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Metschnikowia similis sp. nov. and *Metschnikowia colocasiae* sp. nov., two ascomycetous yeasts isolated from *Conotelus* spp. (Coleoptera: Nitidulidae) in Costa Rica

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Abstract: We describe two related species of *Metschnikowia* isolated from *Conotelus* spp. (Coleoptera: Nitidulidae) of Costa Rican *Convolvulacae*. *Metschnikowia similis* sp. nov. is a sister species to *Metschnikowia dekortorum* (Lachance & Bowles 2002). These two species are distinguishable only by the ability to grow in the presence of 5 % NaCl, the sterility of hybrid asci, and rDNA sequencing. *Metschnikowia colocasiae* sp. nov. is a sister species to *Metschnikowia arizonensis* and can be differentiated from other species by a combination of the utilization of D-gluconate and L-lysine, growth at 34 ºC, and by the lack of ascospore formation in hybrids. The two new species occur in nature as haploid mating types and form acicular ascospores that reach 50 to 100 µm in length.

Taxonomic novelties: *Metschnikowia similis* Lachance & Bowles sp. nov., *M. colocasiae* Lachance & Bowles sp. nov.
Key words: *Conotelus* spp. (Coleoptera: Nitidulidae), *Metschnikowia colocasiae*, *Metschnikowia similis*, new yeast species.

INTRODUCTION

Costa Rica is widely recognized as a "hotspot" of biodiversity. This qualification is as valid for yeasts as it is for plants and animals (Lachance *et al*. 2001d). Four years of field studies in the Guanacaste Province have given rise to the description of several new species from tree exudates (Lachance *et al*. 2001c) and from insects associated with ephemeral flowers (Lachance *et al*. 2001a, b, Lachance & Bowles 2002, Lachance *et al*. 2003, Teixeira *et al*. 2003). We have recently isolated two new *Metschnikowia* species from nitidulid beetles (*Conotelus* spp.) that are frequently found in the flowers of *Convolvulaceae* in the same area. *M. similis* is similar to *M. dekortorum* (Lachance & Bowles 2002) and occupies the same habitat, but is reproductively isolated at the level of spore viability. *Metschnikowia colocasiae* is related to *M. arizonensis* (Lachance & Bowles 2002), but the two form sterile asci when crossed.

MATERIALS AND METHODS

The origin of the strains studied is described in Table 1. All sites were located in Guanacaste Province, Costa Rica. Three were roadside patches of morning glories near the town of Santa Cecilia. *Ipomoea indica* plants were located on Highway 4, 2 km east of the town, *Merremia tuberosa* plants bordered a taro (*Colocasia esculenta*) field 5 km east and 1 km north, and a patch of *Ipomoea batatas* was situated along the road to Estación Orosi, 1 km south of Highway 4. The El Gavilán site was on a dirt road 1 km south of the main road, on a slope of the Volcano Rincón de la Vieja. The Sangregado plants were found 14 km south-east of the village of Nuevo Arenal.

The yeasts were isolated by allowing beetles to walk over plates of YM agar supplemented with 100 µg/mL chloramphenicol. For the 2003 collection, the medium was supplemented in addition with 10 µg/mL cetyldimethylethylammonium bromide (CTAB) which allows the growth of most species in the *Metschnikowia* clade, but inhibits the majority of other yeasts found in this community. Independent isolates from different specimens were treated as separate strains. Representative cultures are preserved in liquid nitrogen in the University of Western Ontario, Department of Biology (formerly Plant Sciences) culture collection, UWOPS. The yeasts were characterized by standard methods (Yarrow 1998).

Evaluation of mating compatibility and ascus formation was performed by mixing pairs of cultures on Yeast Carbon Base (Difco) supplemented with 0.01 % ammonium sulphate and 1.5 % agar, and examining periodically by phase contrast microscopy. Images were recorded electronically. Spore viability was tested by suspending mature asci (3 d at 25 ºC) in sterile water, filtering over a small piece of sterile 20 µm mesh plankton net on blotting paper, and inverting the net on a thin block of YM agar to release the asci.

Table 1. Origin of strains of *Metschnikowia similis* and *M. colocasiae*. Isolates were collected by the authors from specimens of *Conotelus* spp. (Coleoptera: Nitidulidae) in Costa Rica. The strain numbers are those of the UWOPS culture collection.

