Research article

Nectar yeasts of the *Metschnikowia* clade are highly susceptible to azole antifungals widely used in medicine and agriculture*

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Abstract

The widespread use of azole antifungals in medicine and agriculture and the resulting long-persistent residues could potentially affect beneficial fungi. However, there is very little information on the tolerance of non-target environmental fungi to azoles. In this study, we assessed the susceptibility of diverse plant- and insect-associated yeasts from the *Metschnikowia* clade, including several ecologically important species, to widely used medical and agricultural azoles (epoxiconazole, imazalil, ketoconazole and voriconazole). A total of 120 strains from six species were tested. Minimum inhibitory concentrations (MICs) were determined by the EUCAST broth microdilution procedure after some necessary modifications were made. The majority of species tested were highly susceptible to epoxiconazole, ketoconazole and voriconazole (>95% of strains showed MICs≤0.125 mg/l). Most strains were also very susceptible to imazalil, although MIC values were generally higher than for the other azoles. Furthermore, certain *Metschnikowia reukaufii* strains displayed a 'trailing' phenotype (i.e. showed reduced but persistent growth at antifungal concentrations above the MIC), but this characteristic was dependent on test conditions. It was concluded that exposure to azoles may pose a risk for ecologically relevant yeasts from the *Metschnikowia* clade, and thus could potentially impinge on the tripartite interaction linking these fungi with plants and their insect pollinators.

Introduction

Azoles are currently the largest and most widely used class of antifungal agents in clinical medicine (Sheehan *et al.* 1999; Pierce *et al.* 2013; Allen *et al.* 2015), and also represent a mainstay for crop protection and material preservation (Hof 2001; Groenier and Lebow 2006; Price *et al.* 2015). Their mechanism of action is mainly based on the alteration of cell membrane structure and function through interference with the biosynthesis of ergosterol, but alterations in nutrient transport and other pleiotropic effects not yet fully understood have also been described (Ghannoum and Rice 1999; Price *et al.* 2015; Sanguinetti *et al.* 2015).

Recently, azole resistance has been increasingly reported in many fungal pathogens of animals and plants, which is a cause of great public concern (Serfling *et al.* 2007; Chakrabarti 2011; ECDC 2013; Vermeulen *et al.* 2013; Price *et al.* 2015). For some of such pathogens it has been suggested that antifungal resistance can arise during prolonged treatment or, alternatively, through exposure of the microorganism to sub-lethal concentrations of the compounds in the environment (ECDC 2013). Moreover, azole residues can disperse and persist in the environment (Kahle *et al.* 2008; Battaglin *et al.* 2010; Bollmann *et al.* 2014) and potentially affect non-pathogenic or even beneficial fungi, and may therefore have a considerable impact on ecosystem health and functionality. However, there is very little information on the tolerance of non-target fungal species to medical and agricultural azoles, and risk assessments for antifungal use do not take into account their effects on entire fungal communities (Dijksterhuis *et al.* 2011; Dimitrov *et al.* 2014).

The *Metschnikowia* clade (Saccharomycetales) consists of an ancient and diverse group of ascomycetous yeasts harboring around 50 *Metschnikowia* species, the three species of genus *Clavispora* and several asexual forms currently assigned to the genus

Candida (Lachance 2011; Guzmán *et al.* 2013). Members of this clade are adapted to a wide variety of habitats, including flowers and their pollinators (e.g. *Metschnikowia gruessii*, *M. proteae*, and *M. reukaufii*), plant surfaces, fruits and agricultural soils (e.g. *M. pulcherrima*), and aquatic environments (e.g. *M. bicuspidata*) (Lachance 2011; Guzmán *et al.* 2013). Also, some *Metschnikowia* species are being used in agriculture since they are highly effective in the control of plant pathogens (Piano *et al*. 1997; Kurtzman and Droby 2001; Sipiczki 2006). The widespread presence of *Metschnikowia* species in natural and agricultural plant communities makes them a potential accidental target for azole antifungals. However, except for some reference strains from culture collections and a few clinical and agricultural isolates (Jawich *et al.* 2006; Desnos-Ollivier *et al.* 2012; Savini *et al.* 2013; Cordero-Bueso *et al.* 2014), little is known about the possible effects that azoles might have on this ecologically important fungal group.

