papillate, glossy, butyrous colonies with entire margins.

Growth on malt extract agar. After 1 week at room temperature, strains form copious amounts of subglobose chlamydospores with large, easily seen oil droplets. Cross or ‘aeroplane’ configurations are present in strains PYCC 4606, PYCC 4648 and PYCC 4650^T.

Dalmau plate cultures on corn-meal agar. After 1 week at 25 °C, growth under semi-anaerobic conditions showed development of primitive pseudohyphae.

Formation of ascospores. Asci with ascospores were formed by PYCC 4650^T after incubation for 12 days at

Fig. 1. Phase-contrast photomicrographs of *M. vanudenii* PYCC 4650^T grown on dilute V8 agar at 17 °C. Mature ascus containing two filiform ascospores. Scale bar, 10 µm.

17 °C on dilute (1:15) V8 agar (Fig. 1). Asci initially developed from ellipsoid chlamydospores, which further elongated resulting in a cylindrical peduncle shape. Mature asci (6–8 × 25–45 µm) contain two acicular to filiform ascospores, which are noticeably pointed at one end. Most asci are persistent, although a few become deliquescent.

Ascosporeulation was not observed for strains PYCC 4603, PYCC 4606, PYCC 4648 and PYCC 4649. These four strains were mixed pairwise in all possible combinations with just one pair, PYCC 4648 × PYCC 4649, showing evidence of mating. Conjugated cells and apparent zygotes were observed, but ascospores were not seen. Additionally, when this pair of strains was mixed, the frequency of cross-like cells markedly increased over that seen for the individual strains. The observation of conjugants suggests that *M. vanudenii* might be heterothallic with haploid strains showing varying degrees of mating competence.

Physiological and biochemical characteristics

See Table 2.

Etymology. The specific epithet *vanudenii*, Latin gen. of van Uden, was chosen in honour of the late Professor Nicolau van Uden for his important contributions to the study of the yeast genus *Metschnikowia* as well as for his work on the physiology and biochemistry of yeasts.

Origin and deposits. The type strain PYCC 4650^T [UWO(PS) 86AW4.1] was isolated from a ‘walk plate’ sampling of a muscoid fly collected from the common milkweed *Asclepias syriaca* in Guelph, Ontario, Canada. This strain has been deposited (living and dried) in the Portuguese Yeast Culture Collection (PYCC), Centro de Recursos Microbiológicos (CREM), Universidade Nova de Lisboa, Caparica, Portugal, and under number CBS 9134

Table 2. Physiological and biochemical characteristics that differentiate *M. lachancei* sp. nov. from *M. vanudenii* sp. nov.

Species: *M. lachancei* strains PYCC 4605^T and PYCC 5767; *M. vanudenii* strains PYCC 4650^T, PYCC 4603, PYCC 4606, PYCC 4648 and PYCC 4649. All strains tested positive for glucose fermentation. Glucose, sucrose, maltose, arbutin, melezitose and xylitol were assimilated by all strains, but D-ribose, L-arabinose, D-arabinose, L-rhamnose, methyl- α -D-glucoside, melibiose, lactose, raffinose, inulin, starch, *m*-erythritol, galactitol, *m*-inositol, D-glucuronic, DL-lactic acid, citric acid, malic acid, L-tartaric acid and methanol were not. Ethylamine, cadaverine and betaine, as sole nitrogen sources, were assimilated by all strains, but nitrate, nitrite, creatine and creatinine were not. All strains were able to grow in vitamin-free medium, 50 % glucose, 10 % NaCl/5 % glucose and at 30 °C, but were not able to grow in YNB with 0.01 % cycloheximide and at 35 °C. None formed starch-like compounds. Abbreviations: w, weak; v, variable; +, positive; –, negative.

