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ORIGINAL PAPER

Four new species of *Metschnikowia* and the transfer of seven *Candida* species to *Metschnikowia* and *Clavispora* as new combinations

Cletus P. Kurtzman · Christie J. Robnett · Eleanor Basehoar · Todd J. Ward

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Abstract From comparisons of ITS1-5.8S-ITS2 and gene sequences for nuclear D1/D2 LSU rRNA, nuclear SSU (18S) rRNA, translation elongation factor 1-α (EF1-α) and RNA polymerase II subunit 2 (RPB2), the following four new ascosporogenous yeast species were resolved and are described as *Metschnikowia anglica* (NRRL Y-7298^T [type strain], CBS 15342, MycoBank MB 823167), *Metschnikowia leonuri* (NRRL Y-6546^T, CBS 15341, MB 823166), *Metschnikowia peoriensis* (NRRL Y-5942^T, CBS 15345, MB 823164) and *Metschnikowia rubicola* (NRRL Y-6064^T, CBS 15344, MB 823165). The following six species of *Candida* are members of the *Metschnikowia* clade and are proposed for transfer to *Metschnikowia* as new combinations: *Candida chrysomelidarum* (NRRL Y-27749^T, CBS 9904, MB 823223), *Candida gelsemii* (NRRL Y-48212^T, CBS 10509, MB 823192), *Candida kofuensis* (NRRL Y-27226^T, CBS 8058, MB 823195), *Candida picachoensis* (NRRL Y-27607^T, CBS 9804, MB 823197), *Candida pimensis* (NRRL Y-27619^T, CBS 9805, MB 823205) and *Candida rancensis* (NRRL Y-48702^T, CBS 8174, MB 823224). *Candida fructus* (NRRL Y-17072^T, CBS 6380, MB 823206) is transferred to *Clavispora* as a new combination, and *Candida musae* is shown to be a synonym of *C. fructus*. Apparent multiple alleles for ITS, D1/D2, EF1-α and RPB2 were detected in strains of some species.

Keywords *Candida* · *Clavispora* · *Metschnikowia* · Multiple alleles · New combinations · 11 new taxa

Introduction

The ascomycete yeast genus *Metschnikowia* was described more than 100 years ago (Kamienski 1899) and most of the species produce distinctive long, needle-shaped ascospores. Species are common worldwide and often isolated from flowers and insects but some, such as members of the *Metschnikowia bicuspidata* clade, are known to parasitize brine shrimp (*Artemia* spp.), *Daphnia* species and copepods (Lachance 2011, 2016 and references therein). The *Metschnikowia pulcherrima* clade is of particular interest to agriculture because *Metschnikowia pulcherrima* and *Metschnikowia fructicola* are effective as biocontrol agents to inhibit fruit storage rots such as those caused by species of *Penicillium*, *Botrytis* and

Cletus P. Kurtzman: Sadly, Clete Kurtzman died whilst this manuscript was under revision. An appreciation has been published (Lachance 2018).

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certain other fungi (Janisiewicz et al. 2001; Kurtzman and Droby 2001; Piano et al. 1997; Türkel et al. 2014). The mechanism of biocontrol is not entirely clear, but may be partially based on competitive inhibition through sequestration of iron via production of the iron-binding compound pulcherrimin (Oro et al. 2014; Sipiczki 2006). Additionally, *M. fructicola* produces chitinase enzymes in the presence of fungal pathogens (Banani et al. 2015; Wang et al. 2017) and these may assist in biocontrol. Although important for agricultural uses, few biotechnological applications have so far been demonstrated for species of *Metschnikowia,* although one example is stereoconversion of secondary alcohols by *Metschnikowia koreensis* (Meena et al. 2014).

The taxonomy of *Metschnikowia* has been partially clarified by DNA sequence comparisons, which have resulted in better definition of earlier described species recognized from phenotype and the discovery of numerous new species. Lachance (2016) reported that 81 species are now known for the *Metschnikowia* clade, but this clade is recognized from short DNA sequences, such as the D1/D2 domains of the LSU rRNA gene, or from concatenation of a few gene sequences. Consequently, branch support in phylogenetic trees based on these analyses is often weak and species relationships are uncertain. Examples of this problem are shown in the D1/D2 analysis of Lachance et al. (2011), the multilocus analysis of Guzmán et al. (2013) and the analysis given in the present study. However, even with weak branch support, it appears that *Metschnikowia,* as presently recognized, may actually represent several genera. The large-spored species, such as *Metschnikowia aberdeeniae* and *Metschnikowia hawaiiensis*, form a separate clade, the core species of the *Clavispora* clade are separate from neighbors and several nearby species groupings are potential genera. From this, it appears that the taxonomy of this large group of species can only be resolved from whole genome comparisons.

In the present work, four new ascosporogenous species are described and assigned to *Metschnikowia* and seven new combinations are proposed, six *Candida* species in *Metschnikowia* and one *Candida* species in *Clavispora*. These new species and new combinations are closely related to the type species of their respective genera and are expected to be unaffected by any future recircumscription of *Metschnikowia* and *Clavispora*.

Materials and methods

Isolation and phenotypic characterization of strains of the new species to be described

Most strains of the proposed new species examined in this study were isolated over many years by L. J. Wickerham, tentatively identified from phenotype as *Metschnikowia pulcherrima* or *M. reukaufii*, and accessioned into the ARS Culture Collection for future study. Isolation typically consisted of placing a substrate sample into a flask of liquid isolation medium that contained Yeast Nitrogen Base along with glucose, cellobiose, D-xylose, L-rhamnose, erythritol, D-mannitol and calcium 2-keto-gluconate and incubating at 25 °C for 1–2 weeks on a shaker. A loopful of growth from the flask was streaked on YM agar plates from which individual colonies were isolated (Wickerham 1969).

Fermentation and growth tests for strain characterization were conducted in liquid media following standard methods (Kurtzman et al. 2011) and incubated for at least 4 weeks at 25 °C. Individual strains and strain mixtures were tested for ascosporulation on some or all of the following media: YM agar (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, 20 g agar/l H_2O); 5% malt extract agar (50 g malt extract, 30 g agar/l H_2O); RG agar (0.2 g yeast extract, 0.2 g peptone, 1.0 g glucose and 20 g agar/l H_2O); YCBAS agar (11.7 g Difco Yeast Carbon Base, 100 mg $(NH_4)_2SO_4$, 20 g agar/l H_2O), and 1:19 V8 juice agar (equal volumes of deionized water and V8 juice, adjust pH to 5.5 with NaOH, dilute 1:19 with water, agar to 2%, autoclave). Incubation times and temperatures are reported in the new species descriptions.

DNA isolation, sequencing and phylogenetic analysis

Methods for DNA isolation and sequencing of the internal transcribed spacer (ITS) and genes for domains D1/D2 of the nuclear large subunit rRNA (D1/D2), nuclear small subunit rRNA (18S), translation elongation factor-1α (EF-1α) and RNA polymerase II, subunit 2 (RPB2) were previously reported (Kurtzman and Robnett 1998, 2003). The most commonly used primers were: ITS, NS-7A and ITS4; 18S, NS-1AF, NS-3AF, NS-3AR, NS-5.4R,

NS-7BR, NS-8R; D1/D2, NL-1, NL-4; EF-1α, YTEF-1F, YTEF-6AR, EF1-1577F, EF1-1567R, EF1-983F and EF-2218R; RPB2, YRPB2-1F, YRPB2-2F, YRPB2-2.2F, YRPB2-4F, YRPB2-5F, YRPB2-5R, YRPB2-6F, RPB2-6FR, RPB2-7R, YRPB2-8F, YRPB2-8R, YRPB2-9R, YRPB2-9.3R and YRPB2-11R. For phylogenetic analysis, sequences were aligned using Muscle, which is in the MEGA version 7.0.14 software package (Kumar et al. 2016), and the alignments were visually adjusted. Phylogenetic relatedness among species was determined from the maximum likelihood program included in MEGA 7.0.14 using the Hasegawa–Kishino–Yano model, and bootstrap support was determined from 1000 replicates.

Results and discussion

Relatedness among species of the "small-spored" members of *Metschnikowia* and the many *Candida* species that appear associated with them (Daniel et al. 2014; Guzmán et al. 2013; Kurtzman and Robnett 2013; Lachance 2011, 2016; Lachance et al. 2011) was assessed from maximum likelihood analysis of concatenated gene sequences for D1/D2 and EF-1α, and typical of analyses that include few gene sequences, deep nodes in the phylogenetic tree showed weak branch support (Fig. 1). The clade delimited by *Metschnikowia sinensis* and *M. lachancei*, which includes *M. bicuspidata*, type species of the genus *Metschnikowia*, was strongly supported (92% bootstrap support). Placement of certain other species, such as *M. saccharicola* and *M. drosophilae*, which are almost certainly members of *Metschnikowia*, are only weakly supported and continued assignment to the genus will require verification from more robust datasets, such as whole genome sequences.