Floral nectar is a sugary solution essential for the attraction of pollinators that provide a key ecosystem service (Kearns *et al*. 1998; Vanbergen and the Insect Pollinators Initiative 2013). But nectar is also a crucial habitat for nectarivorous members of the *Metschnikowia* clade (Brysch-Herzberg 2004; Herrera *et al*. 2010), and this group of yeasts seems to play important ecological functions, including the attraction of pollinators (Herrera *et al.* 2013; Schaeffer and Irwin 2014; Schaeffer *et al.* 2014; Pozo *et al*. 2015). It is well known that pesticides, chemical residues and azoles can accumulate in floral nectar and may have sublethal effects on plant pollinators (Rortais *et al*. 2005; Desneux *et al*. 2007; Wallner 2009; Blacquière *et al.* 2012; Bernauer *et al*. 2015). However, the impact of such anthropogenic contaminants on nectar yeast communities has not been investigated to date.

In this study we determined the susceptibility of a large collection of strains from the *Metschnikowia* clade isolated from the floral nectar of diverse wild plants and

their insect pollinators to a number of azole antifungals that are widely used in agriculture (epoxiconazole and imazalil) and medicine (ketoconazole and voriconazole). Imazalil and ketoconazole are two imidazoles, i.e. compounds containing two nitrogen atoms in the azole ring, whereas epoxiconazole and voriconazole belong to the triazoles and have three nitrogen atoms in the azole ring (Sheehan *et al.* 1999). Minimum inhibitory concentrations (MICs) were determined by a modification of the reference European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution (BMD) method. In addition, as antifungal susceptibility testing of yeasts from the *Metschnikowia* clade is still rarely performed, we explored the effect of some specific parameters (incubation time, MIC end point and culture medium) on the performance of the EUCAST method for this yeasts group.

Materials and methods

Isolates

A total of 120 strains from the *Metschnikowia* clade, most of which were obtained and identified to the species level by molecular methods in the course of previous studies (Pozo *et al.* 2011, 2012; de Vega *et al.* 2012, 2014; Jacquemyn *et al.* 2013; Lenaerts *et al.* 2015), were tested (Table S1, supplementary materials). Studied strains belonged to the following six species: *Metschnikowia reukaufii* (n = 46), *M. proteae* (n = 23), *M. gruessii* (n = 22), *M. koreensis* (n = 11), *M. caudata* (n = 7) and *Candida rancensis* (n = 11). Most strains originated from the floral nectar of wild plants (87.7% of total, excluding type strains) and insect floral visitors (12.3%) from South Africa (44.7%), Spain (36.0%), Morocco (10.5%) and Belgium (8.8%). In addition, the type strains of the six tested species were also included in the experiments (Table S1). All strains were stored at −80°C as cell suspensions in 25% glycerol stocks.

125

126 **Antifungal susceptibility testing**

127 *In vitro* antifungal susceptibility of strains was determined by the reference EUCAST
128 BMD procedure guidelines for yeasts (Arendrup *et al.* 2012), with some modifications
129 in incubation conditions that had to be made for testing the strains investigated in this
130 study (see below). RPMI 1640 (Sigma-Aldrich, Diegem, Belgium) supplemented with
131 glucose (Sigma-Aldrich) to a final concentration of 20 g/l and buffered with 3-(N-
132 morpholino) propanesulfonic acid (Sigma-Aldrich) to a pH of 7.0 (hereafter referred to
133 as RPMI+2%G) was used as test medium (Arendrup *et al.* 2012). The antifungal agents
134 tested (all purchased from Sigma-Aldrich) were the imidazoles imazalil and
135 ketoconazole, and the triazoles epoxiconazole and voriconazole. Final concentrations of
136 the antifungal agents were in the range of 0.016 to 8 mg/l, and a positive control (i.e.
137 drug-free medium) was included in each test. Assay plates (96 wells, flat-bottom;
138 Thermo Fisher Scientific/Nunc, Roskilde, Denmark) were prepared in batches
139 according to the EUCAST guidelines and stored until used (but always for less than 3
140 months) at –80ºC. Prior to susceptibility testing, frozen strains were subcultured by at
141 least two serial transfers on yeast malt (YM) agar (2.0% agar, 1.0% dextrose, 0.5%
142 peptone, 0.3% yeast extract, 0.3% malt extract; pH 6.2) for 72 to 96 h at 25ºC, so as to
143 check them for purity. Yeast suspensions were prepared in sterile distilled water,
144 adjusted to the density of a 0.5 McFarland standard ($1\text{-}5\cdot10^6$ cells/ml) and further
145 diluted 1/10 in sterile distilled water. Columns 1 to 10 of the test plate contained 100 µl
146 of twofold serial dilutions of the antifungals, column 11 contained 100 µl of drug-free
147 medium, and column 12 corresponded to the sterility controls. One hundred microliters
148 of the working yeast cell suspension were inoculated per well in columns 1 to 11, and
149 100 µl of sterile distilled water per well in column 12. Plates were then covered with a

