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Catching speciation in the act: *Metschnikowia bowlesiae* sp. nov., a yeast species found in nitidulid beetles of Hawaii and Belize

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Abstract We describe the species *Metschnikowia bowlesiae* sp. nov. based on the recovery of six isolates from Hawaii and Belize. The species belongs to the *Metschnikowia arizonensis* subclade of the large-spored *Metschnikowia clade*. The isolates are haploid and heterothallic. Both Hawaiian strains had the mating type h^+ and the Belizean strains were h^-. Paraphyletic species structures observed in some ribosomal DNA sequence analyses suggest that *M. bowlesiae* sp. nov. might represent an intermediate stage in a succession of peripatric speciation events from *Metschnikowia dekortorum* to *Metschnikowia similis* and might even hybridize with these species. The type of *M. bowlesiae* sp. nov. is strain UWOPS 04-243x5 (CBS 12940^T, NRRL Y-63671) and the allotype is strain UWOPS 12-619.1 (CBS 12939^A, NRRL Y-63670).

Keywords Yeast · *Metschnikowia* · Peripatric speciation · Paraphyletic species

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Introduction

It is sometimes said that speciation can be inferred but not observed. Except for instances where isolated reproductive communities secede from the sudden effect of meiotic non-disjunction, the formation of separate gene pools from a single genetic population spans periods that exceed the lifetime of a human observer. However, there come times when the line that divides direct observation from strong inference becomes indistinct. For example, observations of what can reasonably be regarded as intermediate stages of a continuum strongly infer the existence of that continuum. An important component of yeast systematics is the delineation of species as meaningful groups that properly reflect the reproductive discontinuity of natural populations. On rare occasion, with sufficiently intense sampling, it should be possible to observe intermediate stages along the dynamic process of speciation. Recently separated species should show a high degree of relatedness as evidenced by highly similar DNA sequences, but they should also exhibit a residual ability to form hybrids with various degrees of offspring fertility, which in turn will make species delineation that much more difficult. One way to maximize the likelihood of observing intermediate stages in reproductive isolation is to direct one's efforts towards highly speciose groups of organisms that exhibit a clear pattern of sexual cycling. One such group is the large-spored *Metschnikowia* clade.

Metschnikowia hawaiiensis was the first discovered of the many species that now constitute the large-

spored clade (Lachance et al. 1990). The genus *Metschnikowia* shares the common characteristic of forming elongate asci with only two, usually needle-shaped ascospores (Lachance 2011). The large-spored clade consists of beetle-associated yeasts that share a common mating system and are found in nature as separate haploid mating types. Clade members often exhibit strong biogeographies. *M. hawaiiensis* is endemic to Hawaii as are three other described and two undescribed relatives known to occur in association with Hawaiian endemic nitidulid beetles of the genus *Prosopeus* (Lachance et al. 2005). The Hawaiian subclade is joined by another that comprises seven described and three undescribed species found mostly in association with species of *Conotelus*, a genus of nitidulid beetles that are native to the Americas (Fidalgo-Jiménez et al. 2008 and references therein). Three members of the New World yeast subclade occur in Central America and two of these also occur in Hawaii, where they were introduced through the adventive beetle *Conotelus mexicanus* in the mid-twentieth century (Lachance et al. 2003a). The aforementioned yeast species are joined next by a subclade of four described and one undescribed species (Lachance 2011; Guzmán et al. 2013) typified by *Metschnikowia arizonensis* (Lachance and Bowles 2002). This species is exceptional among Neotropical congeners in that it came from nitidulids of the genus *Carpophilus* (instead of *Conotelus*) and is known so far only from cactus flowers in a single locality in central Arizona. Other subclade members were discovered in Costa Rica (Lachance and Bowles 2004) and one (*Metschnikowia colocasiae*) was also found in Brazil. We now describe a new member of the *M. arizonensis* subclade based on two isolates recovered in Hawaii and four collected in Belize. The new species is named *Metschnikowia bowlesiae* sp. nov. in honour of the late Jane M. Bowles.