The new species proposed for *Metschnikowia* in the current study are members of the *M. bicuspidata* clade, as are six *Candida* species proposed for transfer to *Metschnikowia*. An additional species, *Candida fructus*, is closely related to *Clavispora lusitaniae*, type species of *Clavispora*, and will be transferred to that genus. From the analysis presented in Fig. 1 and that of Guzmán et al. (2013), *Metschnikowia* and *Clavispora* appear to be separate genera. Multiple alleles were detected for certain genes in some species, and this will be discussed relative to species circumscription.

Description of new species of *Metschnikowia*

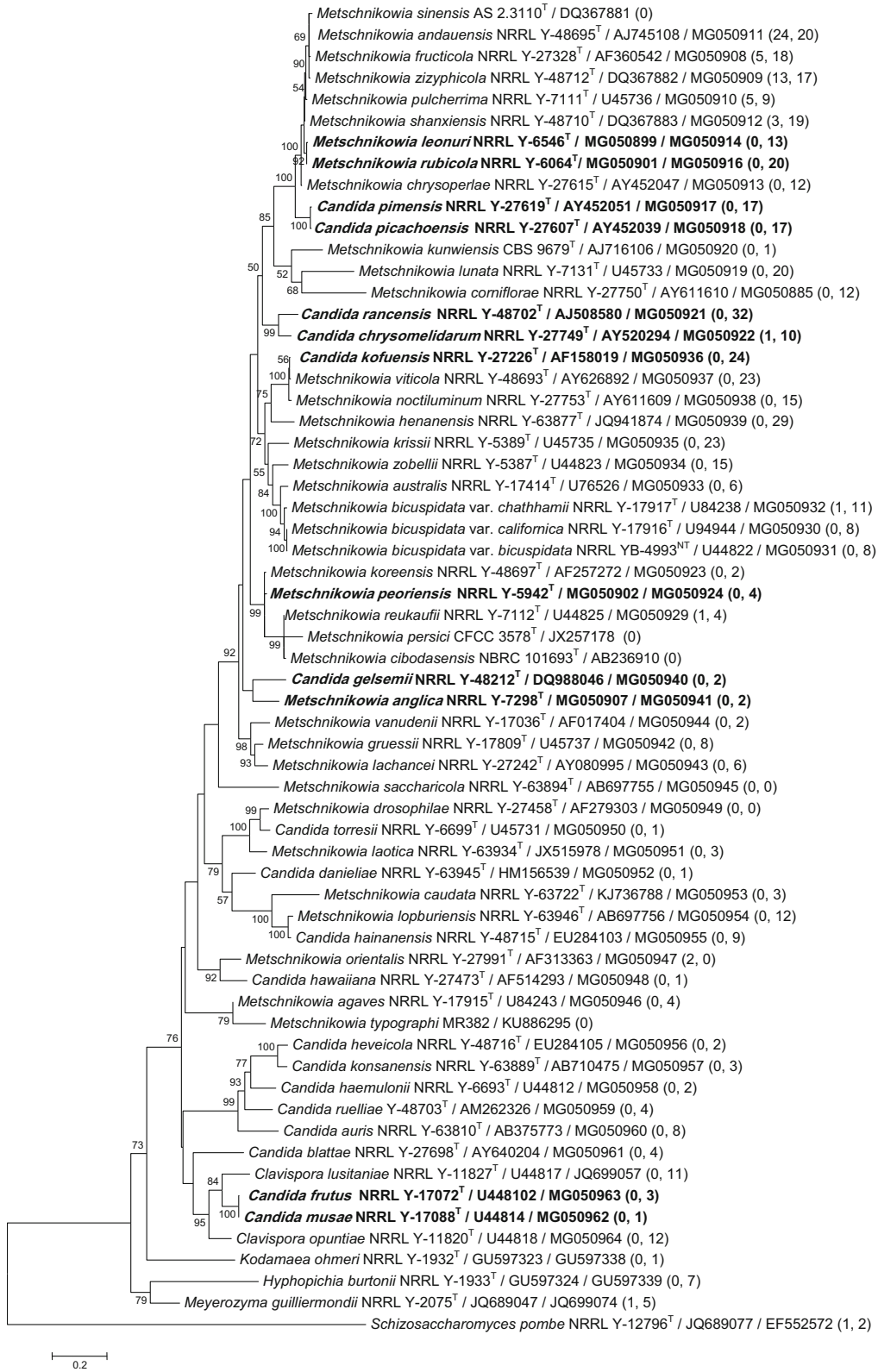
Metschnikowia peoriensis C. P. Kurtzman, C. J. Robnett & E. Basehoar sp. nov

Cell morphology

After 2 days on YM agar at 25 °C, cell morphology ranges from spherical (3–8 μm) to ellipsoidal (2–3 × 3–8 μm). Cell division is by multilateral budding and cells occur singly and in budded pairs (Fig. 2a). After 7 days at 25 °C, poorly differentiated pseudohyphae formed under the coverglass on a Dalmau plate with corn meal agar (Fig. 2b), but true (septate) hyphae were not detected. Colony growth is white, semi-glistening, butyrous in texture and low with a slightly irregular margin. Strains did not form pulcherrimin on either YM agar or V8 juice agar.

Ascosporic state

Eleven of the 18 known strains of *M. peoriensis* form ascospores, six represent mating types and one that has not been observed to form ascospores or to conjugate with either mating type (Table 1). Ascosporulation was observed on 1:19 V8 juice agar after incubation at 15 °C. NRRL Y-5942 and several other strains formed abundant asci with ascospores after 7 days, but other strains formed asci only after 4 weeks. Asci are unconjugated and develop from the large "pulcherrima" cells or "chlamydospores" that are abundantly present and assume a sphaeropedunculate shape (Fig. 2d). Asci measure 25–36 μm in length with the basal portion having a diameter of 5–8 μm. Asci are persistent and produce two closely paired acicular ascospores. Conjugation between mating types was tested on 1:19 V8 juice agar with incubation at 15 °C. Conjugants were usually detected within 2–3 days following mixing of complementary strains (Fig. 2c). Cultures with conjugants did not form asci, but when these cultures were transferred to YM agar, incubated for 3 days at 25 °C, and then transferred back to V8 agar and incubated at 15 °C, a limited number of asci with ascospores formed. Apparently, growth on YM agar replenished cellular nutrient levels. The most