150 sterile lid to prevent the medium from evaporating and incubated at 25ºC. Although the
151 EUCAST method recommends an incubation temperature of 35 ± 2ºC, we selected 25ºC
152 as this is a common growth temperature for *Metschnikowia* species and most tested
153 strains were unable to grow or only showed poor growth at 35 ± 2ºC (Lachance 2011;
154 de Vega *et al.* 2012, 2014; Pozo *et al.* 2012). Assay plates were read
155 spectrophotometrically (530 nm) after 24, 48 and 72 h of incubation. For the slow-
156 growing species *M. caudata* (de Vega *et al.* 2014), incubation was extended for 24
157 additional hours (i.e. 96 h in total; see Results). All strains were tested at least twice on
158 different days and *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019,
159 which are two quality control strains recommended by the EUCAST method, were
160 included in each series of experiments. Additionally, in order to assess possible
161 differences in the performance of the EUCAST method related to the test medium,
162 susceptibility tests were repeated for a selection of 37 strains (the seven *M. caudata*
163 strains available, and six strains representative of different regions and/or hosts for each
164 of the remaining five species) using non-synthetic YM broth (1.0% dextrose, 0.5%
165 peptone, 0.3% yeast extract, 0.3% malt extract; pH 6.2) instead of synthetic
166 RPMI+2%G.
167
168 **Data analysis**
169 Since no reference MIC end point has yet been established for susceptibility testing of
170 azoles against *Metschnikowia* clade strains, end points of ≥50% and ≥90% of reduction
171 in turbidity compared to the azole-free control well (i.e. partial and almost complete
172 inhibition of growth, respectively) were determined. Essential agreement (EA) between
173 the MIC values determined at different incubation times, or by using different test
174 media (i.e. RPMI+2%G and YM broth) and reading end points (50% vs. 90%) was

175 defined as discrepancy of no more than ±2 two-fold dilutions (Cuenca-Estrella *et al.*
176 2010). When necessary, high off-scale MIC results were converted to the next highest
177 concentration and low off-scale MIC results were left unchanged (Pfaller *et al.* 2011).
178 Discrepancies between MIC values were classified as non-substantial differences (NSD,
179 discrepancies of three or four two-fold dilutions) or substantial differences (SD,
180 discrepancies of >4 two-fold dilutions) (Cuenca-Estrella *et al.* 2010). Where relevant,
181 differences among the MIC data distributions were evaluated by the Friedman's test
182 followed by Bonferroni post-hoc comparisons, as implemented in Statgraphics
183 Centurion XVII (Statpoint Technologies, Inc., Warrenton, VA, USA). The critical *p*-
184 value was set at <0.05.
185

186 ## Results

187 **Optimization of the EUCAST method for yeast strains from the *Metschnikowia***
188 **clade**

189 The EUCAST method for antifungal susceptibility testing recommends incubating
190 microdilution plates for 24 ± 2 h, after which the absorbance at 530 nm of azole-free
191 control wells should be >0.2 (Arendrup *et al.* 2012). If required, test plates can be
192 further incubated for 12–24 h, but failure to reach the threshold absorbance after 48 h is
193 considered to represent a failed test (Arendrup *et al.* 2012). A strict application of these
194 stringent criteria was not possible in the present study, as most tested strains (including
195 those used for quality control) displayed poor growth in RPMI+2%G medium after 24 h
196 of incubation at 25ºC and, in some cases, an absorbance value >0.2 was not reached
197 until 72 h (Figure 1). In addition, although all *C. rancensis* strains and the quality
198 control strains displayed enough growth in RPMI+2%G after 48 h (Figure 1, Table 1
199 and Table S2, supplementary materials), absorbance values for some strains were still

200 rather low (e.g. mean ± S.D. = 0.30 ± 0.02 for *C. rancensis* SA16, and 0.31 ± 0.03 for
201 isolate SA25). Notably, none of the seven *M. caudata* isolates included in the study
202 consistently grew in RPMI+2%G even after extended incubation up to 96 h post-
203 inoculum (Figure 1).