Methods

Yeast isolation and characterization

Four specimens each of *C. mexicanus* and *Prosopeus subaeneus* were recovered from *Ipomoea indica* flowers along the trail of Manuka State Park, Island of Hawaii, in June 2004. The beetles were placed on YM/chloramphenicol agar plates and allowed to walk for 5–10 min. Uncharacteristically, only three samples yielded yeasts and these included, in addition to a strain of *Metschnikowia lochheadii* (introduced), a strain of *Candida oleophila*, two strains of an undescribed relative of *Candida cylindracea* and a strain of an undescribed relative of *Candida fructus*. Two samples yielded isolates of *M. bowlesiae* sp. nov. (Table 1). We conducted a much more intense search in the same locality in 2012, only to find a small number of beetles that were devoid of yeasts. The Belize collection, carried out in February 2012, was primarily an effort to recover large numbers of *Metschnikowia santaceciliae* isolates for the purpose of studying their genetic structure in physical space. One hundred and four specimens of *Conotelus* sp. from flowers of various plants were collected in the vicinity of Indian Church, San Carlos, and the Lamanai Archaeological Reserve, in the district of Orange Walk, Belize. These yielded primarily the expected Central American natives, *M. santaceciliae* and *M. lochheadii*, and the Neotropical *Candida* (iter. nom. *Metschnikowia*) *ipomoeae* as well as the six isolates listed in Table 1. Cultures were maintained frozen in liquid nitrogen in the UWOPS yeast culture collection of the University of Western Ontario. Growth characteristics were determined by replica plating following standard methods (Kurtzman et al. 2011). Cardinal growth temperatures were determined at the Microbiology Unit of the Biotron, University of Western Ontario.

Species assignment was based on a combination of mating reactions and DNA sequencing. Crosses were prepared by mixing pairs of strains on nitrogen-poor agar media such as YCBY (Yeast Carbon Base, Difco, with 0.01 % yeast extract). Low magnification (10 × 10) microscopy of the plates themselves allowed direct observation of asci as early as 6 h after mixing. Material from successful mixes was examined under oil immersion (10 × 100) after 2–3 days incubation at 25 °C. As various degrees of inter-specific plasmogamy occur across the large-spored *Metschnikowia* clade, it is possible to assign to each strain a standard mating type (h^+ or h^-), where h^+ is the mating type of the type strain of *M. hawaiiensis*, the first-discovered member of the clade.

Sequence analyses

Barcode sequences (ITS/5.8S-D1/D2 LSU rRNA gene region) were determined with various primer combinations that included NS7a, NL5a, NL1, and NL4

Table 1 New yeast isolates considered in this study

Strain number UWOPS	Mating type	Substrate	Locality
Metschnikowia bowlesiae			
04-243x5[T] CBS 12940[T] NRRL Y-63671[T]	h^+	*C. mexicanus* in *I. indica*	Manuka State Park, Hawaii
04-251.3		*P. subaeneus* in *I. indica*	
12-607.3	h^-	*Conotelus* sp. in *Ipomoea crinicalyx*	Lamanai Outpost, Belize
12-611.1	h^-	*Conotelus* sp. in *Cochlosperma vitifolium*	Lamanai runway, Belize
12-619.1[A] CBS 12939[A] NRRL Y-63670[A]	h^-	*Conotelus* sp. in *I. indica*	
12-653.3	h^-	*Conotelus* sp. in *Ipomoea crinicalyx*	Boundary Road to Lamanai Reserve, Belize
Metschnikowia dekortorum			
12-619.2	h^+	*Conotelus* sp. in *I. indica*	Lamanai runway, Belize
12-653.5	h^+	*Conotelus* sp. in *Ipomoea crinicalyx*	Boundary Road to Lamanai Reserve, Belize

[T] Type strain

[A] Allotype

(Kurtzman and Robnett 2003) as well as IT1 and IT2 (Lachance et al. 2001). Complete rRNA cistron sequences were determined using these primers plus others as detailed by Rosa et al. (2007). Ribosomal intergenic spacer (IGS) sequences were obtained with primer IG2 (Lachance et al. 2000) and the special primer MetIG1bf (5′-GAGTAGCCTTGTTGTTACGATCC-3′), which was designed to recognize a DNA segment at the far 3′ end of the LSU rRNA gene that is conserved among large-spored *Metschnikowia* species. Sequences were aligned and trees constructed using the programs included in MEGA5 (Tamura et al. 2011). The DualBrothers 1.1.3 (Suchard et al. 2003) and Mr. Bayes (Ronquist et al. 2012) plugins of the program Geneious 7.0.3 were used, respectively, to detect recombination and construct Bayesian trees.