Species	Locality	Plant Species	Date	Mating type	Number
M. similis	Santa Cecilia	*Ipomoea indica* (purple)	23 Feb. 2003	h^-	03-133.4[A]
			5 Dec. 2001	h^+	01-518a6
		Ipomoea batatas	22 Feb. 2001	h^-	01-138a3
	El Gavilán	*I. batatas*	26 Feb. 2003	h^-	03-190.2
				h^+	03-192.2[T]
				h^+	03-193.3
	Sangregado	*I. batatas*	24 Feb. 2003	h^+	03-158.2
				h^+	03-161.2
				h^+	03-163.2
M. colocasiae	Santa Cecilia	*I. indica* (purple)	23 Feb. 2003	h^+	03-134.2[T]
		Merremia tuberosa	23 Feb. 2003	h^+	03-147.2
			26 Feb. 2003	h^-	03-202.1[A]

[A]Allotype. [T]Type culture.

A sterile coverslip was placed on top of the block and the asci were examined periodically under the microscope.

The D1/D2 variable domains of the large subunit ribosomal DNA were amplified by PCR from whole cells as described previously (Lachance *et al*. 1999). The amplified DNA was concentrated and cleaned on Qiaquick PCR columns (Qiagen, Mississauga, Ontario) and sequenced in an ABI sequencer at the John P. Robarts Research Institute, London, Ontario. Sequence alignment and tree construction were done with the program DNAMAN, Version 4.15 (Lynnon BioSoft, Vaudreuil, Québec).

RESULTS

Metschnikowia similis Lachance & Bowles, **sp. nov.** MycoBank MB500017. Figs 1, 2.

Etymology: The epithet *similis* (si'mi.lis) L. nom. sing. fem. adj. *similis*, "similar", refers to the similarity between the species and its sister species, *M. dekortorum*.

In medio liquido post dies tres cellulae singulae aut binae, ovoidae aut cylindracae (5–8 × 2–3 μm). Cultura in agaro malti post dies 14 (17 ºC), alta-convexa cum centro depresso, rugosa, candida, cohaerens. In agaro farinae *Zeae maydis* post dies 14 pseudomycelium simplex formatur. Post sex horas cellulae stirpium interfertilium mixtarum in agaro carbone suppleto fundamento tubi junctionis formantur. Post dies tres, asci cylindracei (50–70 μm) videntur. Asci stabiles sunt. Ascosporae aculeatae. Glucosum et trehalosum (lente) fermentantur, at non sucrosum, galactosum, maltosum et cellobiosum. Glucosum, sucrosum, galactosum, trehalosum (aliquando exigue), maltosum, melezitosum, methyl-α-D-glucosidum (lente aut exigue), cellobiosum (aliquando lente), salicinum (variabiliter), L-sorbosum, D-xylosum (aliquando exigue), ribitolum (lente et variabiliter), xylitolum (aliquando exigue), mannitolum (exigue), glucitolum (aliquando exigue), acidum succinicum (exigue), glucono-Δ-lactonum (exigue et variabiliter), 2-ketogluconas et N-acetylglucosaminum assimilantur, at non inulinum, raffinosum, melibiosum, lactosum, amylum solubile, L-rhamnosum, L-arabinosum, D-arabinosum, D-ribosum (aliquando exigue), methanolum, ethanolum (aliquando exigue), 1-propanolum, 2-propanolum, glycerolum, erythritolum, galactitolum, inositolum, acidum lacticum, acidum citricum, acidum DL-malicum, acidum gluconicum, acidum D-glucuronicum, D-glucosaminum (aliquando lente), acetonum, ethylacetatum et hexadecanum (aliquando exigue). Ethylaminum et cadaverinum (exigue) assimilantur at non nitratum, nitritum, and L-lysinum. Vitamina ad crecentiam necessaria sunt. Crescit ad 30 ºC, at non 34 ºC aut 4 ºC.

Habitat: *Conotelus* spp. e *Convolvulaceae* in Costa Rica. **Typus** Asci et ascosporae combinationis UWOPS 03-192.2. × UWOPS 01-133.4 in herbario Universitatis Ontariensis Occidentalis, London, praeservatus. Ex-type UWOPS 03-192.2, ex-allotype UWOPS 01-133.4, in collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 9737 et CBS 9738 depositae.

In yeast extract (0.5 %) glucose (2 %) broth after 3 d at 25 ºC, the cells are elongate, occur singly or in parent-bud pairs, and measure 5–8 × 2–3 μm (Fig. 1a). On malt agar after 2 wk at 17 ºC, colonies are raised and convex with a central depression, with an irregular margin, rugose, white, and cohesive. In Dalmau plate cultures on cornmeal agar after 2 wk, chains of elongated cells forming undifferentiated branches are observed (Fig. 1b). After 6 h on Yeast Carbon Base agar with 0.01 % ammonium sulphate, mixed cells of complementary mating types give rise to short conjugation tubes. Conjugated pairs, zygotes, and immature asci are also present. After 2–3 d, mature asci are visible.