204 In contrast, when tested in YM broth, the quality control strains and all strains
205 tested except those belonging to *M. caudata* reached absorbance values >0.2 in just 24 h
206 (Figure 1). Further incubation in YM broth resulted in most cases in saturated
207 absorbance values in the drug-free wells (data not shown), thus resulting in unreliable
208 MIC determination. For *M. caudata*, enough growth level for reliable MIC
209 determination was not reached until 48–96 h, depending on the strain and test plate
210 (Figure 1).

211 Voriconazole MIC values obtained in RPMI+2%G medium for the quality
212 control strains fell within the acceptable ranges provided in the EUCAST reference
213 document (0.03–0.25 mg/l for *C. krusei* ATCC 6258 and 0.015–0.06 mg/l for *C.*
214 *parapsilosis* ATCC 22019; Arendrup *et al.* 2012) or, for a minority of tests, differed in
215 ≤2 two-fold dilutions (Table 1; Table S2, supplementary materials). Obviously, due to
216 the methodological modifications described in previous paragraphs, this comparison of
217 voriconazole MICs for the quality control strains is tentative (the acceptable ranges
218 given by the EUCAST document only refer to the 50% inhibition end point and
219 incubation at 37ºC for 24 h). The reliability of epoxiconazole, imazalil and ketoconazole
220 MIC determinations in RPMI+2%G could not be assessed, as acceptable MIC ranges
221 are not yet available for these antifungals; nevertheless, repeated assays for these
222 compounds yielded consistent results (data not shown). The same can be said for MIC
223 determinations in YM broth.

In view of these results, it was concluded that for all species except *M. caudata* the optimal test conditions for azole MIC determination by the EUCAST procedure are 72 h of incubation in RPMI+2%G or 24 h of incubation in YM broth. In the particular case of *M. caudata*, MIC values can only be reliably determined after 96 h of incubation in a nutrient rich medium such as YM broth.

In vitro susceptibility to azole antifungals of yeasts of the *Metschnikowia* clade

Table 2 shows the azole MIC distributions for the studied strains in RPMI +2%G medium (or YM broth, in the case of *M. caudata*). In general, epoxiconazole, ketoconazole and voriconazole were very active against all species tested and, regardless of the end point considered for MIC determination, >95% of strains were susceptible at concentrations ≤0.125 mg/l. Notably, in most cases there were no significant differences in the median MICs of epoxiconazole, ketoconazole and voriconazole (p >0.05 in all pair-wise comparisons except epoxiconazole vs. ketoconazole and epoxiconazole vs. voriconazole for *M. reukaufii* and the 90% inhibition end point). In contrast, median MICs for imazalil were generally higher (p <0.05 in all pair-wise comparisons except imazalil vs. voriconazole for *M. caudata* and the 90% inhibition end point), and MIC distributions depended largely on the species and endpoint criteria. For example, only 68.3% and 27.5% of the total number of isolates were susceptible to ≤0.125 mg/l of imazalil when the partial (≥50%) and almost complete (≥90%) inhibition end points were considered, respectively.

An excellent EA (100%) was observed for most species-azole combinations between the MIC values obtained by the two end point criteria considered (Table 2). A notable exception was *M. reukaufii*, which yielded discrepant results for all tested antifungals: 1 NSD and 2 SD for epoxiconazole, 8 NSD for imazalil, and 1 SD for

ketoconazole and voriconazole (Table 2). Interestingly, one particular isolate (6.3.2-Y2, obtained in 2012 in Belgium from floral nectar of *Pulmonaria officinalis*) displayed discrepant results for all tested antifungals. In addition, two NSD were observed for *M. caudata* and imazalil.