Results and discussion

Species delineation and phylogeny

Because large-spored *Metschnikowia* species occur in nature as haploid strains with fixed mating types, a species delineation based on mating success can be applied. Mating between strains of different species in the *M. arizonensis* subclade results in the formation of diverse structures ranging from conjugation tubes or simple zygotes to asci exhibiting varying degrees of maturity and is dependent to some degree on the phylogenetic divergence of the species in question (Lachance and Bowles 2004). The delineation of the boundary separating the sister species *Metschnikowia dekortorum* and *M. similis* was initially problematic. Specifically, strain 01-138a3 was designated as the allotype of *M. dekortorum* based on the observation of occasional two-spored asci in crosses with the type of the species (01-142b3) and in spite of a substantial amount of divergence (two gaps and 8–9 substitutions) in the D1/D2 barcode sequences (Lachance and Bowles 2002). Conspecificity of strains that differ significantly in D1/D2 sequences is not unprecedented, as exemplified by the 32 substitutions observed among successfully mating strains of *Clavispora lusitaniae*, also a member of the Metschnikowiaceae (Lachance et al. 2003b). A later examination of all available strains tentatively assigned to *M. dekortorum* (Lachance and Bowles 2004) indicated that crosses between strains with nearly identical sequences produced a large proportion of asci with two spores that readily germinated when transferred to fresh medium. Some empty or sterile, single-spored asci were also

observed. In contrast, crosses between strains with significantly different sequences tended to form mostly empty or single-spored asci and only rarely two-spored asci, all of which were sterile. As a result of those experiments, Lachance and Bowles (2004) described *M. similis* as distinct from *M. dekortorum* and transferred strain 01-138a3 to *M. similis*. They concluded that an abundance of two-spored asci could serve as proxy to infer ascospore viability, in agreement with Marinoni and Lachance (2004), who found that the mostly single ascospores produced in crosses between the two varieties of *Metschnikowia continentalis* were inviable. For that reason the varieties (*continentalis* and *borealis*) were elevated to the rank of species.

Mating experiments performed in the present study clearly separated the type (03-192.2) and allotype (03-133.4) of *M. similis* from strains of other subclade members, as inter-specific crosses led without exception to single-spored (Fig. 1a) or empty asci. Crosses between *M. bowlesiae* sp. nov. and *M. dekortorum* also gave rise to single-spored (Fig. 1b) and empty asci, but a few two-spored asci (Fig. 1c) were also present, similar to what was reported by Lachance and Bowles (2004) for mixtures of *M. dekortorum* and *M. similis*. This prompted us to examine more systematically the mating success of several strains of *M. bowlesiae* sp. nov. and *M. dekortorum* (Table 2). Crosses between compatible pairs of *M. bowlesiae* yielded fertile asci (Fig. 1d), as did most but not all crosses among strains of *M. dekortorum*. Crosses between various strains of *M. bowlesiae* sp. nov. and *M. dekortorum* produced mostly empty or single-spored asci, with rare two-spored asci. Asci arising from crosses between *M. bowlesiae* sp. nov. and the other two species often showed evidence of lysis in their wall (Fig. 1a, b), further evidence that genetic incompatibilities prevent the formation a mature, fertile asci, and effectively isolate these species postzygotically.

It is of interest that two beetle specimens (12-619 and 12-653) both yielded the h^- mating type of *M. bowlesiae* sp. nov. and the h^+ mating type of *M. dekortorum*. The latter species was previously known only from records in Guanacaste Province, Costa Rica, where it is sympatric with *M. similis*. Based on their joint occurrence in the same beetles, it is likely that *M. bowlesiae* sp. nov. and *M. dekortorum* do mate in nature, which may in some cases allow for occasional introgression. However, one would also expect frequent mating between the two species to reduce their evolutionary fitness. Any mechanism that favours the erection of reproductive boundaries, for example assortative mating or hybrid sterility, would be advantageous and result in a positive feedback loop that promotes speciation.