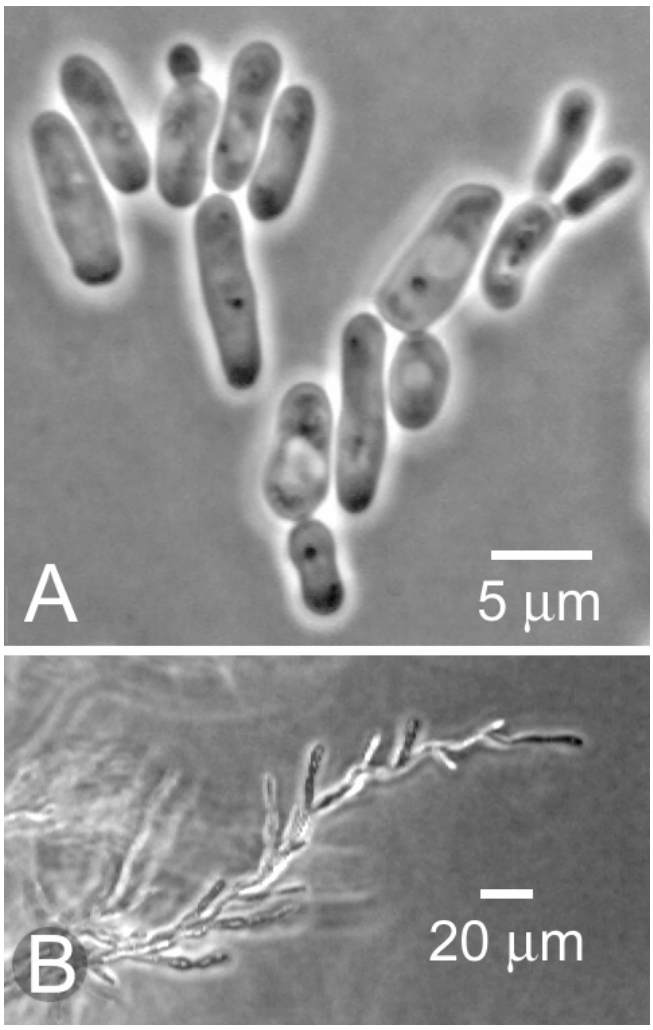

Fig. 1. Phase contrast photomicrographs of vegetative cells (A) and pseudomycelium (B) of *M. similis* strain 03-190.2.

Asci are large and cylindrical, and most retain vestiges of the original zygote (Fig. 2a). Two acicular ascospores, 50 to 70 µm by 1.3–1.5 µm, are formed. Evanescence was not observed.

Glucose and trehalose are fermented slowly; sucrose, galactose, maltose, and cellobiose were not fermented. Glucose, sucrose, galactose, trehalose (sometimes weakly), maltose, melezitose, methyl- α-D-glucoside (slow or weak), cellobiose (sometimes slowly), salicin (variable), L-sorbose, D-xylose (sometimes weakly), ribitol (slowly and variable), xylitol (sometimes weakly), mannitol (weakly), glucitol (sometimes weakly), succinic acid (weakly), gluconoΔ-lactone (weakly and variable), 2-ketogluconic acid, and N-acetylglucosamine are assimilated, but not inulin, raffinose, melibiose, lactose, soluble starch, L-rhamnose, L-arabinose, D-arabinose, D-ribose (sometimes weakly), methanol, ethanol (sometimes weakly), 1-propanol, 2-propanol, glycerol, erythritol, galactitol, inositol, lactic acid, citric acid, DL-malic acid, gluconic acid, D-glucuronic acid, D-glucosamine (sometimes slowly), acetone, ethyl acetate, and hexadecane (sometimes weakly).

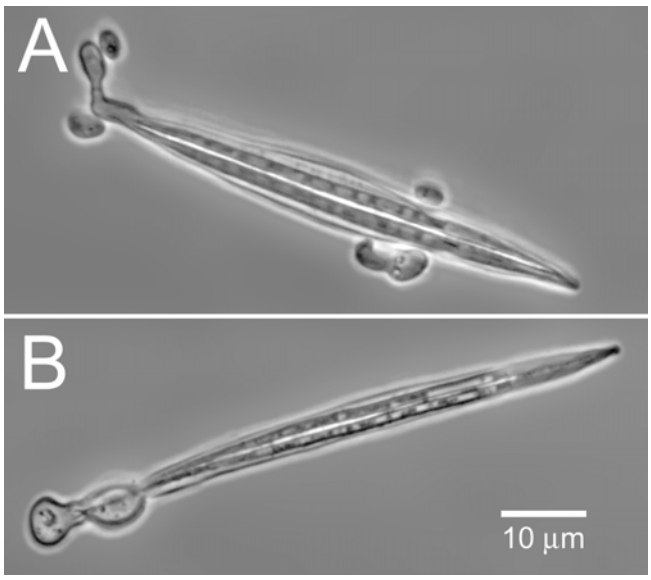

Fig. 2. Phase contrast photomicrographs of asci of *M. similis* strains 03-190.2 × 03-192.2 (A) and *M. colocasiae* strains 00-134.2 × 00-202.1 (B).