Regarding the comparison of test media, for most species, azole antifungal and end point combinations, the EA between the MIC results determined after 72 h of incubation in plates containing RPMI+2%G or 24 h in plates containing YM broth was 100% (Table 3). Discrepancies in MIC results due to the test medium when the partial inhibition end point criterion was considered were only observed for imazalil and the species *M. gruessii* and *M. koreensis*, for which three out of the six isolates tested in each case yielded NSDs (Table 3). Non-significant differences were also obtained for the same three *M. gruessii* and a single *M. reukaufii* isolate when tested for imazalil susceptibility considering the almost complete inhibition end point (Table 3). Notably, isolate 6.3.2-Y2 of *M. reukafii* yielded SDs in the 90% end point MIC results when tested for epoxiconazole, ketoconazole and voriconazole susceptibility (Table 3).

Discussion

Controlling fungal pathogens is paramount to ensuring human and animal health, food security and preservation of wood and other materials (Hof 2001; ECDC 2013; Price *et al.* 2015). However, the widespread use of azole antifungals in agriculture and medicine is leading to a significant accumulation of azole residuals in the environment (Kahle *et al.* 2008; Battaglin *et al.* 2010), which poses a threat for the composition and/or functioning of fungal communities harboring non-target fungi (Dijksterhuis *et al.* 2011; Dimitrov *et al.* 2014). In spite of this, current knowledge about yeast antifungal susceptibility profiles is mostly limited to species responsible for human infections

(Desnos-Ollivier *et al.* 2012), and there is very scarce information on the tolerance of environmental fungi to antifungal compounds. To contribute to fill this research gap, this study has provided novel information on the azole susceptibility of plant- and insect-associated strains from the *Metschnikowia* clade. To do so, we first had to optimize the EUCAST broth microdilution method of antifungal susceptibility testing for *Metschnikowia* clade yeasts.

Apart from setting the incubation temperature to 25ºC, which is optimal for members of the *Metschnikowia* clade (see Materials and Methods), the scarce growth displayed by most tested species in RPMI+2%G necessitated extended incubation of test plates (72 h, instead of the 24 h recommended by the EUCAST method) for reliable determination of azole MICs. Alternatively, adequate growth for MIC determination was obtained in just 24 h when RPMI+2%G was substituted for nutrient rich YM broth. Nevertheless, *M. caudata* was particularly recalcitrant to azole susceptibility testing, and MIC values for this species could only be determined when YM broth was used as test medium and plates were read after 96 h of incubation.

In general, most strains included in the present study were highly susceptible to broad-spectrum imidazole and triazole antifungals of widespread use in clinical and agricultural settings. These findings are in line with the observation of Desnos-Ollivier *et al.* (2012), who tested 62 *Metschnikowia* isolates (belonging to 36 different species) from reference culture collections and found no resistance to the medical azoles fluconazole, itraconazole, posaconazole and voriconazole. Nevertheless, for a few *M. reukaufii* strains included in our study the azole MICs determined at a 90% inhibition end point were several two-fold dilutions higher than those obtained using the partial inhibition criterion. This observation points to the occurrence of a 'trailing' phenotype in some *Metschnikowia* strains, which is defined as the manifestation of reduced but

299 persistent growth in broth dilution tests with azole agents at antifungal concentrations
300 above the MIC (Lee *et al.* 2004). Curiously, the trailing phenotype of *M. reukaufii* only
301 appeared when susceptibility tests were performed in RPMI+2%G but not when these
302 were carried out in YM broth, thus confirming that this effect depends on species and
303 strain-specific characteristics, as well as on different methodological aspects
304 (Arthington-Skaggs *et al.* 2002; Agrawal *et al.* 2007; Coenye *et al.* 2008).

305 It is worth noting that MICs for the imidazole imazalil for our strain collection
306 were generally higher than those observed for the other azoles tested. A similar result
307 was reported by Dijksterhuis *et al.* (2011) after performing toxicity tests to determine
308 the effects of azoles and other fungicides on aquatic fungi and oomycetes. The reason
309 for this higher susceptibility to imazalil is still unknown, but it might be due to a longer
310 exposure to imazalil residues and/or the presence of higher concentrations of these in
311 the environment. Indeed, imazalil has been extensively used in agriculture since the
312 1970s, while epoxiconazole was introduced twenty years later (Morton and Staub 2008;
313 Price *et al.* 2015). Typical uses of imazalil include field, glasshouse and indoor
314 application by diverse methods (e.g. spraying, dipping, waxing) for the pre- and post-
315 harvest control of diverse fungal pathogens (EFSA 2010). Moreover, apart from its
316 agricultural applications, imazalil is used (sometimes under the synonym enilconazole)
317 in veterinary medicine as a topical broad-spectrum antimycotic and also in some
318 countries as a fungicide formulation for the disinfection of farm buildings (EMEA
319 1998), which constitute potential additional sources for environmental contamination
320 (Kahle *et al.* 2008). Although most strains tested in this study were obtained from
321 natural plant communities located relatively far from agricultural fields and human
322 settlements, the presence of azole residues in these environments cannot be excluded
323 and should be evaluated in future.