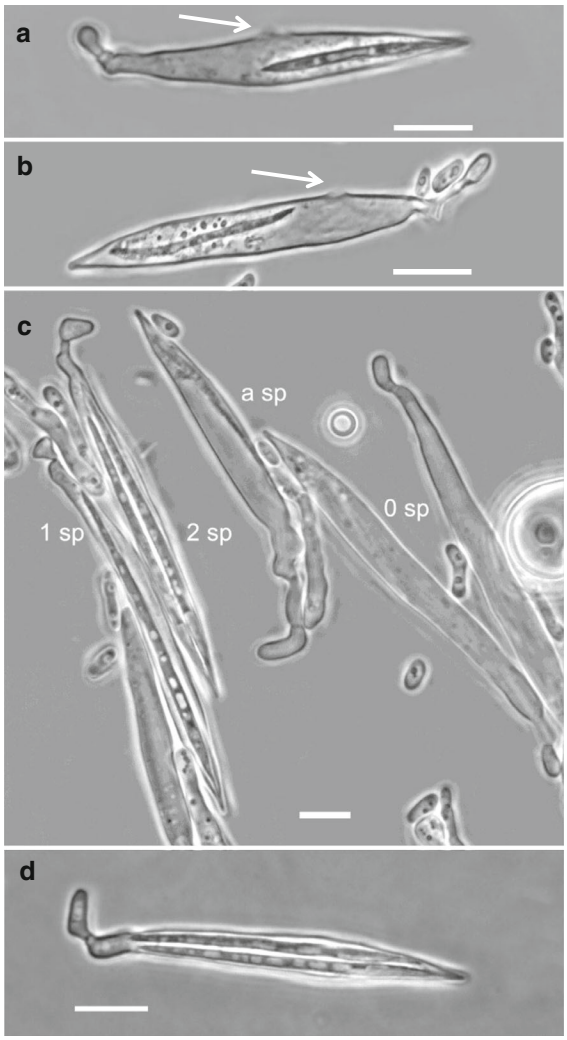


Fig. 1 Ascus formation following mating among various yeast strains. Phase contrast micrographs of mated cells after 2–3 days at 25 °C on YCBY agar. **a** *M. similis* 03-192.2^T × *M. bowlesiae* sp. nov. 12-653.3; **b** *M. dekortorum* 03-167b3 × *M. bowlesiae* sp. nov. 12-619.1^A; **c** *M. dekortorum* 01-142b3^T × *M. bowlesiae* sp. nov. 12-611.1; **d** *M. bowlesiae* sp. nov. 04-243x5^T × 12-619.1^A. *Arrows* point to lysis in ascus walls. Different ascus types are labeled as single spores (1 sp), two-spored (2 sp), empty (0 sp), or containing an aborted spore (a sp). *Scale bars* show 10 μm

As one discovers species that are more and more recently diverged, one expects to find the task of

Table 2 Mating reactions among selected strains of *M. bowlesiae* and *M. dekortorum*

	h^+				
	M. bowlesiae	*M. dekortorum*			
	04-243x5^T	01-142b3^T	01-521b3	03-167b3	12-619.2
h^-					
M. bowlesiae					
12-607.3	2 (1)	0 (1)	0 (1)	0 (1)	0 (1)
12-611.1	2 (1)	0 (1)	1 (0)	0 (1)	0 (1)
12-619.1^A	2 (1)	0 (1)	1 (0)	0 (rare)	1 (0)
M. dekortorum					
03-172.2^A	1 (0)	2 (0)	1 (0)	2 (1)	2 (1)
03-168a2	0 (1)	1 (0)	2 (0)	2 (1)	2 (0)
01-522a5	0 (1)	2 (1)	2 (0)	2 (0)	2 (0)

The largest class of asci (2, two-spored; 1, single-spored; 0, empty) is given, followed in parentheses by the second most abundant class

delimiting the species in a non-trivial, non-arbitrary manner more and more arduous. In such a case, speciation is genuinely perceived as a dynamic process, a work in progress. As closely related heterothallic species gradually become isolated reproductively, the differentiation among DNA sequences used to recognize the species phylogenetically is also bound to be progressive and might even be subject to reversals due to hybridization and introgression. In the present case, regardless of analytical method or dataset, *M. bowlesiae* sp. nov., *M. dekortorum*, and *M. similis* formed together a well-defined clade, and so the kinship of the three species is not in question. However, the within-clade species boundaries in sequence space are not unequivocal. For example, the barcoding sequences comprising the rRNA gene cluster region spanning the internal transcribed spacers, the 5.8S rRNA gene, and the D1/D2 domains of the large subunit rRNA gene for all currently available strains of *M. dekortorum*, *M. similis*, and *M. bowlesiae* sp. nov. did not yield consistent patterns when analysed by different inference methods. Although the neighbour-joining method recovered three subclades that matched the patterns obtained in mating experiments, statistical support (bootstrap values of 79–100 %, Fig. 2a) was not as high as one would like for all subclades. Trees obtained from the same alignment but using maximum likelihood, maximum parsimony, or Bayesian analyses did not support exactly the same hierarchical topology, although members of each of the three species remained neighbours. The pattern elicited by Bayesian analysis (Fig. 2b) even suggested a peripatric speciation sequence with *M. bowlesiae* sp. nov. emerging from *M. dekortorum* and in turn giving rise to *M. similis*. Such a scenario is plausible and also consistent with the observation that overall sequence polymorphism is highest in *M. dekortorum* and lowest in *M. similis*, with an intermediate value for the new species. In spite of the systematist's common aversion for paraphyletic taxa, during early stages of peripatric speciation, the stem ancestral (stem) species is bound to remain paraphyletic with respect to the new, emerging species. The present case is to our knowledge the first suggestion in yeasts of such a three-species succession. The inference, however, is only as good as the strength of the underlying phylogenetic signal, which in the present case, is not strong. In fact, closer inspection of the sequences shows that the various informative regions produced somewhat conflicting signals. The ITS1, D1, and D2 regions each generated a clear signature for all strains of *M. similis,* but not for the other two species. The ITS2 region separated *M. dekortorum* from the rest, but left the other two species unresolved from one another. These patterns were examined further with a DualBrothers test, which failed to identify clear instances of recombination. The first two trees, based primarily on the D2 domain, placed the new species within *M. dekortorum*. The third tree, based primarily on the 5′ segment of the ITS1, recovered with strong support the pattern suggested by mating compatibility and the complete alignment (Fig. 2a).