Characteristic	<i>M. lachancei</i>	<i>M. vanudenii</i>
Assimilation of carbon compounds:		
D-Galactose	+	–/w
L-Sorbose	w	v
D-Glucosamine	w	v
D-Xylose	+	v
α,α -Trehalose	+	v
Cellobiose	+	v
Salicin	+	v
Glycerol	–	v
Ribitol	w	v
D-Glucitol	–	v
D-Mannitol	+	v
D-Glucono-1,5-lactone	+	v
2-Keto-D-gluconic acid	+	v
D-Gluconic acid	+	v
Succinic acid	w	–
Ethanol	–	v
N-Acetyl-D-glucosamine	+	v
Assimilation of nitrogen compounds:		
D-Glucosamine	–	v
L-Glutamic acid	+	v
Lipolytic activity:		
Tween 80	v	–
Other:		
Hydrolysis of urea	+	–
nDNA G + C content (mol%)	46.9 $\pm$ 0.4 (PYCC 4605 ^T)	44.4 $\pm$ 0.6 (PYCC 4650 ^T)

in the Centraalbureau voor Schimmelcultures in Utrecht, The Netherlands.

Latin diagnosis of *Metschnikowia lachancei* Giménez-Jurado, Kurtzman, Starmer & Spencer-Martins sp. nov.

In medio liquido cum dextroso et peptono et extracto levedinis post dies 3 ad 25 °C cellulae sunt ellipsoideae aut cylindrateae (3–7 $\times$ 8–3 μ m), singulae, binae vel aggregatae, gemmationem multipolarem reproducentes. Breves pseudohyphae formatur. Chlamydosporae non formatur. Post dies 7 ad 25 °C sedimentum parcum formatur. Cultura in medio agaros cum dextroso et peptono et extracto levedinis post dies 7, albida vel crenea cum margo crenatus. In agaros farinae Zea

mays post dies 7 ad 25 °C pseudohyphae vera cum conidia globosa formatur. In agaros V8 dilutus post dies 3–4 ad 17 °C asci clavatispedunculati, pedunculi cylindrateae (3–6 $\times$ 14–23 μ m), 2-spori in quoque asco. Ascosporae clavatus vel pyriformis. Fermentatio glucosum. Assimilat glucosum, D-galactosum, D-glucosaminum, D-xylosum, sucrosom, maltosom, α,α -trehalosom, cellobiosom, salicinum, arbutinum, melezitosom, glycerolum, xylitolum, D-sorbitolum, D-mannitolum, glucono- δ -lactonum, 2-ketogluconatum, acidum gluconicum, D-glutamatum et N-acetyl-glucosaminum, et ad non L-sorbosom, D-ribosom, L-arabinosom, D-arabinosom, L-rhamnosom, ethanolum, methanolum, methyl- α -D-glucosidum, melibiosom, lactosom, raffinolum, inulinum, amyllum solubile, erythritolum, ribitolum, D-glucitolum, myo-inositolum, acidum glucoronicum, acidum lacticum, acidum

succinicum, acidum citricum, acidum malicum et acidum tartaricum. Assimilat L-lysini, ethylamini, cadaverini, beta-nini, D-glutaminum ad non kallii nitratis, sodii nitrosi, D-glucosaminum, creatino nec creatinino. Vitaminae externae ad crescentiam necessariae sunt. Crescit in 30 °C sed non in 35 °C. Materiae amyloidea non formatur. Ureum finditur. Ubiquinonum majus Q-9. Proportio molaris guanini plus cytosini in acidi deoxyribonucleati 46.9 mol%.

Typus: PYCC 4605^T, ex flore, in collectione zymotica Centraalbureau voor Schimmelcultures (CBS 9131^T), Trajectum ad Rhenum, praeservatus.

Description of *Metschnikowia lachancei* Giménez-Jurado, Kurtzman, Starmer & Spencer-Martins sp. nov.

Growth in glucose (2%) yeast extract (0.5%) peptone (1%) broth. After 3 days at 25 °C, vegetative cells are ellipsoidal to cylindrical (3–7 × 8–13 µm) and occur in pairs, tetrads or small chains. Cell division occurs by multilateral budding. Sparse slender pseudohyphae are formed. Chlamydospores are not formed. Some cells contain refractile globules. After 1 week at 25 °C sediment is formed.

Growth on glucose (2%) yeast extract (0.5%) peptone (1%) agar. After 1 week at room temperature, the streak culture is cream to off-white coloured, smooth, glossy and with a lobate margin.