Floral nectar is a valuable reward for pollinators, and extensive research work has been carried out to understand its composition, availability and secretion patterns (Nicolson and Thornburg 2007; Brandenburg *et al.* 2009; Heil 2011; Lievens *et al.* 2015). More recently, there has been a growing interest in studying the role of floral nectar as a habitat for eukaryotic and prokaryotic microorganisms, and the effects these might have on nectar chemistry, pollinator behavior and sexual plant reproduction (see Pozo *et al.* 2015 for an updated review). In particular, it was found that *Metschnikowia* yeasts are widespread in the floral nectar of diverse plant families, and some species such as *M. reukaufii* could have a relevant role in attracting pollinators and influencing their foraging behaviour (Herrera *et al.* 2013; Schaeffer and Irwin 2014; Schaeffer *et al.* 2014). Another emerging focus of interest is the study of the presence of anthropogenic contaminants in floral nectar, and the impact of these on declining pollinator populations and, eventually, on plant reproduction. For example, it has been that demonstrated that some insecticides such as the neonicotinoids are relatively common in nectar and can alter the physiology and behavior of pollinators (Blacquière *et al.* 2012; Stanley *et al.* 2015). Although some studies have reported the presence of trace amounts of certain azoles in pollen and nectar collected by foraging honey bees shortly after field applications and over a prolonged time afterwards (e.g. Wallner 2009), to the best of our knowledge, no study has analyzed so far the possible effect of these antifungal compounds on the nectar microorganisms-plant-pollinator system. In any case, given the high susceptibility to azoles of nectar yeasts from the *Metschnikowia* clade found in this study, it seems clear that future risk assessments of the use of antifungals should pay attention to the nectar microbiota.

In summary, results of this study provide compelling evidence that exposure to azoles may pose a risk for ecologically important yeasts from the *Metschnikowia* clade,

and thus could potentially have detrimental effects on ecosystem dynamics and key services including plant pollination. This adds yet another source of concern for the long-term persistence of healthy plant-pollinator systems in natural communities. A next step would be to study the *in planta* effects of azoles on *Metschnikowia* yeasts, as well as to determine the actual ecological consequences of the in vitro results here reported.

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Tables

Table 1. Range of minimum inhibitory concentrations (MICs) obtained for quality control strains.[a]

Strain	Test medium[b]	Antifungal (*n*)[c]	≥50% inhibition end point			≥90% inhibition end point		
			24 h	48 h	72 h	24 h	48 h	72 h
Candida krusei ATCC 6258	RPMI+2%G	EPZ (23)	ND(65.2%), ≤0.016–0.125	0.031–0.125	0.063–0.25	ND(65.2%), 0.125–0.25	0.25–0.5	0.5–1
		IZL (22)	ND(54.5%), 0.063–0.5	0.5–2	1–4	ND(54.5%), 1–2	4	8
		KTZ (22)	ND(63.6%), ≤0.016–0.25	0.031–0.25	0.063–0.25	ND(63.6%), 0.25– 0.5	0.5–1	0.5–1
		VCZ (22)	ND(45.5%), 0.031–0.125	0.125–0.25	0.125–0.5	ND(45.5%), 0.25	0.25–0.5	0.5– 1
	YM broth	EPZ (9)	0.125–0.25	1	1– 2	0.5	1–2	1–2
		IZL (10)	1–4	4–8	8–>8	2–8	4–8	8–>8
		KTZ (9)	0.25–1	0.5–2	0.5–4	0.5–2	0.5–2	0.5–4
		VCZ (9)	0.5	1–2	1–2	0.5–1	1–2	2
Candida parapsilosis ATCC 22019	RPMI+2%G	EPZ (33)	ND(45.5%), ≤0.016– 0.031	0.063–0.125	0.063–0.25	ND(45.5%), ≤0.016–0.25	0.25–0.5	0.25–0.5
		IZL (30)	ND(36.7%), 0.125–0.25	0.25–0.5	0.5–1	ND(36.7%), 1–2	2–4	4–8
		KTZ (33)	ND(36.4%), ≤0.016–0.031	≤0.016–0.031	≤0.016–0.063	ND(36.4%), 0.031–0.063	0.063–0.125	0.125–0.25
		VCZ (33)	ND(30.3%), ≤0.016	≤0.016–0.031	0.031	ND(30.3%), 0.031–0.063	0.031–0.063	0.063–0.125
	YM broth	EPZ (8)	0.031–0.063	0.125–0.25	0.25–0.5	0.25	1	2–4
		IZL (6)	0.25– 1	1–4	2–8	1– 4	4–8	8–>8
		KTZ (7)	≤0.016	≤0.016–0.063	0.031–0.125	≤0.016–0.063	0.031–0.25	0.063– 1
		VCZ (8)	0.031	0.063	0.063– 0.125	0.063–0.125	0.125–0.25	0.25–0.5