Ethylamine hydrochloride and cadaverine (weak) are utilized as nitrogen sources, but not nitrate, nitrite, and L-lysine.

Vitamins are necessary for growth. Growth in the absence of amino acids is positive. Growth occurs slowly at 30 ºC, but not at 34 ºC. Growth at 4 ºC is negative. Liquefaction of gelatin is negative. Casein hydrolysis is negative. Hydrolysis of Tween 80 is negative. Acid production on chalk agar is negative. Growth in the presence of 5 % NaCl is negative or weak; at 10 % negative. Growth in the presence of 50 % glucose is negative. Growth in the presence of cycloheximide 0.01 % is negative. Growth in the presence of digitonin 8 mg/L is negative. Growth in the presence of acetic acid 1 % is negative. Growth in the presence of 10 mg/L CTAB is positive; at 50 mg/L negative. Growth in the presence of ethanol 6 % is negative. Starch production is negative. The diazonium blue B reaction is negative.

Habitat: In nitidulid beetles of the genus *Conotelus* found in flowers of *Convolvulaceae* in Costa Rica.

Notes: The ex-type culture of *M. similis* is strain UWOPS 03-192.2 (h^+). Strain UWOPS 03-133.4 (h^-) is the designated allotype. In compliance with Art. 8.1 of the International Code of Botanical Nomenclature (Greuter *et al*. 2000), the holotype is a fixed preparation of asci obtained from these cultures, maintained in the Herbarium of the University of Western Ontario, London, Ontario. The ex-types were isolated from specimens of *Conotelus* sp. on *Ipomoea batatas* near El Gavilán, Guanacaste Province, Costa Rica.

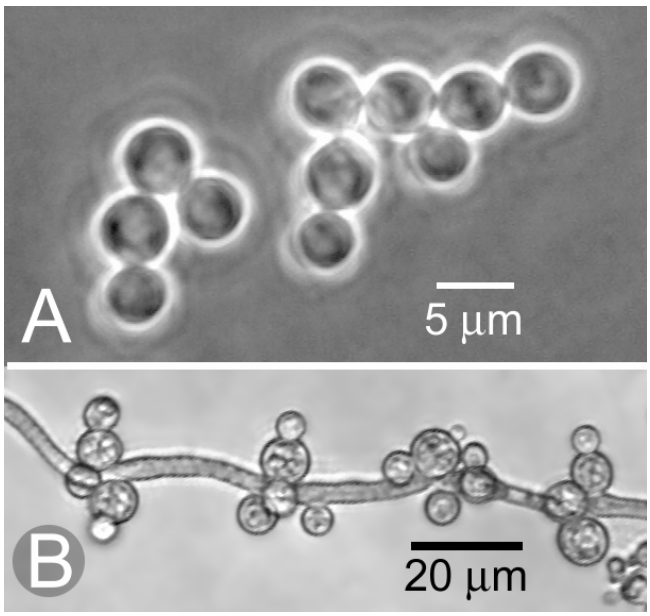

Fig. 3. Phase contrast photomicrographs of vegetative cells (A) and pseudomycelium (B) of *M. colocasiae* strain 03-134.2

They have been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strains CBS 9737 (= UWOPS 03-192.2 = NRRL Y-27627) and CBS 9738 (= UWOPS 03-133.4 = NRRL Y-27628).

Metschnikowia colocasiae Lachance & Bowles, **sp. nov.** MycoBank MB500018. Figs 2, 3.

Etymology: The epithet *colocasiae* (co.lo.ca'siae) L. gen. sing. fem. n. *colocasiae*, "of *Colocasia*", refers to one of the collection sites, on the border of a field in cultivation with *Colocasia esculenta* (Araceae), the taro plant.