The strongest sequence-based support for mutual exclusion of the three species was phenetic. The pairwise number of substitutions among strains of each species ranged from 0 to 12 in *M. bowlesiae* sp. nov., 0 to 13 in *M. similis* and 0 to 15 in *M. dekortorum*, and the interspecies divergence values ranged from 15 to

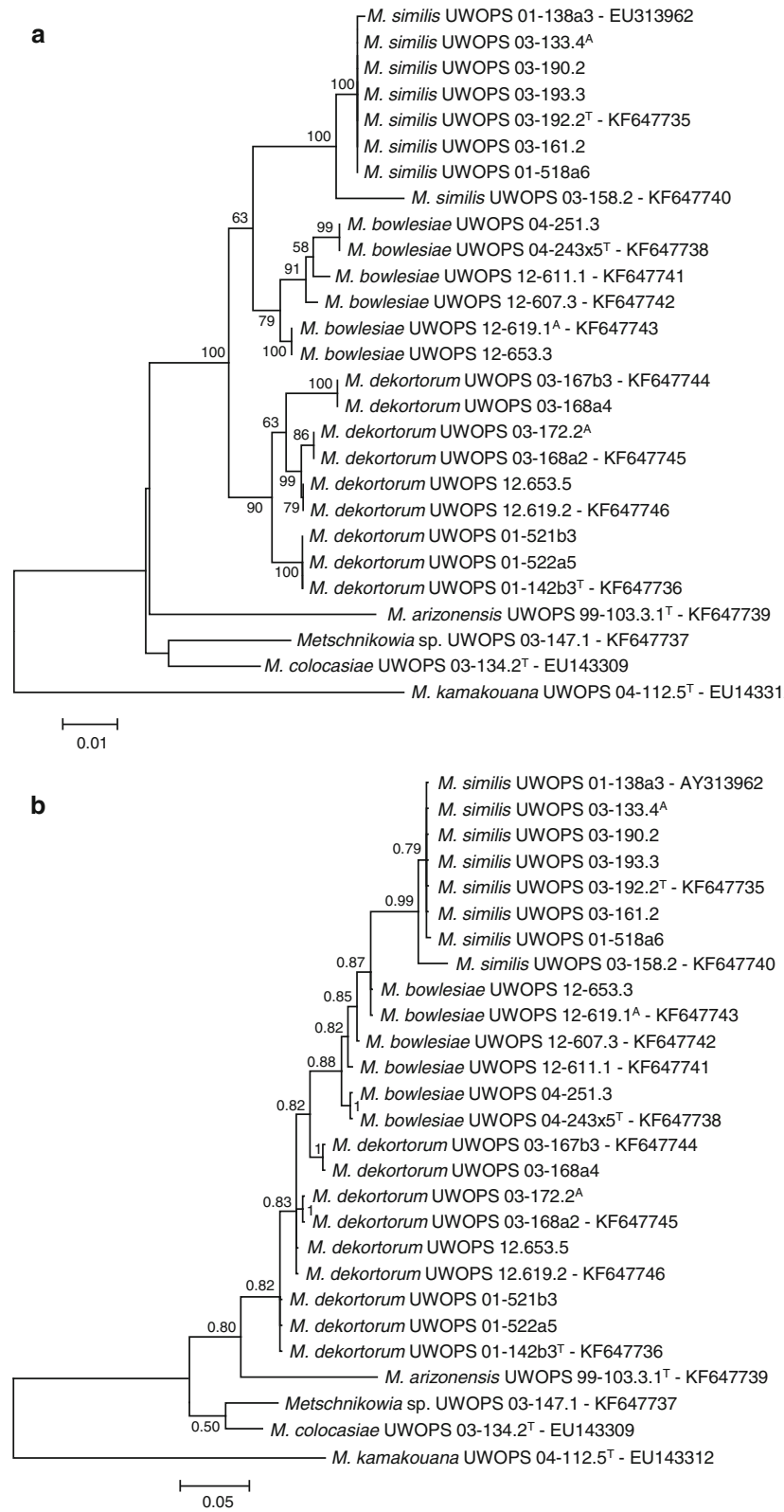


Fig. 2 Phylograms of all known strains of *M. bowlesiae* sp. nov., *M. dekortorum*, and *M. similis*, the type strains of other clade members, and *M. kamakouana* as outgroup, based on **a** neighbour-joining and **b** Bayesian analyses of the ITS-D1/D2 region of the ribosomal cluster. The alignment consisted of 791 positions. Bootstrap values above 50 % (**a**) and clade posterior probabilities (**b**) are shown. The *scale bars* show patristic distances. The neighbour-joining analysis used Kimura's two-parameter transform, whereas the Bayesian analysis followed the general time reversible model. Four chains of 1,100,000 trees were sampled at a rate of 1/200 after a burn-in of 100,000 trees. Both analyses allowed for heterogeneous rates following a Γ distribution with five categories

23 (*M. bowlesiae–M. dekortorum*), 19 to 33 (*M. bowlesiae–M. similis*), and 26 to 34 (*M. dekortorum–M. similis*). Additionally, an analysis of the alignment used in Fig. 2 with the program TCS (Clement et al. 2000) correctly assigned all sequences to networks that corresponded to the proposed species structure at the 95 % parsimony criterion (not shown).

We sequenced the ribosomal IGS region of representatives of all phylotypes identified in Fig. 2. The abundance of internal repeats caused the sequences to vary in quality, particularly in the Belizean strains of *M. bowlesiae* sp. nov. This notwithstanding, good quality alignments were obtained for the 5′ and 3′ ends of all sequences, yielding a gap-free alignment for 637 of 1,946 aligned positions. A neighbour-joining tree (Online Resource 1) constructed from these data confirmed the group structure elicited by the ITS-D1/D2 alignment and showed a strong separation of the three species, with bootstrap values of 99–100 % for each of the three clades. The same species topology (not shown) was also recovered by maximum likelihood (species bootstraps 86–100 %), maximum parsimony (species bootstraps 93–100 %) and Bayesian analysis (posterior probabilities 0.87–1.0). A Dual-Brothers analysis of these data identified eight informative domains with varying phylogenetic signals. Among the associated topologies, trees 1, 3, 5, and 