Metschnikowia sinensis AS 2.3110T / DQ367881 (0)
Metschnikowia andauensis NRRL Y-48695T / AJ745108 / MG050911 (24, 20)
Metschnikowia fructicola NRRL Y-27328T / AF360542 / MG050908 (5, 18)
Metschnikowia zizyphicola NRRL Y-48712T / DQ367882 / MG050909 (13, 17)
Metschnikowia pulcherrima NRRL Y-7111T / U45736 / MG050910 (5, 9)
Metschnikowia shanxiensis NRRL Y-48710T / DQ367883 / MG050912 (3, 19)
Metschnikowia leonuri NRRL Y-6546T / MG050899 / MG050914 (0, 13)
Metschnikowia rubicola NRRL Y-6064T/ MG050901 / MG050916 (0, 20)
Metschnikowia chrysoperlae NRRL Y-27615T / AY452047 / MG050913 (0, 12)
Candida pimensis NRRL Y-27619T / AY452051 / MG050917 (0, 17)
Candida picachoensis NRRL Y-27607T / AY452039 / MG050918 (0, 17)
Metschnikowia kunwiensis CBS 9679T / AJ716106 / MG050920 (0, 1)
Metschnikowia lunata NRRL Y-7131T / U45733 / MG050919 (0, 20)
Metschnikowia corniflorae NRRL Y-27750T / AY611610 / MG050885 (0, 12)
Candida rancensis NRRL Y-48702T / AJ508580 / MG050921 (0, 32)
Candida chrysomelidarum NRRL Y-27749T / AY520294 / MG050922 (1, 10)
Candida kofuensis NRRL Y-27226T / AF158019 / MG050936 (0, 24)
Metschnikowia viticola NRRL Y-48693T / AY626892 / MG050937 (0, 23)
Metschnikowia noctiluminum NRRL Y-27753T / AY611609 / MG050938 (0, 15)
Metschnikowia henanensis NRRL Y-63877T / JQ941874 / MG050939 (0, 29)
Metschnikowia krissii NRRL Y-5389T / U45735 / MG050935 (0, 23)
Metschnikowia zobellii NRRL Y-5387T / U44823 / MG050934 (0, 15)
Metschnikowia australis NRRL Y-17414T / U76526 / MG050933 (0, 6)
Metschnikowia bicuspidata var. chathhamii NRRL Y-17917T / U84238 / MG050932 (1, 11)
Metschnikowia bicuspidata var. californica NRRL Y-17916T / U94944 / MG050930 (0, 8)
Metschnikowia bicuspidata var. bicuspidata NRRL YB-4993NT / U44822 / MG050931 (0, 8)
Metschnikowia koreensis NRRL Y-48697T / AF257272 / MG050923 (0, 2)
Metschnikowia peoriensis NRRL Y-5942T / MG050902 / MG050924 (0, 4)
Metschnikowia reukaufii NRRL Y-7112T / U44825 / MG050929 (1, 4)
Metschnikowia persici CFCC 3578T / JX257178 (0)
Metschnikowia cibodasensis NBRC 101693T / AB236910 (0)
Candida gelsemii NRRL Y-48212T / DQ988046 / MG050940 (0, 2)
Metschnikowia anglica NRRL Y-7298T / MG050907 / MG050941 (0, 2)
Metschnikowia vanudenii NRRL Y-17036T / AF017404 / MG050944 (0, 2)
Metschnikowia gruessii NRRL Y-17809T / U45737 / MG050942 (0, 8)
Metschnikowia lachancei NRRL Y-27242T / AY080995 / MG050943 (0, 6)
Metschnikowia saccharicola NRRL Y-63894T / AB697755 / MG050945 (0, 0)
Metschnikowia drosophilae NRRL Y-27458T / AF279303 / MG050949 (0, 0)
Candida torresii NRRL Y-6699T / U45731 / MG050950 (0, 1)
Metschnikowia laotica NRRL Y-63934T / JX515978 / MG050951 (0, 3)
Candida danieliae NRRL Y-63945T / HM156539 / MG050952 (0, 1)
Metschnikowia caudata NRRL Y-63722T / KJ736788 / MG050953 (0, 3)
Metschnikowia lopburiensis NRRL Y-63946T / AB697756 / MG050954 (0, 12)
Candida hainanensis NRRL Y-48715T / EU284103 / MG050955 (0, 9)
Metschnikowia orientalis NRRL Y-27991T / AF313363 / MG050947 (2, 0)
Candida hawaiiana NRRL Y-27473T / AF514293 / MG050948 (0, 1)
Metschnikowia agaves NRRL Y-17915T / U84243 / MG050946 (0, 4)
Metschnikowia typographi MR382 / KU886295 (0)
Candida heveicola NRRL Y-48716T / EU284105 / MG050956 (0, 2)
Candida konsanensis NRRL Y-63889T / AB710475 / MG050957 (0, 3)
Candida haemulonii NRRL Y-6693T / U44812 / MG050958 (0, 2)
Candida ruelliae Y-48703T / AM262326 / MG050959 (0, 4)
Candida auris NRRL Y-63810T / AB375773 / MG050960 (0, 8)
Candida blattae NRRL Y-27698T / AY640204 / MG050961 (0, 4)
Clavispora lusitaniae NRRL Y-11827T / U44817 / JQ699057 (0, 11)
Candida frutus NRRL Y-17072T / U448102 / MG050963 (0, 3)
Candida musae NRRL Y-17088T / U44814 / MG050962 (0, 1)
Clavispora opuntiae NRRL Y-11820T / U44818 / MG050964 (0, 12)
Kodamaea ohmeri NRRL Y-1932T / GU597323 / GU597338 (0, 1)
Hyphopichia burtonii NRRL Y-1933T / GU597324 / GU597339 (0, 7)
Meyerozyma guilliermondii NRRL Y-2075T / JQ689047 / JQ699074 (1, 5)
Schizosaccharomyces pombe NRRL Y-12796T / JQ689077 / EF552572 (1, 2)
0.2

◀ **Fig. 1** Phylogenetic relationships among species of *Metschnikowia*, *Clavispora* and related clades determined from maximum likelihood analysis of concatenated gene sequences for D1/D2 LSU rRNA and EF-1α. GenBank accession numbers following strain numbers are D1/D2 and EF-1α, respectively. Bootstrap values > 50% are given at nodes and based on 1000 replicates. *Schizosaccharomyces pombe* was the designated outgroup species. Numbers of ambiguous nucleotides recorded for each sequences (D1/D2, EF-1α) are given in parentheses. Taxa in bold font represent new species and new combinations proposed in this study

active mating pair was NRRL Y-5931 (a) and NRRL Y-5934 (α).

Fermentation and growth reactions for this species are given in Table 2.

Type

NRRL Y-5942 is designated as the type strain of *Metschnikowia peoriensis.* The strain is preserved as a metabolically inactive lyophilized preparation (the holotype) at the ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA, and an isotype is preserved at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands as CBS 15345. MycoBank number: MB 823164.

Complementary mating types are NRRL Y-5931 (CBS 15336), mating type a, and NRRL Y-5934 (CBS 15337), mating type α. NRRL Y-5942, an ascosporogenous isolate, was isolated from the flower of a purple aster (*Aster novi-belgii*) collected in Peoria, Illinois, USA. Sources of other strains are given in Table 1.

Etymology

The species epithet *peoriensis* pertains to Peoria, Illinois, USA, the location that provided most of the strains examined in this study.

Ecology

Metschnikowia peoriensis is predominantly known from flowers of red clover (*Trifolium pretense*) and purple aster (*Aster novi-belgii*) collected in Peoria, Illinois, USA, and from two isolates obtained from wild blackberries (*Rubus* sp.) collected in Michigan, USA (Table 1). Because of the association with flowers, the species may be insect disseminated.

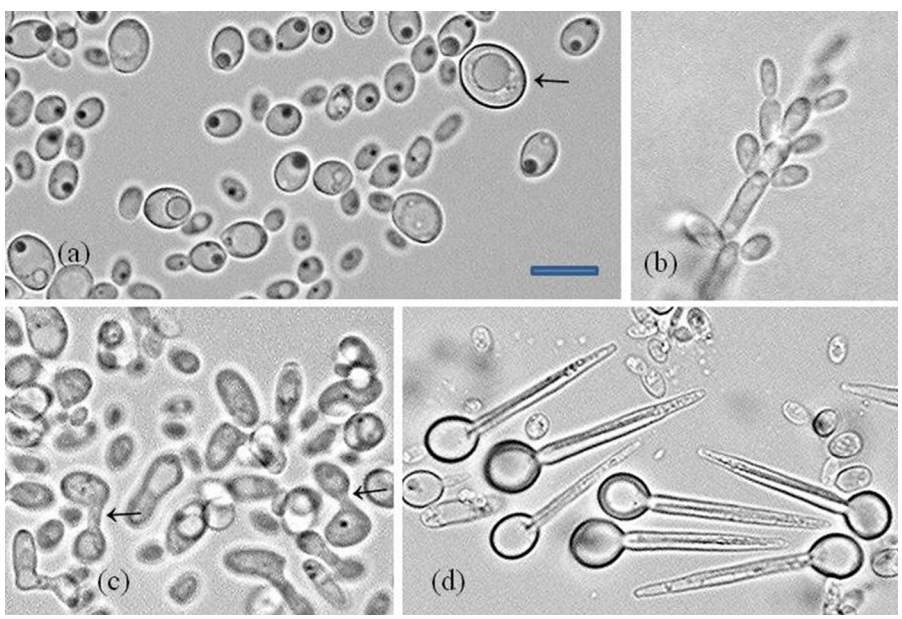


Fig. 2 *Metschnikowia peoriensis,* NRRL Y-5942^{T}. **a** Budding cells and a chlamydospore (arrow), YM agar, 2 days, 25 °C. **b** Simple pseudohypha, Dalmau plate, cornmeal agar, 7 days, 25 °C. **c** NRRL Y-5931 × NRRL Y-5934. Conjugating cells (arrows), 1:19 V8 juice agar, 12 days, 15 °C. **d** NRRL Y-5942. Asci with ascospores, 1:19 V8 juice agar, 9 days, 15 °C. Bar, 10 μm, all photographs