In medio liquido post dies tres cellulae singulae, binae, aut catenatae, sphaeroideae vel ovoideae, 3–5 × 3–4 µm. Cultura in agaro malti post dies 14 (17 ºC), convexa vel elevata convexa, hebes et verucosa, candida, cohaerens. In agaro farinae *Zeae maydis* post dies 14 pseudomycelium hyparum cylindracearum et blastosporae sphaeroideae formantur. Post sex horas cellulae stirpium interfertilium mixtarum in agaro carbone suppleto fundamento tubi junctionis formantur. Post dies tres, asci cylindracei (70–100 µm) videntur. Asci stabiles. Ascosporae aculeatae. Glucosum et trehalosum (lente) fermentantur, at non sucrosum, galactosum, maltosum et cellobiosum. Glucosum, sucrosum, galactosum, trehalosum, maltosum, melezitosum, cellobiosum (lente), salicinum (exigue), L-sorbosum, D-xylosum (lente), glycerolum (exigue), ribitolum (lente), xylitolum, mannitolum, glucitolum, acidum succinicum, acidum gluconicum, glucono-Δ-lactonum (lente), 2-ketogluconatum et N-acetylglucosaminum assimilantur, at non inulinum, raffinosum, melibiosum, lactosum, methyl-α-D-glucosidum, amylum solubile, L-rhamnosum, L-arabinosum, D-arabinosum, D-ribosum, methanolum, ethanolum, 1-propanolum, 2-propanolum, erythritolum, galactitolum, inositolum, acidum lacticum, acidum citricum, acidum DL-malicum, acidum D-glucuronicum, D-glucosaminum, acetonum, ethylacetatum et hexadecanum. Ethylaminum et cadaverinum (lente) assimilantur at non nitratum, nitritum et L-lysinum. Vitamina ad crescentiam necessaria. Crescit 34 ºC (aliquando lente), at non 37 ºC aut 4 ºC.

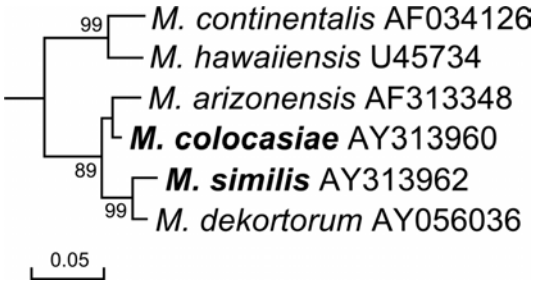

Fig. 4. Phylogram of selected *Metschnikowia* species based on neighbour-joining analysis of the D1/D2 domains of the large subunit rDNA. *M. hibisci* (AF034128, not shown) was used as outgroup solely for rooting purposes. The scale bar shows 5 % substitutions. Bootstrap values were obtained from 1000 pseudo-replicates.

Habitat: *Conotelus* spp. ad *Convolvulaceas* in Costa Rica.

Typus Asci et ascosporae combinationis UWOPS 03-134.2 × UWOPS 03-202.1 in herbario Universitatis Ontariensis Occidentalis, London, praeservatus. Ex-type UWOPS 03-134.2, ex-allotype UWOPS 03-202.1, in collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 9739 et CBS 9740 depositae sunt.

In yeast extract (0.5 %) glucose (2 %) broth after 3 days at 25 ºC, the cells are spheroidal, occur singly, in pairs, or in short chains, and measure 3–5 × 3–4 µm (Fig. 3a). On malt agar after 2 wk at 17 ºC, colonies are convex or raised, dull, rugose, or convoluted, white, and cohesive. In Dalmau plate cultures on cornmeal agar after 2 weeks, a differentiated pseudomycelium with a cylindrical axis and clusters of spherical blastoconidia are observed (Fig. 3b). After six h on Yeast Carbon Base agar with 0.01 % ammonium sulphate, mixed cells of complementary mating types give rise to short conjugation tubes. Conjugated pairs, zygotes, and immature asci are also present. After 2–3 d, mature asci are visible. Asci are large, thin, cylindrical, and most retain vestiges of the original zygote (Fig. 2b). Two acicular ascospores, 70–100 × 0.8–1.1 µm, are formed. Evanescence was not observed. Glucose and trehalose are fermented slowly; sucrose, galactose, maltose, and cellobiose were not fermented. Glucose, sucrose, galactose, trehalose, maltose, melezitose, cellobiose (slowly), salicin (weakly), L-sorbose, D-xylose (slowly), glycerol (weakly), ribitol (slowly), xylitol, mannitol, glucitol, succinic acid, gluconic acid, glucono-Δ-lactone

(slowly), 2-ketogluconic acid, and N-acetylglucosamine are assimilated, but not inulin, raffinose, melibiose, lactose, methyl-α-D-glucoside, soluble starch, L-rhamnose, L-arabinose, D-arabinose, D-ribose, methanol, ethanol, 1-propanol, 2-propanol, erythritol, galactitol, inositol, lactic acid, citric acid, DL-malic acid, D-glucuronic acid, D-glucosamine, acetone, ethyl acetate, and hexadecane. Ethylamine hydrochloride and cadaverine (slowly) are utilized as nitrogen sources, but not nitrate, nitrite, and L-lysine.