8 showed *M. similis* emerging out of *M. bowlesiae* sp. nov. as suggested by the Bayesian analysis shown in Fig. 2b. Tree 2 included some phylotypes of the new species into *M. dekortorum*, tree 4 intermixed all three species, and tree 7 scattered various phylotypes of the new species among its two sisters. These observations are not inconsistent with the possible effects of hybridization. Only tree 6 supported the species structure suggested by mating success and consistent with the trees presented in Fig. 2a and Online Resource 1. As it happens, the domain that supported this topology is located in a conserved region that probably coincides with the RNA polymerase I promoter of the external transcribed spacer, which is known to be highly species-specific (Russell and Zomerdijk 2005). We are not unaware that the ribosomal gene cluster has idiosyncrasies of its own and that a multi-locus study will be required to test the criterion of genealogical concordance. An analysis of the type strains based on the entire rRNA gene cluster (Online Resource 2) also placed *M. bowlesiae* sp. nov. as a sister species to *M. similis*.

Growth responses

Members of the *M. arizonensis* clade are difficult to characterize as to their growth abilities because of the considerable variation observed among strains. The variability found in *M. arizonensis* itself has been noted before (Lachance and Bowles 2002) and the difficulty of separating *M. dekortorum* and *M. similis* on growth tests has been discussed elsewhere (Lachance and Bowles 2004). *M. dekortorum* strain 12-653.5, isolated jointly with a strain of *M. bowlesiae* sp. nov. (12-653.3), exhibited very poor growth on all media, which adds to the known variability of the species and could be attributed to the ill effects of hybridization, although we have no direct evidence of this. The responses for that strain are not taken into account in Table 3. The four Belize isolates of *M. bowlesiae* sp. nov. were relatively homogeneous in their growth responses, but tended to be less vigorous than the Hawaiian isolates in the assimilation of several carbon sources. Interestingly, the Hawaiian strains failed to grow at 32 °C, whereas the Belizean strains grew vigorously at 33 °C and weakly at 34 °C. Lower maximum growth temperatures for yeasts that are native to Hawaii have been noted not only between large-spored *Metschnikowia* species (Lachance et al. 2005), but also within other yeast species such as *Saccharomyces paradoxus* (unpublished observations of this laboratory).