Table 1 Strains of *Metschnikowia peoriensis* and neighboring species examined

NRRL No.	Other No.	Nucleotide differences with NRRL Y-5942^T and length of each DNA sequence compared[a,b]				Ascospore formation[c]	Mating type[d]	Source
		ITS1-5.8S-ITS2 (278 nt)	D1/D2 (499 nt)	EF-1α (890 nt)	RPB2 (2049 nt)			
Metschnikowia peoriensis								
Y-723		nd	0 (2n)	0 (5n)	nd	−	α	Flower, unidentified plant, Peoria, IL, USA
Y-5824		1s (0n)	0 (0n)	0 (4n)	2s (10n)	++		Flower, red clover (*Trifolium pratense*), Peoria, IL
Y-5930		3 (2s–1i–0n)	0 (0n)	0 (7n)	5s (0n)	−	a	Unknown substrate, Peoria, IL
Y-5931	CBS 15336	2 (1s–1i–3n)	0 (0n)	0 (5n)	4s (5n)	−	a	Unknown substrate, Peoria, IL
Y-5932		1 (0s–1i–4n)	0 (0n)	0 (5n)	4s (6n)	+		Unknown substrate, Peoria, IL
Y-5933		0 (1n)	0 (0n)	0 (5n)	1s (0n)	+		Flower, red clover, Peoria, IL
Y-5934	CBS 15337	4 (2s–2i–12n)	0 (1n)	1s (5n)	5s (0n)	−	α	Flower, white clover (*Trifolium repens*), Peoria, IL
Y-5941	CBS 15343	11 (8s–3i–3n)	0 (0n)	0 (0n)	1s (0n)	+		Flower, red clover, Peoria, IL
Y-5942^T	CBS 15345	– (0n)	– (0n)	– (5n)	– (3n)	+++		Flower, purple aster (*Aster novi-belgii*), Peoria, IL
Y-5943		1s (0n)	0 (0n)	0 (5n)	1s (5n)	++		Flower of red clover, Peoria, IL
Y-5944		1s (0n)	0 (0n)	0 (4n)	1s (0n)	−	?	Flower, purple aster, Peoria, IL
Y-5945		1s (1n)	0 (0n)	0 (4n)	1s (0n)	++		Flower, purple aster, Peoria, IL
Y-5946		1s (0n)	0 (0n)	0 (5n)	1s (3n)	++		Flower, purple aster, Peoria, IL
Y-5957	CBS 15338	11 (9s–2i–1n)	0 (0n)	0 (5n)	1s (0n)	−	α	Single colony isolate from NRRL Y-5941
Y-5958	CBS 15339	0 (0n)	0 (0n)	0 (5n)	1s (0n)	−	a	Single colony isolate from NRRL Y-5941
Y-6044		2 (1s–1i–0n)	0 (0n)	1s (3n)	5s (2n)	+		Wild blackberry (*Rubus* sp.), MI
Y-6045		7s (1n)	0 (0n)	0 (4n)	4s (4n)	++		Wild blackberry, MI
YB-2271		2 (1s–1i–0n)	0 (0n)	nd	nd	++		Aphids, weeping willow (*Salix* sp.), Peoria, IL
Metschnikowia koreensis								
Y-48697^T	CBS 8854	15 (12s–3i–1n)	6s (0n)	6s (3n)	64s (0n)	nd	nd	Flower, *Lilium* sp., South Korea
Metschnikowia cibodasensis								
	NBRC 101693^T	25 (19s–6i)	33s	nd	nd	nd	nd	Flower, *Saurauia pendula*, West Java, Indonesia
Metschnikowia reukaufii								
Y-7112^T	CBS 5834	26 (23s–3i–0n)	37s (1n)	22s (4n)	231s (24n)	nd	nd	Flower, fireweed (*Epilobium angustifolium*), Fort Smith, NWT, Canada

Table 1 continued

NRRL No.	Other No.	Nucleotide differences with NRRL Y-5942[T] and length of each DNA sequence compared[a,b]				Ascospore formation[c]	Mating type[d]	Source
		ITS1-5.8S-ITS2 (278 nt)	D1/D2 (499 nt)	EF-1α (890 nt)	RPB2 (2049 nt)			
Metschnikowia persici								
	CFCC 3578[T]	52 (41s–11i)	45 (34s–11i)	nd	nd	nd	nd	Peach fruit surface, China

[a]*Metschnikowia cibodasensis* NBRC 101693: ITS sequence, AB236918; D1/D2 sequence, AB236910 (Sjamsuridzal et al. (2013). *Metschnikowia persici* CFCC 3578: ITS sequence, KF052619; D1/D2 sequence, JX257178 (Wang et al. 2017). *Metschnikowia peoriensis* Y-5942[T]: ITS sequence, MG050889; D1/D2 sequence, MG050902; EF-1α sequence, MG050924; RPB2 sequence, MG050967

[b]s, substitution; i, indel; n, unresolved nucleotide; nd, not determined

[c]−, ascospores not formed; +, infrequent ascospore formation; ++, frequent ascospore formation; +++, abundant ascospore formation. NRRL Y-5944 neither formed ascospores nor reacted to the mating types

[d]Strains used for mating type determination: NRRL Y-5931, a; NRRL Y-5934, α

Relatedness to other Metschnikowia species

On the basis of maximum likelihood analysis of concatenated gene sequences of D1/D2 and EF-1α, *M. peoriensis* is most closely related to *M. koreensis* followed by *M. cibodasensis*, *M. reukaufii* and *M. persici* (Fig. 1). *M. peoriensis* differs from *M. koreensis* by substitutions in D1/D2 (6), EF-1α (6) and RPB2 (64) (Table 1). For these three sequences, there are essentially no differences between strains of *M. peoriensis* (Table 1), and unresolved nucleotides in the sequences for EF-1α and RPB2 usually occur in the same position for all strains. In contrast, the ITS1-5.8S-ITS2 sequence shows, for some strains, as many as 11 differences and all or nearly all occur in ITS1 (Table 1, Fig. 3). An examination of amplicons generated with primers NS-7A and ITS-4 shows two bands for the strains and because nearly all of the nucleotides can be resolved, it appears that the more concentrated amplicon determines the observed sequence. The forward primer Y5942-ITS1 (5′ CTCTAAATATTATATCTTC) was synthesized to match the NRRL Y-5942 ITS1 variable region and when used to generate an amplicon with primer ITS-4, still resulted in generation of two bands (Fig. 3). From this, it is concluded that more than two ITS1 alleles are present and resolution will only come from cloning this region of DNA. The occurrence of unresolved nucleotides is consistent with this observation. Resolution of the ITS1 sequence heterogeneity may be helped by an examination of NRRL Y-5957 and NRRL Y-5958. According to ARS Culture Collection records, these strains were derived as individual colonies from a streak plate of NRRL Y-5941. We have demonstrated that the strains represent complementary mating types and that NRRL Y-5957 has the substituted sequence shown by NRRL Y-5941, whereas the sequence of NRRL Y-5958 differs and matches that of the type strain, which we term "unsubstituted". Despite ITS heterogeneity, little or no intraspecific variability was shown for the other genes sequenced and the 18 strains assigned to *M. peoriensis* appear to be members of the same species.

Phenotypic differences are not sufficient to resolve *M. peoriensis* from other species of *Metschnikowia*. The key to species provided by Lachance (2011) places *M. peoriensis* among members of the *M. pulcherrima* clade, which are unresolved, demonstrating

Table 2 Fermentation and growth reactions of proposed new species of *Metschnikowia*

Species	*M. anglica*	*M. peoriensis*	*M. rubicola*	*M. leonuri*
Fermentation				
Glucose	+	+	+	+
Galactose	−	−	−	−
Sucrose	−	−	−	−
Maltose	−	−	−	−
Lactose	−	−	−	−
Raffinose	−	−	−	−
Trehalose	−	−	−	−
Growth[a]				
Glucose	+	+	+	+
Inulin	−	−	−	−
Sucrose	+	+	+	+
Raffinose	−	−	−	−
Melibiose	−	−	−	−
Galactose	+	+/−	+	+
Lactose	−	−	−	−
Trehalose	+	+	+	+
Maltose	+	+	+	+
Melezitose	+	+	+	+
Methyl-α-D-glucoside	w	−	+	+
Soluble starch	−	−	−	−
Cellobiose	+	+/−	+	−
Salicin	w	+	+	+
L-Sorbose	+	+/−	−	+
L-Rhamnose	−	−	−	−
D-Xylose	w	+/w	+/w	w
L-Arabinose	−	−	−	−
D-Arabinose	−	−	−	−
D-Ribose	−	+	w	+
Methanol	−	−	−	−
Ethanol	−	+/w	+/w	w
Glycerol	+	+	+	+
Erythritol	−	−	−	−
Ribitol	+	+	+/w	+
Galactitol	−	−	−	−
D-Mannitol	+	+	+	+
D-Glucitol	+	+	+	+
myo-Inositol	−	−	−	−
DL-Lactate	−	−	−	−
Succinate	+	+/w	w	w
Citrate	−	−	w/−	−
D-Gluconate	+	+	+	+
D-Glucosamine	−	+	−	w
N-acetyl-D-glucosamine	+	+	+	+
Hexadecane	+	+	+/w	−
Nitrate	−	−	−	−
Vitamin-free	−	−	−	−
2-Keto-D-gluconate	+	+	+	+
5-Keto-D-gluconate	−	−	−	−
Saccharate	−	−	−	−
10% NaCl/5% glucose	w	+	+	+
Starch formation	−	−	−	−
Growth at 37 °C	−	−	−	−

[a]−, negative; +, positive; w, weak

that identification of species must rely on comparison of DNA sequences.