Dalmau plate cultures on corn-meal agar. After 1 week at 25 °C, growth under semi-anaerobic conditions shows the presence of slender pseudohyphae. Cells become elongated and eventually form branching pseudohyphae with terminal spherical conidia. Branching occurs from the quadrants of the parent cell with secondary branching occurring quite often. No crosswalls form between hyphal cells.

Formation of ascospores. Asci with ascospores were observed in cultures of PYCC 4605^T after 3–4 days incubation at 17 °C in dilute (1:15) V8 agar medium. Asci are clavate in shape and develop directly from vegetative cells. Some asci develop directly after conjugation of two cells followed by differentiation at one end of the conjugant giving rise to a long cylindrical peduncle. Mature asci (3–6 × 14–3 µm) usually contain two spindle-shaped ascospores, side by side, with their pointed ends oriented towards the peduncle or elongated portion of the ascus (Fig. 2), but occasionally asci with one protruding ascospore are observed. Asci occur either in pairs or groups of short chains, show different stages of development and are usually persistent. Older asci show ascospores with swollen ends containing visible vacuolar and lipidic material. No ascospores were observed in strain PYCC 5767.

Fig. 2. Phase-contrast photomicrograph of *M. lachancei* PYCC 4605^T. Formation of asci containing clavate ascospores, after 1 week at 17 °C in dilute (1:14) V8 agar. Scale bar, 10 µm.

Physiological and biochemical characteristics

See Table 2.

Etymology. The specific epithet *lachancei*, Latin gen. of Lachance, was chosen in honour of Professor Marc-André Lachance, in recognition of his important contribution to yeast taxonomy and the discovery of exotic species of the yeast genus *Metschnikowia*.

Origin and deposits. The type strain PYCC 4605^T [= UWO(PS) 7ASB2.3^T] was isolated from nectar of the flower *Asclepias syriaca* in South Bombay, NY, USA. This strain has been deposited (living and dried) in the PYCC CREM, Universidade Nova de Lisboa, Caparica, Portugal, and under number CBS 9131 in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Table 3. DNA relatedness (%) among isolates of *M. vanudenii* and *M. lachancei* and the type strains of *M. gruessii* and *M. reukaufii*

Strains: 1, *M. vanudenii* PYCC 4650^T; 2, *M. vanudenii* PYCC 4649; 3, *M. vanudenii* PYCC 4648; 4, *M. vanudenii* PYCC 4606; 5, *M. vanudenii* PYCC 4603; 6, *M. lachancei* PYCC 4605^T; 7, *M. gruessii* PYCC 4382^T; 8, *M. reukaufii* PYCC 4446^T. Data are means of two or more determinations. ND, Not determined.

Strains	1	2	3	4	5	6	7	8
1	100	85	100	94	ND	0	2	19
2		100	88	90	ND	7	4	21
3			100	ND	ND	ND	7	30
4				100	76	ND	0	3
5					100	ND	1	0
6						100	12	23

Fig. 3. Neighbour-joining tree with the Kimura two-parameter distance correction showing phylogenetic placement of *M. vanudenii* and *M. lachancei* among species of the genus *Metschnikowia*, related *Candida* species and reference species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (outgroup species in the analysis). Bootstrap values (1000 replications) are given for nodes with >50% support. T, Type strain; NT, neotype strain. Culture collection prefixes: Y and YB, ARS Culture Collection (NRRL); CBS, Centraalbureau voor Schimmelcultures; KCTC, Korean Collection for Type Cultures.

G+C content and nDNA relatedness

Initial characterization of the two new species was from mol% G+C content, which revealed that they separated into two groups, *M. lachancei* with 46.9 mol% and *M. vanudenii* with a strain mean of 43.5 mol%. This latter value is similar to the G+C content of *M. reukaufii* (Table 1). nDNA reassociation experiments demonstrated *M. vanudenii*, *M. lachancei* and *M. guessii* to be separate species (Table 3), which is consistent with relationships determined from rDNA sequence analysis (Fig. 3).

Phylogeny

Phylogenetic analysis of the D1/D2 domain of the large subunit rDNA revealed that *M. vanudenii* and *M. lachancei* fit within the *Metschnikowia* clade (Fig. 3). The two new species form a distinct lineage with *M. guessii* and cluster

with those members of *Metschnikowia* that produce small ascospores.

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