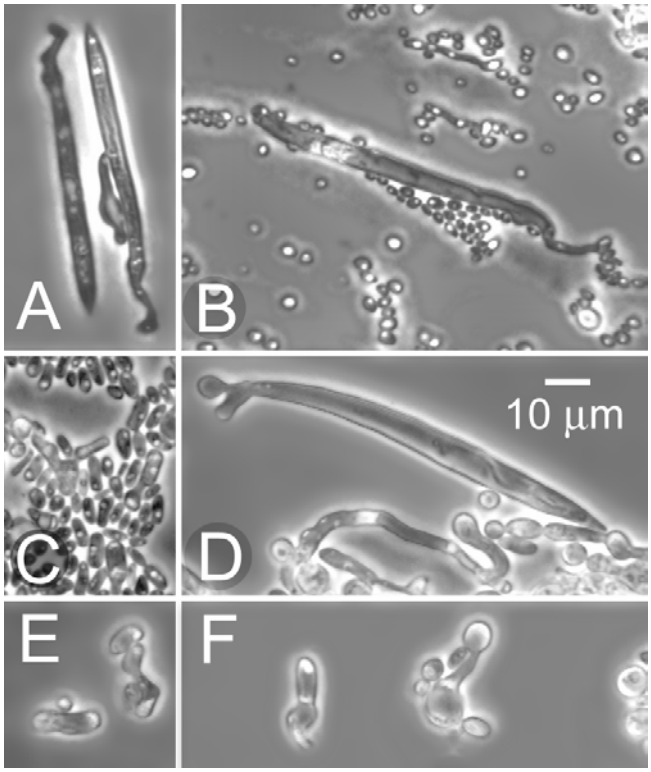

Fig. 5. Phase contrast photomicrographs of interspecific matings between *M. colocasiae* strains 03-134.2 (h^+; A, C, E) and 03-202.1 (h^-; B, D, F) crossed with *M. arizonensis* strains 99-103.4 (h^-; A) and 99-103.3.1 (h^+; B), *M. similis* strains 03-190.2 (h^-; C) and 03-192.2 (h^+; D), and *M. dekortorum* strains 03-168a2 (h^-; E) and 03-167b3 (h^+; F). A, B, D. Immature asci. C, E, F. Zygotes, not developing into asci.

Vitamins are necessary for growth. Growth in the absence of amino acids is positive. Growth at 34 ºC is positive or slow; 37 ºC negative. Growth at 4 ºC is negative. Liquefaction of gelatin is negative. Casein hydrolysis is weak. Hydrolysis of Tween 80 is slow. Acid production on chalk agar is negative. Growth in the presence of 5 % NaCl is negative. Growth in the presence of 50 % glucose is weak. Growth in the presence of cycloheximide 0.01 % is negative. Growth in the presence of digitonin 8 mg/L is negative. Growth in the presence of acetic acid 1 % is negative. Growth in the presence of 50 mg/L CTAB is positive. Growth in the presence of ethanol 6 % is negative. Starch production is negative. The diazonium blue B reaction is negative.

Habitat: In nitidulid beetles of the genus *Conotelus* found in flowers of *Convolvulaceae* in Costa Rica.

Notes: The ex-type culture of *M. colocasiae* is strain UWOPS 03-134.2 (h^+), isolated from a specimen of *Conotelus* sp. on *Ipomoea indica* near Santa Cecilia, Guanacaste Province, Costa Rica. The allotype is strain UWOPS 03-202.1 (h^-), isolated from a specimen of *Conotelus* sp. on *Merremia tuberosa* near a taro field in the vicinity of Santa Cecilia. In compliance with Art. 8.1 of the International Code of Botanical Nomenclature (Greuter *et al*. 2000), the holotype is a fixed preparation of asci obtained from these cultures, maintained in the Herbarium of the University of Western Ontario, London, Ontario. The ex-types have been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strains CBS 9739 (= UWOPS 03-134.2 = NRRL Y-27629) and CBS 9740 (= UWOPS 03-202.1 = NRRL Y- 27630).