Ecology and biogeography

The recovery of *M. dekortorum* in Belize was not unexpected, given that the species was previously known from Costa Rica, as were *M. lochheadii*, *M. santaceciliae*, and *C. ipomoeae*. In view of the richness of Mesoamerican biodiversity, the discovery of a new species that is closely related to *M. dekortorum* or *M. similis* is also not surprising. The finding of four, genetically distinct isolates of one mating type of *M. bowlesiae* sp. nov. in Belize and two isolates of the other mating type in Hawaii, on the other hand, is an unlikely result that is more difficult to explain. The presence of *M. lochheadii* and *C. ipomoeae* in Hawaii has been attributed to the recent introduction of the Mesoamerican nitidulid beetle, *C. mexicanus* (Lachance et al. 2003a), and it is not inconceivable that the same introduction also vectored *M. bowlesiae* sp. nov. from the American continent.

Table 3 Growth responses of *M. bowlesiae* sp. nov. The results for *M. dekortorum* and *M. similis* are added for comparison

Growth test	*M. dekortorum*	*M. similis*	*M. bowlesiae*
Galactose	+/s	+	+
Trehalose	+/s	+/w	+/w
Methyl-α-D-glucoside	–	s/w	–
Cellobiose	+/w	+/s	+/w
Salicin	+	v	w/s
Xylose	+	+/w	s/w
D-Ribose	s/–	–/w	–
Ethanol	w/–	w/–	w
Ribitol	s/–	s/–	w/s
Xylitol	v	+/w	s/w
Mannitol	s/–	w	s/w
Glucitol	w	+/w	w/+
Succinic acid	s/–	w	w/–
Citric acid	w/–	–	s/–
Gluconolactone	s/–	w/–	w/–
2-Ketogluconic acid	+/w	+	w/+
Glucosamine	–/w	–/s	–
Hexadecane	w/–	–/w	s
Growth at 32 °C	+	+/w	±
Growth at 34 °C	–/w	–	w/–
Tween 80 hydrolysis	–	w	s/–
NaCl 5 %	w	–	w
CTAB 50 mg/L	–	–	–/s

The following tests gave positive responses (+) for all three species: fermentation of glucose, assimilation of glucose, sucrose, maltose, melezitose, L-sorbose, and *N*-acetylglucosamine as carbon sources, utilization of ethylamine hydrochloride and cadaverine as nitrogen sources, growth on amino acid-free medium, and resistance to 10 mg/L CTAB. The following tests gave negative responses (−) for all three species: assimilation of inulin, raffinose, melibiose, lactose, soluble starch, L-arabinose, D-arabinose, L-rhamnose, methanol, 1-propanol, 2-propanol, glycerol, erythritol, galactitol, inositol, D-glucuronic acid, lactic acid, malic acid, D-gluconic acid, acetone, and ethyl acetate as carbon sources, utilization of nitrate, nitrite, and L-lysine as nitrogen sources, growth on vitamin-free medium, growth at 4 °C, growth at 37 °C, hydrolysis of gelatin or casein, acid production on chalk agar, resistance to 10 % NaCl, 50 % glucose, 0.01 % cycloheximide, 8 mg/L digitonin, 1 % acetic acid, and 6 % ethanol, starch production, and the diazonium blue B colour reaction. Other responses are reported as weak (w), slow (s), or variable (v)

The new species, by virtue of its less vigorous growth, would not have shared the invasive propensities of *M. lochheadii* and *C. ipomoeae*. The possibility that the new species is a relict of a more ancient introduction, analogous to that of the endemic Hawaiian subclade containing *M. hawaiiensis* and others, is less likely. The recovery of two isolates, one from a native beetle and another from an adventive species should not be over-interpreted in view of the low abundance of beetles at the Manuka site.

Description of *M. bowlesiae* Lachance sp. nov.