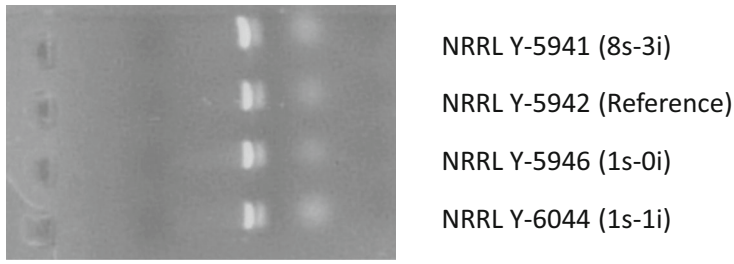
Metschnikowia rubicola C. P. Kurtzman, C. J. Robnett & E. Basehoar sp. nov

Cell morphology

After 2 days on YM agar at 25 °C, cell morphology ranges from ellipsoidal (3–5 × 4–10 μm) to elongate (2–3 × 5–14 μm). Cell division is by multilateral budding and cells occur singly and in budded pairs (Fig. 4a). After 7 days at 25 °C, simple pseudohyphae were formed under the coverglass on a Dalmau plate with corn meal agar (Fig. 4b), but true (septate) hyphae were not detected. Colony growth is low, white, semi-glistening, butyrous in texture and with a smooth, slightly irregular margin. Five strains formed pulcherrimin on either YM agar or V8 juice agar.

Ascosporic state

Seventeen strains of *M. rubicola* were examined in this study and of these, only two formed ascospores (Table 3). Seven of the strains represent mating types and the remaining eight strains gave ambiguous mating responses. Ascosporulation was observed on

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Y-5942   ITS1 - GATCATTAAAAAAATATTATACAACACTTTTAGGAAAAAAACTCTAAATATTATATCTTC
                ||||||||||||||||||||||||||||||||||||||||||| ||   ||||||   |  ||
Y-5941          GATCATTAAAAAAATATTATACAACACTTTTAGGAAAAAACCTN--AATATTTCTTTNTC

      AACAAAAGTTTAAAAAA-N - 5.8S - AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAA
       |   ||||||||||||            ||||||||||||||||||||||||||||||||||||||||
      NATCTAAGTTTAAAAAAAN          AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAA

      GAACGCAGCGAATTGCGATACGTAATATGACTTGCAGACGTGAATCATTGAATTTTTGAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      GAACGCAGCGAATTGCGATACGTAATATGACTTGCAGACGTGAATCATTGAATTTTTGAA

      CGCACATTGCGCCTTAAGGTATTCCTCAAGGCATGCGTGGATGAGCGATATTN - ITS2 - TACTCTC
      |||||||||||||||||||||||||||||||||||||||||||||||||||           |||||||
      CGCACATTGCGCCTTAAGGTATTCCTCAAGGCATGCGTGGATGAGCGATATTN           TACTCTC