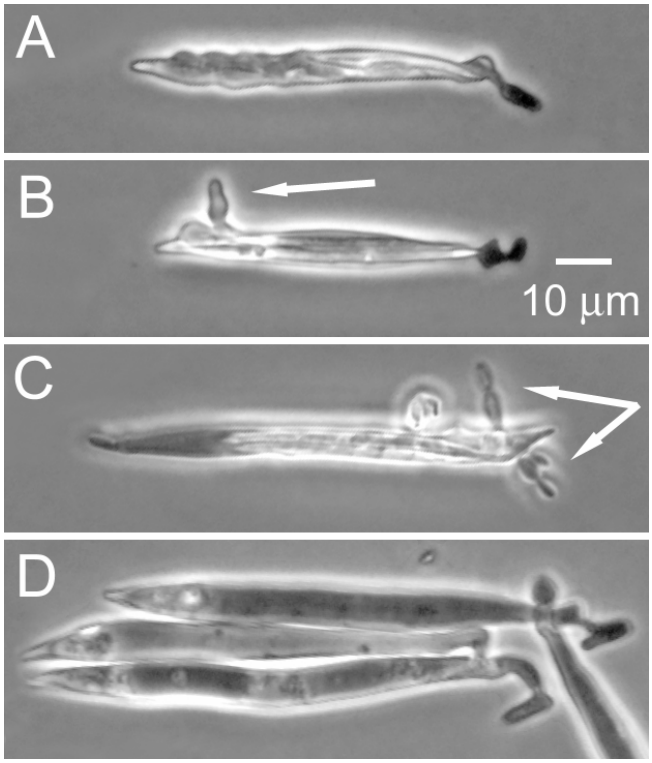

Fig. 6. Phase contrast photomicrographs of ascospore germination in *M. similis* 03-133.4 × 03-192.2 (A, B), *M. dekortorum* 03-167b3 × 03-172.2 (C), and sterile asci produced by the interspecific cross 03-133.4 × 03-167b3 (D). Germination is evidenced by an increase in ascospore refringence, swelling and fragmentation of the spore, and budding through the intact ascus (arrows).

Phylogenetic placement and species delineation

The D1/D2 large subunit rDNA regions were sequenced for two strains of *M. colocasiae* and four strains of *M. similis*. Conspecific strains had identical sequences, for which reason only one representative sequence was deposited for each species (AY313960 and AY313962).

Table 2. A comparison of growth responses that differ between *Metschnikowia dekortorum*, *M. similis*, and *M. colocasiae*. Other results are given in the text.

Growth test	*M. dekortorum*	*M. similis* sp. nov.	*M. colocasiae* sp. nov.
Galactose	+/s	+	+
Trehalose	+/s	+/w	+
Methyl-α-D-glucoside	–	s/w	–
Cellobiose	+/w	+/s	s
Salicin	+	v	w
Xylose	+	+/w	s
D-ribose	s/–	–/w	–
Ethanol	w	w/–	–
Glycerol	–	–	w
Ribitol	s/–	s/–	s
Xylitol	v	+/w	+
Mannitol	s	w	+
Glucitol	w	+/w	+
Succinic acid	s/–	w	+
Citric acid	w/–	–	s
Gluconic acid	–	–	+
Gluconolactone	s/–	w/–	s
2-Ketogluconic acid	+/w	+	+
Glucosamine	–/w	–/s	–
Hexadecane	w/–	–/w	–
Growth at 30 ºC	s	s	+
Growth at 34 ºC	–/w	–	+/s
Casein hydrolysis	–	–	w
Tween 80 hydrolysis	–	–	s
Cadaverine	w	w	s
NaCl 5 %	+	–/w	–
Glucose 50 %	–	–	w
CTAB 50 mg/L	–	–	+

The phylogram in Fig. 4 shows that the new species are sister species to *M. arizonensis* and *M. dekortorum*, respectively. Together, these form a stable sister clade to other large-spored *Metschnikowia* species (Lachance & Bowles 2002), represented here by *M. continentalis* and *M. hawaiiensis*. Addition of other species to the analysis did not affect the topology of the species included in Fig. 4. *Metschnikowia similis* and *M. dekortorum* differed by 2 gaps and by 8 or 9 substitutions, depending on how the gapped positions were aligned. *M. colocasiae* and *M. arizonensis* differed by 9 substitutions and 11 gaps.

When suitable combinations of mating types of *M. colocasiae* were mixed with those of the other three members of the subclade, sterile asci were formed with *M. arizonensis* (Fig. 5a, b) and *M. similis* h^- (Fig. 5d), but mixtures with *M. similis* h^+ (Fig. 5c) or *M. dekortorum* (Fig. 5e, f) usually gave rise to rare zygotes that did not develop into asci. *M. colocasiae* is thus defined on the basis of reproductive isolation. All compatible mating type combinations of *M. similis* and *M. dekortorum* formed abundant asci, but only the intraspecific mixtures produced a substantial proportion (*ca*. one third) of asci containing two viable spores (Table 3). All spores in single-spored asci failed to germinate after 3 d on YM agar. Examples of germinating ascospores and sterile asci are shown in Fig. 6. Ascospore germination is evidenced by an increase in refringence, swelling of ascospores, and bud formation within (Fig. 6a) or across (Fig. 6b, c) the ascus wall. The absence of spore germination in hybrid asci defines *M. similis* as a separate species.