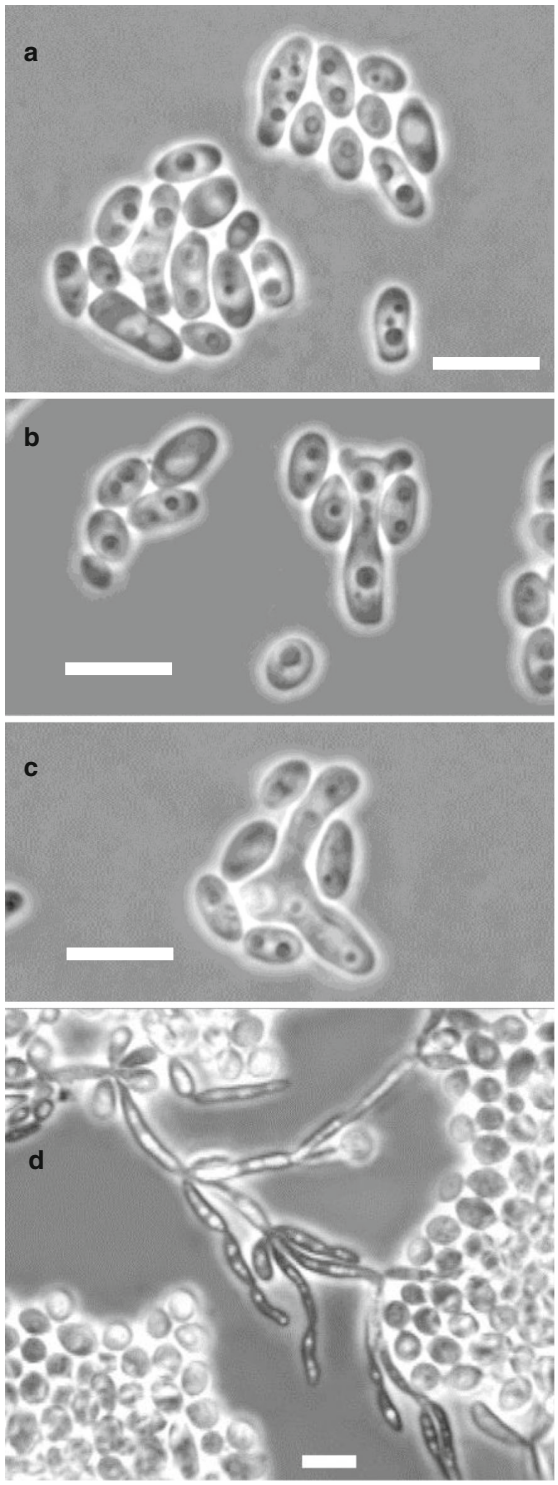
Metschnikowia bowlesiae (bowles'i.ae. N.L. gen. f. n. *bowlesiae* of Bowles, in honour of the late Jane Margaret Bowles, botanist and ecologist, in recognition to her contributions to the discovery of many yeast species, including *M. bowlesiae* sp. nov.).

After 3 days at 25 °C on YM agar, the cells are ovoid to ellipsoid, 2–4 × 5−8 µm, and occur singly or in mother-bud pairs (Fig. 3a). Larger cells with wide protuberances may occur (Fig. 3b, c) singly or in large clumps. After 1 week the colonies are low-convex and slightly umbonate to punctiform. The margin can be entire or papillate. The colonies range in size from less than 1 mm in some strains to 3 mm in others. Growth tends to be more vigorous in strains of h^+ mating type. In slide cultures on YCBY agar after 2 weeks at 25 °C, short chains of pseudohyphal cells are formed (Fig. 3d).

Asci (Fig. 1d) arise from the conjugation of cells of complementary mating types, reaching nearly full size 8 h after mixing agar media. The asci are fusiform (6–8 × 50–60 µm) and typically contain two aciculate spores (1–2 × 40–50 µm). Vestiges of the original conjugated cells are usually present. Ascospore maturity is reached after 2 days at 25 °C. Ascus formation occurs on a large variety of media but is generally easier to observe under conditions of nitrogen limitation (e.g., YCBY agar).

Growth responses are given in Table 3. Based on the assimilation results, fermentation was tested on glucose, sucrose, galactose, trehalose, maltose, and cellobiose. Gas production from glucose began after 5–6 days and the Durham tube was full by 7–8 days. Gas was not produced from any other sugar tested.

The type is strain UWOPS 04-243x5^T, recovered from a specimen of *C. mexicanus* (Coleoptera: Nitidulidae) in a flower of *I. indica*, in Manuka State Park, Hawaii. It has been deposited in the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, under number CBS 12940^T



◀**Fig. 3** Growth forms of *M. bowlesiae* sp. nov. The phase contrast micrographs show budding cells after 3 days on YM agar (**a**–**c**) and pseudohyphae after 2 weeks on YCBY agar (**d**), all at 25 °C. Strains 04-243x5^{T} (**a**, **b**) and 12-619.1^{A} (**c**, **d**). *Scale bars* show 10 μm

(NRRL Y-63671). It has the mating type h^{+}. The designated allotype, of mating type h^{-}, is UWOPS 12-619.1^{A} (CBS 12939^{A}, NRRL Y-63670), isolated from a specimen of *Conotelus* sp. in a flower of *I. indica*, in Lamanai, Orange Walk, Belize. The name is registered under MycoBank number MB 805995.

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