      AAACCTTCGGTTTGGTCTTGTAACCACAAAATATCAAATGGCTGTA
      |||||||||||||||||||||||||||||||||||||||||||||
      AAACCTTCGGTTTGGTCTTGTAACCACAAAATATCAAATGGCTGTA
```

Fig. 3 Amplicons of ITS1-5.8S-ITS2 from four strains of *Metschnikowia peoriensis* using primers Y5942-ITS1 and ITS4. Each amplification produced two bands, typical of amplifications using primers NS-7A and ITS-4. Primer Y5942-ITS1 was designed to match the ITS1 sequence of NRRL Y-5942 with the expectation that NRRL Y-5942, NRRL Y-5946 and NRRL Y-6044 would only produce single bands and that NRRL Y-5941 would produce no amplicons because of its many differences with the NRRL Y-5942 sequence. See text for discussion

1:19 V8 juice agar after incubation at 15 °C for 2–3 months. Asci are unconjugated and develop from the large "pulcherrima" cells that are commonly present and that assume a sphaeropedunculate shape (Fig. 4d). Asci measure 21–38 µm in length with the basal portion having a diameter of 4–8 µm. In contrast to many species of *Metschnikowia*, the basal portion of the ascus is somewhat elongated, rather than spherical (Fig. 4d). Asci are persistent and produce two closely paired acicular ascospores. Conjugation between mating types was tested on 1:19 V8 juice agar with incubation at 15 °C. Conjugants were usually detected within 2–3 days following mixing of complementary strains (Fig. 4c). The most active mating pair was NRRL Y-6148 (a) and NRRL Y-6066 (α).

Fermentation and growth reactions for this species are given in Table 2.

Type

NRRL Y-6064 is designated as the type strain of *Metschnikowia rubicola.* The strain is preserved as a metabolically inactive lyophilized preparation (the holotype) at the ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA, and an isotype is preserved at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands as CBS 15344. MycoBank number: MB 823165. Complementary mating types are NRRL Y-6148 (CBS 15347), mating type a, and NRRL Y-6066 (CBS 15340), mating type α. NRRL Y-6064, an ascosporogenous isolate, was isolated from a thimbleberry (*Rubus parviflorus*) growing in Sweet Home, Oregon, USA. Sources of other strains are given in Table 3.

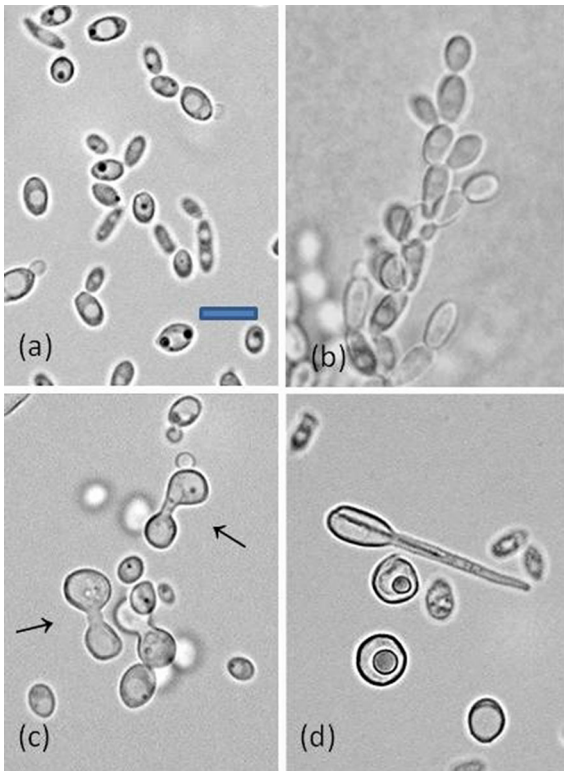

Fig. 4 *Metschnikowia rubicola,* NRRL Y-6064^T. **a** Budding cells, YM agar, 2 days, 25 °C. **b** Simple pseudohypha, Dalmau plate, cornmeal agar, 7 days, 25 °C. **c** NRRL Y-6148 × NRRL Y-6066, conjugating cells (arrows), 1:19 V8 juice agar, 4 weeks, 15 °C. **d** NRRL Y-6064, ascus with an ascospore, 1:19 V8 juice agar, 12 weeks, 15 °C. Bar, 10 µm, all photographs

Etymology

The species epithet *rubicola* refers to isolation of many of the strains from fruit of the thimbleberry, *Rubus parviflorus*, which was growing in Cape Perpetua and Sweet Home, OR, USA.

Ecology

Metschnikowia rubicola is presently known only from the state of Oregon, USA, and has been isolated primarily from thimbleberries and the flowers of red clover (Table 3). Presumably the species is insect disseminated.

Relatedness to other Metschnikowia species

On the basis of maximum likelihood analysis of concatenated sequences of D1/D2 and EF-1α, *M. rubicola* is most closely related to *M. leonuri* followed by *M. shanxiensis*, and *M. chrysoperlae* (Fig. 1). The relatedness between *M. rubicola* and *M. leonuri* will be discussed in the description of the species, which follows. *M. rubicola* differs from *M. shanxiensis* and *M. chrysoperlae* by substitutions in ITS, D1/D2, EF-1α (only for *M. chrysoperlae*) and RPB2 (Table 3). Although the number of substitutions with neighboring species is rather small, the 17 strains of *M. rubicola* do not differ from one another in the four sequences compared (Table 3). Notable is the sequence of the EF-1α gene, which for strains of *M. rubicola* contains 16–20 unresolved nucleotides (Table 3). Neighboring taxa *M. leonuri*, *M. shanxiensis* and *M. chrosoperlae* also have numerous unresolved positions in this gene sequence, which suggests the presence of two divergent copies. Of 20 unresolved nucleotide positions for NRRL Y-6064 and 18 for NRRL Y-6065, 18 are at the same location for each strain.

Fermentation and assimilation reactions of *M. rubicola* are so similar to other members of the *M. pulcherrima* clade that separation of species from phenotype is not certain.

Metschnikowia leonuri C. P. Kurtzman, C. J. Robnett & E. Basehoar sp. nov

Cell morphology

After 2 days on YM agar at 25 °C, cell morphology ranges from spherical (3–7 µm) to ovoidal (3–6 × 4–7 µm). Cell division is by multilateral budding and cells occur singly and in budded pairs (Fig. 5a). After 7 days at 25 °C, infrequent, poorly differentiated pseudohyphae formed under the coverglass on a Dalmau plate with corn meal agar (Fig. 5b), but true (septate) hyphae were not detected. Colony growth is low, white, semi-glistening, butyrous in texture and with irregular margins. Strains were not observed to form pulcherrimin on either YM agar or V8 juice agar.

Table 3 Strains of *Metschnikowia rubicola* and neighboring species examined

NRRL No.	Other No.	Nucleotide differences with NRRL Y-6064^T and nucleotide length of each DNA sequence compared[a,b]				Ascospore formation[c]	Mating type[d]	Source
		ITS1-5.8S-ITS2 (278 nt)	D1/D2 (499 nt)	EF-1α (890 nt)	RPB2 (2049 nt)			
Metschnikowia rubicola								
Y-6064^T	CBS 15344	− (0n)	− (0n)	− (19n)	− (0n)	++		Thimbleberry (*Rubus parviflorus*), Sweet Home, OR, USA
Y-6065		0 (0n)	0 (0n)	0 (18n)	0 (0n)	–	α?	Thimbleberry, Cape Perpetua, near Waldport, OR
Y-6066	CBS 15340	0 (0n)	0 (0n)	0 (16n)	0 (3n)	–	α	Thimbleberry, Cape Perpetua, OR
Y-6067		0 (0n)	0 (0n)	0 (20n)	0 (2n)	–	a?	Thimbleberry, Cape Perpetua, OR
Y-6069		0 (0n)	0 (0n)	0 (19n)	0 (0n)	–	α?	Unidentified flower, Corvallis, OR
Y-6146		0 (0n)	0 (0n)	0 (20n)	0 (3n)	–	α	Thimbleberry, Sweet Home, OR
Y-6147		0 (0n)	0 (0n)	0 (20n)	0 (1n)	+		Salad berry (?), Cape Perpetua, OR
Y-6148	CBS 15347	0 (1n)	0 (0n)	0 (2n)	0 (1n)	–	a	Thimbleberry, Sweet Home, OR
Y-6149		0 (0n)	0 (0n)	0 (20n)	0 (1n)	–	α	Flower, red clover (*Trifolium pratense*), Sweet Home, OR
Y-6257		0 (1n)	0 (0n)	0 (19n)	0 (1n)	–	α	Thimbleberry, Sweet Home, OR
Y-6258		0 (0n)	0 (0n)	0 (20n)	0 (1n)	–	?	Thimbleberry, Sweet Home, OR
Y-6259		0 (0n)	0 (0n)	0 (20n)	0 (0n)	–	?	Flower, red clover, Sweet Home, OR
Y-6260		0 (4n)	0 (0n)	0 (19n)	0 (0n)	–	?	Flower, red clover, Sweet Home, OR
Y-6261		0 (3n)	0 (0n)	0 (18n)	0 (0n)	–	?	Flower, red clover, Sweet Home, OR
Y-6262		0 (3n)	0 (0n)	0 (19n)	0 (0n)	–	?	Flower, red clover, Sweet Home, OR
Y-6273		0 (0n)	0 (0n)	0 (19n)	0 (0n)	–	a	Flower, red clover, Sweet Home, OR
Y-6274		0 (0n)	0 (0n)	0 (20n)	0 (0n)	–	a	Flower, red clover, Sweet Home, OR
Metschnikowia leonuri								
Y-6464		21 (20s, 1i, 0n)	0 (0n)	3s (18n)	17s (63n)	++		Flower, peppermint plant (*Mentha* sp.), Peoria, IL USA
Y-6546^T	CBS 15341	21 (20s, 1i, 0n)	0 (1n)	3s (16n)	nd	+++		Motherwort (*Leonurus cardiaca*) plant, Peoria, IL
Metschnikowia shanxiensis								
Y-48710^T	AS 2.3112	24s (0n)	2s (0n)	0 (22n)	21s (29n)	nd		Jujube fruit (*Zizyphus jujuba*), Jiaocheng, Shanxi Province, China

Table 3 continued

NRRL No.	Other No.	Nucleotide differences with NRRL Y-6064[T] and nucleotide length of each DNA sequence compared[a,b]				Ascospore formation[c]	Mating type[d]	Source
		ITS1-5.8S-ITS2 (278 nt)	D1/D2 (499 nt)	EF-1α (890 nt)	RPB2 (2049 nt)			
Metschnikowia chrysoperlae								
Y-27615[T]	CBS 9803	18 (16s, 2i, 0n))	3s (0n)	5s (12n)	34s (42n)	nd		Eggs, green lacewing (*Chrysoperla* sp.), Tucson, AZ USA

[a]*Metschnikowia shanxiensis* AS 2.3112: ITS sequence, DQ367883; D1/D2 sequence, DQ367883 (Xue et al. 2006). *Metschnikowia chrysoperlae* NRRL Y-27615: ITS sequence, AY494783; D1/D2 sequence, AY452047. *Metschnikowia rubicola* Y-6064[T]: ITS sequence, MG050888; D1/D2 sequence, MG050901; EF-1α sequence, MG050916; RPB2 sequence, MG050966

[b]s, substitution; i, indel; n, unresolved nucleotide; nd, not determined

[c]−, ascospores not formed; +, infrequent ascospore formation; ++, frequent ascospore formation; +++, abundant ascospore formation

[d]Strains used for mating type determination: NRRL Y-6148, a; NRRL Y-6066, α.; a?, α? indicate a possible conjugation with the mating tester strains; ? indicates no response with either mating type

Ascosporic state