DISCUSSION

Beetles in the genus *Conotelus* almost invariably carry a large population of yeasts in their gut. The yeast species belong to a restricted group with affinities in the genera *Metschnikowia* and *Wickerhamiella* (Lachance & Bowles 2002, Lachance *et al*. 2001b, d, 2003). The species follow a regional distribution characterized by a higher diversity in the tropics than in temperate or subtropical areas. The two species described here raise to seven the number of members of beetle-associated species in the large-spored *Metschnikowia* clade found in Guanacaste Province of Costa Rica. The others are *M. lochheadii*, *M. santaececiliae*, *M. dekortorum*, *Candida ipomoeae*, and an undescribed *Metschnikowia* species represented by strain UWOPS00-154.1 (Lachance *et al*. 2001d).

Table 3. Ascus germination in crosses involving *Metschnikowia similis* (strains 03-192.2 h^+ and 03 133.4 h^-) and *Metschnikowia dekortorum* (strains 03-167b3 h^+ and 03-172.2 h^-). Mature asci (YCB agar, 3 d) were enriched by filtration and transferred to YM agar for microscopic observation after 1 and 2 d. All germinating spores were found in two-spored asci.

Cross	Total number of asci	Asci with non- germinating spores			Asci with germinating spores
		Spores per ascus			
		0	1	2	
M. similis $h^+ \times h^-$	43	14	15	1	13
M. dekortorum $h^+ \times h^-$	28	4	15	0	9
M. similis $h^+ \times$ *M. dekortorum* h^-	63	58	4	1	0
M. dekortorum $h^+ \times$ *M. similis* h^-	243	236	7	0	0

Only four additional taxa are known worldwide, namely *M. borealis* (Nearctic), *M. continentalis* (Brazil), *M. hawaiiensis*, and *C. kipukae* (Hawaii). The basis for regional specificity is not yet understood.

The reproductive boundaries of the new species are clearly defined on the basis of ascus fertility or sterility (Table 3, Figs 5, 6), and the divergence in D1/D2 sequences (Fig. 4) provides additional support of species divergence. However, *M. similis* is difficult to distinguish by other means from its sister, *M. dekortorum*. Indeed, strains 01-138a3 and 01-518a6 (Table 1) were mistakenly reported as representatives of *M. dekortorum* in the description of that species (Lachance & Bowles 2002). As shown in Table 2, the intraspecific variation at the level of growth characteristics equals or exceeds the variation observed between the species. At this time, the only character that appears consistent is growth on YM agar containing 5 % NaCl, which is always positive in *M. dekortorum* and negative or occasionally weak in *M. similis*. This test is not part of the standard set used in most laboratories. The formation of abundant asci, some of which may contain ascospores, in hybrid crosses between the two species further leads to confusion. Identification is therefore performed most expediently by rDNA sequencing.

Metschnikowia colocasiae is easily distinguished from its sister species, *M. arizonensis*, as the latter performs very poorly on most growth tests (Lachance & Bowles 2002). However, the growth profile is typical of that seen in many other *Metschnikowia* species, and identification on that basis requires examining combinations of characters including growth on D-gluconic acid, utilization of L-lysine as sole nitrogen source, and maximum growth temperature. The morphology of the colonies, the vegetative cells, and the pseudomycelium provide additional criteria. Again, rDNA sequencing is the most convenient approach for identification.

During the preparation of this paper, it was brought to our attention that other strains of *M. colocasiae* had been isolated independently in two localities in Brazil. Ten strains from flowers of two unspecified plant species were recovered by C.C.C. Ruivo (Universidade Estadual Paulista Júlio de Mesquita Filho, Instituto de Biociências de Rio Claro). They were identified by matching of D1/D2 sequences with our GenBank entry AY313960. Other strains were isolated by C.A. Rosa (Universidade Federal de Minas Gerais, Belo Horizonte) from flowers in the west-central state of Tocantins. The strains were reported to resemble *Candida ipomoeae* in colony morphology, but to differ slightly for the latter in physiological characteristics. We crossed strains UFMG Toc121.1 and UFMG Toc203.1 with the type and allotype cultures and found them to represent mating types h^- and h^+, respectively.

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