Seven strains of *M. leonuri* are presently known and only one (NRRL Y-412) did not form ascospores (Table 4). This strain was tested with mating types of *M. rubicola* (NRRL Y-6066, NRRL Y-6148) but neither conjugations nor asci were observed in the mixtures after 2 months. For the other strains, ascosporulation was observed on 1:19 V8 juice agar after incubation at 15 °C for 1 week. Asci are unconjugated and develop from the large "pulcherrima" cells that are commonly present and assume a sphaeropedunculate shape (Fig. 5c). Asci measure 23–30 µm in length with the basal portion having a diameter of 6–8 µm. Asci are persistent and produce two closely paired acicular ascospores. It was not determined if the species is heterothallic.

Fermentation and growth reactions for this species are given in Table 2.

Type

NRRL Y-6546 is designated as the type strain of *Metschnikowia leonuri*. The strain is preserved as a metabolically inactive lyophilized preparation (the holotype) in the ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA, and an isotype is preserved at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands as CBS 15341. MycoBank number: MB 823166. NRRL Y-6546, an ascosporogenous isolate, was isolated from a motherwort plant (*Leonurus cardiaca*) growing in Peoria, Illinois, USA. Sources of other strains are given in Table 4.

Etymology

The species epithet *leonuri* refers to *Leonurus cardiaca* (mortherwort), the plant from which the type strain was isolated.

Ecology

Metschnikowia leonuri is presently known from flowers, a mushroom and the gummy exudate from a wild cherry tree, all from Midwestern USA (Table 4). If there is habitat specificity, flowers would seem to be the primary site.

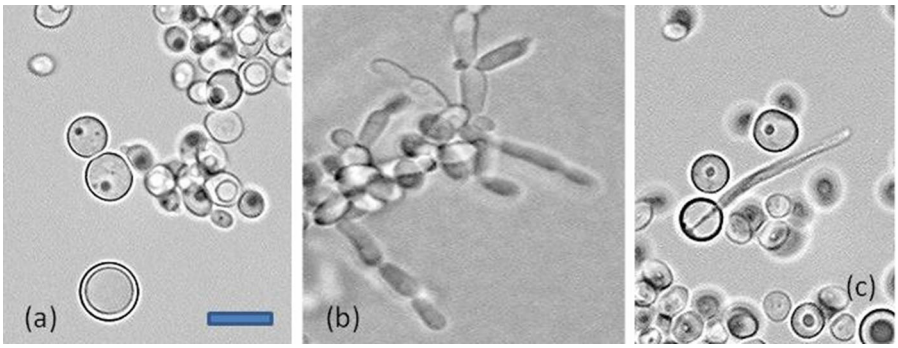

Fig. 5 *Metschnikowia leonuri*, NRRL Y-6546^T. **a** Budding cells, YM agar, 2 days, 25 °C. **b** Simple pseudohypha, Dalmau plate, cornmeal agar, 7 days, 25 °C. **c** Ascus with an ascospore, 1:19 V8 juice agar, 11 days, 15 °C. Bar, 10 μm, all photographs

Relatedness to other Metschnikowia species

When initially characterized from D1/D2 sequences, strains of *M. leonuri* were grouped with *M. rubicola* because they showed no nucleotide differences. However, small differences were noted in sequences for ITS, EF-1α and RPB2 (Table 4). These differences are not great, but they exceed differences ordinarily seen for strains of a species, suggesting that strains of *M. leonuri* should not be considered as divergent members of *M. rubicola.* The two species also show minor phenotypic differences, such as ascus shape and growth differences on cellobiose, L-sorbose, D-glucosamine and hexadecane. Strains of *M. rubicola* are known only from Oregon, whereas strains of *M. leonuri* are presently known only from Midwestern USA. Taken together, these small differences suggest the presence of two species.

As with *M. rubicola*, the EF-1α gene sequence for strains of *M. leonuri* shows 15–18 unresolved nucleotide positions, most of which are shared positions indicating the presence of two divergent copies of the EF-1α gene. In addition, the two available RPB2 sequences exhibit numerous unresolved nucleotides. NRRL Y-6463 has 55 unresolved positions, whereas NRRL Y-6464 has 63. Of these, 52 positions are shared. Consequently, it appears that *M. leonuri* may represent a hybrid species.

Metschnikowia anglica C. P. Kurtzman, C. J. Robnett & E. Basehoar sp. nov

Cell morphology

After 2 days on YM agar at 25 °C, cell morphology ranges from spherical (3–5 μm) to ellipsoidal (3–5 × 4–7 μm). Cell division is by multilateral budding and cells occur singly and in budded pairs (Fig. 6a). After 7 days at 25 °C, pseudohyphae and true (septate) hyphae were absent from under the coverglass on a Dalmau plate with corn meal agar. Colony growth is low, white, semi-glistening, butyrous in texture with a slightly irregular margin. Pulcherrimin was not observed on either YM agar or V8 juice agar.

Ascosporic state

A single strain is known for *M. anglica* and it forms ascospores, although sparingly. Ascosporulation was observed on 1:19 V8 juice agar after incubation at 15 °C for 11 weeks. Asci are unconjugated and develop from the large "pulcherrima" cells that are commonly present and assume a sphaeropedunculate shape (Fig. 6b). Asci measure 18–25 μm in length with the basal portion having a diameter of 5–6 μm. Asci are persistent and produce two closely paired acicular ascospores. It was not determined if the species is heterothallic.

Fermentation and growth reactions for this species are given in Table 2.

Type

NRRL Y-7298 is designated as the type strain of *Metschnikowia anglica.* The strain is preserved as a metabolically inactive lyophilized preparation (the holotype) in the ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA, and an isotype is preserved at the Westerdijk Fungal Biodiversity Institute, Utrecht, The

Table 4 Strains of *Metschnikowia leonuri* and neighboring species examined

NRRL No.	Other No.	Nucleotide differences with NRRL Y-6546^T and nucleotide length of each DNA sequence compared[a,b]				Ascospore formation[c]	Mating type[d]	Source
		ITS1-5.8S-ITS2 (279 nt)	D1/D2 (499 nt)	EF-1α (890 nt)	RPB2 (2049 nt)			
Metschnikowia leonuri								
Y-412		0 (2n)	0 (5n)	4s (18n)	nd	−	?	Unidentified mushroom, MN, USA
Y-6463		0 (0n)	0 (0n)	0 (16n)	1s (55n)	+		Flower, red clover (*Trifolium pretense*), Peoria, IL, USA
Y-6464		0 (0n)	0 (1n)	0 (18n)	− (63n)	++		Flower, peppermint plant (*Mentha* sp.), Peoria, IL
Y-6546^T	CBS 15341	− (0n)	− (0n)	− (16n)	nd	+++		Motherwort (*Leonurus cardiaca*) plant, Peoria, IL
Y-6556		0 (0n)	0 (0n)	0 (18n)	nd	++		Flower, peppermint plant, Peoria, IL
Y-6566		0 (0n)	0 (0n)	0 (18n)	nd	+++		Flower, Shasta daisy (*Leucanthemum superbum*), near Ontonagon, MI, USA
YB-786		0 (0n)	0 (7n)	4s (15n)	nd	+		Gum, wild cherry (*Prunus avium*), Peoria, IL
Metschnikowia rubicola								
Y-6064^T	CBS 15344	21 (20s, 1i, 0n)	0 (0n)	3s (20n)	17s (0n)	++		Thimbleberry (*Rubus parviflorus*), Sweet Home, OR, USA
Metschnikowia shanxiensis								
Y-48710^T	AS 2.3112	4 (3s, 1i, 0n)	5s (3n)	4s (22n)	9s (29n)	nd		Jujube fruit (*Zizyphus jujuba*), Jiaocheng, Shanxi Province, China
Metschnikowia chrysoperlae								
Y-27615^T	CBS 9803	15 (10s, 5i, 0n)	3s (0n)	0 (12n)	1s (42n)	nd		Eggs, green lacewing (*Chrysoperla* sp.), Tucson, AZ, USA

[a]*Metschnikowia shanxiensis* AS 2.3112: ITS sequence, DQ367883; D1/D2 sequence, DQ367883. *Metschnikowia chrysoperlae* NRRL Y-27615: ITS sequence, AY494783; D1/D2 sequence, AY452047. *Metschnikowia leonuri* Y-6546^T: ITS sequence, MG050886; D1/D2 sequence, MG050899, EF-1α sequence, MG050914. Strain differences for RPB2 were based on the sequence from NRRL Y-6464 (MG050965)

[b]s, substitution, i, indel; n, unresolved nucleotide; nd, not determined

[c]−, ascospores not formed; +, infrequent ascospore formation; ++, frequent ascospore formation; +++, abundant ascospore formation

[d]Non-ascosporogenous strain NRRL Y-412 was tested with mating types of *M. rubicola* (NRRL Y-6066, NRRL Y-6148) but neither conjugations nor asci were observed in the mixtures after 2 months

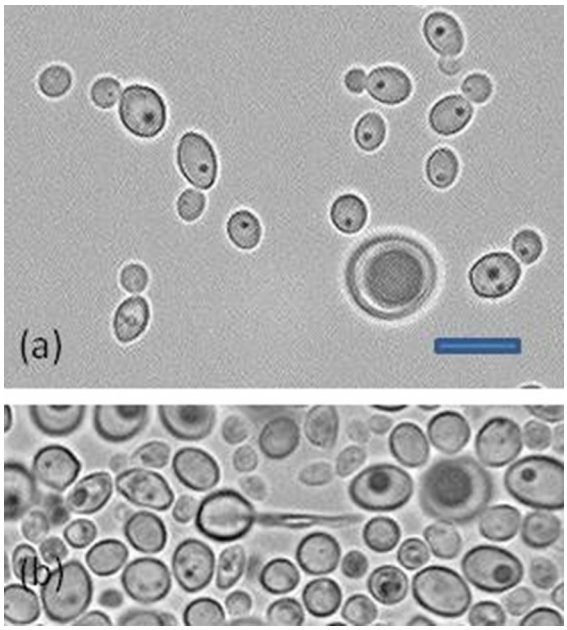

Fig. 6 *Metschnikowia anglica*, NRRL Y-7298^T. **a** Budding cells and a chlamydospore, YM agar, 2 days, 25 °C. **b** Ascus with an ascospore, 1:19 V8 juice agar, 11 weeks, 15 °C. Bar, 10 μm, both photographs

Netherlands as CBS 15342. MycoBank number: MB 823167. NRRL Y-7298 is an ascosporogenous isolate of the strain designated 489/1 *Candida pulcherrima*, which was received in February 1972 from Peter K. C. Austwick, Nuffield Institute of Comparative Medicine, Zoological Society of London, Regents Park, London, United Kingdom. The source of the isolate was not given.

Etymology

The species epithet *anglica* refers to England (UK), the apparent country of origin for the type strain.

Ecology

Unknown.

Relatedness to other Metschnikowia species

On the basis of maximum likelihood analysis of concatenated D1/D1 and EF-1α sequences, *M. anglica* is most closely related to *Candida gelsemii* (Fig. 1). The two species differ from one another by 44 substitutions and 30 indels in D1/D2 and 46 substitutions in EF-1α. Of the four gene sequences determined (ITS1-5.8S-ITS2, D1/D2, EF-1α, RPB2), unresolved nucleotides were found only in EF-1α, which exhibited two.

Because of similar fermentation and assimilation reactions, resolution of *M. anglica* from other species of *Metschnikowia* is uncertain when employing phenotype.

As required by the new code of nomenclature (*International Code of Nomenclature for algae, fungi, and plants* (Melbourne Code), McNeill et al. 2012), the following *Candida* species are transferred to *Metschnikowia* as new combinations because of their phylogenetic placement in that genus.

Metschnikowia chrysomelidarum (N.H. Nguyen, S.O. Suh, C.K. Erbil & M.Blackwell) C.P. Kurtzman, C.J. Robnett & E. Basehoar, *f.a.*, comb. nov

Basionym: *Candida chrysomelidarum* N.H. Nguyen, S.O. Suh, C.K. Erbil & M. Blackwell (2006). Mycol. Res. 110: 352.
Type strain: NRRL Y-27749, CBS 9904.
MycoBank No.: MB 823223.

Metschnikowia gelsemii (M.A. Lachance) C.P. Kurtzman, C.J. Robnett & E. Basehoar, *f.a.*, comb. nov

Basionym: *Candida gelsemii* M.A. Lachance (2007). Antonie van Leeuwenhoek 92: 40 (Manson et al. 2007).
Type strain: NRRL Y-48212, CBS 10509.
MycoBank No.: MB 823192.

Metschnikowia kofuensis (K. Mikata, K. Ueda-Nishimura, S. Goto, C.P. Kurtzman, M. Suzuki, D. Yarrow & T. Nakase) C.P. Kurtzman, C.J. Robnett & E. Basehoar, *f.a.*, comb. nov

Basionym: *Candida kofuensis* K. Mikata, K. Ueda-Nishimura, S. Goto, C.P. Kurtzman, M. Suzuki, D. Yarrow & T. Nakase (1999). Microbiol. Cult. Coll. 15: 51.
Type strain: NRRL Y-27226, CBS 8058, IFO 10931.
MycoBank No.: MB 823195.

Metschnikowia picachoensis (S.O. Suh, C.M. Gibson & M. Blackwell) C.P. Kurtzman, C.J. Robnett & E. Basehoar, *f.a.*, comb. nov

Basionym: *Candida picachoensis* S.O. Suh, C.M. Gibson & M. Blackwell (2004). Int. J. Syst. Evol. Microbiol. 54: 1885.
Type strain: NRRL Y-27607, CBS 9804.
MycoBank No.: MB 823197.

Metschnikowia pimensis (S.O. Suh, C.M. Gibson & M. Blackwell) C.P. Kurtzman, C.J. Robnett & E. Basehoar, *f.a.*, comb. nov

Basionym: *Candida pimensis* S.O. Suh, C.M. Gibson & M. Blackwell (2004). Int. J. Syst. Evol. Microbiol. 54: 1887.
Type strain: NRRL Y-27619, CBS 9805.
MycoBank No.: MB 823205.

Metschnikowia rancensis (C. Ramírez & A. González) C.P. Kurtzman, C.J. Robnett & E. Basehoar, *f.a.*, comb. nov

Basionym: *Candida rancensis* C. Ramírez & A. González (1984). Mycopathologia 88: 101.
Type strain: NRRL Y-48702, CBS 8174.
MycoBank No.: MB 823224.

Candida species proposed for transfer to the genus *Clavispora* as a new combination

For reference taxa, several species of the *Clavispora* clade were included in the phylogenetic analysis shown in Fig. 1. Besides *Clavispora lusitaniae*, type species of the genus, *Cl. opuntiae* is present along with *Candida fructus* and *C. musae*. These latter two species were proposed to be conspecific based on identical D1/D2 DNA sequences (Kurtzman and Robnett 1998), and Lu et al. (2004) found the two species to have identical ITS1-5.8S-ITS2 DNA sequences, thus further suggesting conspecificity. However, the two species were reported to differ by 14 substitutions and seven indels in the 18S rRNA gene sequence (Suzuki and Nakase 1999), indicating that the two taxa might not be conspecific. In the present study, DNA sequences for the two species were determined for 18S, EF-1α and RPB2 genes and repeated for D1/D2 and ITS. The only difference found was one substitution among 2148 nucleotides in the RPB2 gene sequence. The differences in the SSU sequences reported earlier were not detected. From these sequence comparisons, we conclude that *C. fructus* and *C. musae* are conspecific. GenBank accession numbers for *C. fructus*/*C. musae* sequences, respectively, are the following: D1/D2, U44810/U44814; ITS1-5.8S-ITS2, MG050897/MG050898; 18S rRNA, MG050883/MG050884; EF-1α, MG050963/MG050962; RPB2, MG050974/MG050975.

In a further comparison, type strains of *C. fructus* (NRRL Y-17072) and *C. musae* (NRRL Y-17088) were examined for ascospore formation following incubation alone and as a mixture on YM, 5% malt extract, YCBAS, RG and 1:19 V8 juice agar media at 15 and 25 °C with weekly microscopic inspections for 2 months. Neither conjugations nor ascospores were observed.

Both species were described in the same publication (Nakase 1971) and assigned to the genus *Torulopsis*, but later transferred to the genus *Candida* (Yarrow and Meyer 1978). *Candida musae* (Nakase) S.A. Meyer & Yarrow, 1978, *Torulopsis fructus* Nakase, 1971, and *Torulopsis musae* Nakase, 1971 (synonym) were listed as synonyms of *Candida fructus* (Offord and Kirk, 2017). In keeping with the requirements of the new code of nomenclature (*International Code of Nomenclature for algae, fungi, and plants* (Melbourne Code), McNeill et al. 2012), the following new combination is proposed for the name *Candida fructus*.

Clavispora fructus (T. Nakase) C.P. Kurtzman, C.J. Robnett & E. Basehoar, *f.a.*, comb. nov

Basionym: *Torulopsis fructus* T. Nakase (1971). J. Gen. Appl. Microbiol. 17: 415.
Type strain: NRRL Y-17072, CBS 6380, IFO 1581.
MycoBank No.: MB 823206.

Synonyms:
Torulopsis fructus T. Nakase (1971).
Candida fructus (T. Nakase) S.A. Meyer & D. Yarrow (1978).
Torulopsis musae T. Nakase (1971).
Candida musae (T. Nakase) S.A. Meyer & D. Yarrow (1978).

Conclusions

Most yeast species are now routinely identified from unique sequences in the D1/D2 domains of the nuclear LSU rRNA gene and/or from divergence in ITS1-5.8S-ITS2 sequences. The preceding sequences have been viewed as reliable indicators of species identification, and strains of a species generally show no more than 0.5–1.0% sequence divergence (Kurtzman and Robnett 1998; Kurtzman et al. 2011; Vu et al. 2016). However, some exceptions have been noted. For example, Peterson and Kurtzman (1991) found no divergence between certain species of *Saccharomyces*, which later proved to be hybrids with each sharing the same gene for D1/D2, and Lachance et al. (2003) discovered strains of *Clavispora lusitaniae* that showed greater than expected D1/D2 divergence.

The findings of Sipiczki et al. (2013) are of particular interest to the current study. Sipiczki et al. (2013) observed that D1/D2 sequences from *M. fructicola* and *M. andauensis* contained a number of ambiguous nucleotides and wondered if these were the result of sequencing errors in the original studies (Kurtzman and Droby 2001; Molnár and Prillinger 2005) or resulted from other reasons. A repeat of the earlier work again gave a number of ambiguous nucleotides, which suggested that copies of divergent genes were present. Cloning revealed very few D1/D2 copies with identical sequences and the number of substitutions in different D1/D2 copies for *M. andauensis* ranged from 1 to 18, and substitutions in copies for *M. fructicola* ranged from 2 to 25. A neighbor-net analysis of cloned sequences showed that these species share a continuous pool of diverse repeats that appear to evolve by reticulate evolution. We observed ambiguous nucleotides in D1/D2 sequences for *M. zizyphicola*, *M. pulcherrima* and *M. shanxiensis* (Fig. 1). These three species along with *M. fructicola* and *M. andauensis* are members of the same subclade in *Metschnikowia*. ITS1-5.8-ITS2 sequences among strains of *M. peoriensis* show considerable variability, and based on preliminary data, it appears that multiple divergent copies of the ITS sequences are present, perhaps mimicking the results of the D1/D2 study of Sipiczki et al. (2013). Future work will need to include cloning of the ITS sequences to verify this premise.

In addition to the variation in ITS sequences found for *M. peoriensis*, we detected a substantial number of ambiguous nucleotides in the EF-1α sequence for *M. rubicola*, *M. leonuri* and other species of the *M. pulcherrima* clade as well as some nearby species (Fig. 1), which suggests the presence of two or more divergent copies of this gene. The presence of divergent copies of the RPB2 gene is also likely for strains of *M. leonuri* (Table 4).

The occurrence of hybrid species among the yeasts is poorly understood at this time, but studies of *Saccharomyces* indicate that some species show considerable introgression of genetic material from their neighbors (Hittinger et al. 2015; Dujon and Louis 2017). Consequently, from a taxonomic perspective, what degree of hybridization or non-introgressive genome chimerisation (Karanyicz et al. 2017) disqualifies a strain from being regarded as a distinct species? Clearly, there are no good answers at this time and thorough analysis of genome sequences will be required to provide guidance. Based on the apparent occurrence of multi-copy EF-1α genes in the *M. pulcherrima* clade, all species in this clade may be genomic chimeras. With the addition of *M. rubicola* and *M. leonuri*, there are now nine known species in the clade (Fig. 1), and this provides an opportunity to examine species boundaries in a clade well separated from the *Saccharomyces* clade, which has served as a model for studies in speciation, molecular genetics and evolution.

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