[a] For each quality control strain and combination of test conditions (test medium, antifungal compound, incubation time –24, 48 and 72 h–, and inhibition end point), the range of MIC values (in mg/l) obtained in this study is given. In some cases, the percentage of tests in which the actual MIC value could not be determined (ND) due to scarce growth (i.e. absorbance at 530 nm ≤0.2; Arendrup *et al.* 2012) is also provided.

[b] RPMI+2%G, RPMI 1640 supplemented with glucose and buffered with 3-(N-morpholino) propanesulfonic acid (see main text); YM broth, yeast malt broth.

[c] EPZ, epoxiconazole; IZL, imazalil; KTZ, ketoconazole; VCZ, voriconazole. The number of tests performed (*n*) in each case is shown within parentheses.

Table 2. Distribution of minimum inhibitory concentrations (MICs) for azole antifungals determined by the EUCAST broth microdilution method for yeast strains of the *Metschnikowia* clade.

Species (no. of strains tested)	Antifungal[a]	End point[b]	MIC distribution (mg/l)[c]										EA[d]	NSD[e]	SD[e]
			≤0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8			
Metschnikowia reukaufii (46)	EPZ	50%	42*	3	1								93.5%	1 (2.2%)	2 (4.3%)
		90%	20	14*	7	2	1	1	1						
	IZL	50%	1	2	2	22*	18	1					82.6%	8 (17.4%)	0
		90%			2	1	11	21*	11						
	KTZ	50%	46*										97.8%	0	1 (2.2%)
		90%	39*	4	2					1					
	VCZ	50%	46*										97.8%	0	1 (2.2%)
		90%	41*	3	1							1			
Metschnikowia proteae (23)	EPZ	50%	23*										100%	0	0
		90%	23*												
	IZL	50%			7	15*	1						100%	0	0
		90%				13*	10								
	KTZ	50%	23*										100%	0	0
		90%	21*	2											
	VCZ	50%	23*										100%	0	0
		90%	22*	1											
Metschnikowia gruessii (22)	EPZ	50%	21*		1								100%	0	0
		90%	19*	2		1									
	IZL	50%		1	16*	4			1				100%	0	0
		90%			1	14*	6			1					
	KTZ	50%	21*		1								100%	0	0
		90%	21*			1									
	VCZ	50%	21*		1								100%	0	0
		90%	20*	1			1								
Candida	EPZ	50%	2	5*	4								100%	0	0

rancensis (11)		90%	1	2	5*	3								
	IZL	50%				1	3	4*	3			100%	0	0
		90%					1	7*		2	1			
	KTZ	50%	4	7*								100%	0	0
		90%		6*	4	1								
	VCZ	50%	5	5*	1							100%	0	0
		90%	2	3	6*									
Metschnikowia koreensis (11)	EPZ	50%	11*									100%	0	0
		90%	6*	4	1									
	IZL	50%			1	7*	3					100%	0	0
		90%				2	2	7*						
	KTZ	50%	9*	2								100%	0	0
		90%	8*	3										
	VCZ	50%	10*	1								100%	0	0
		90%	9*	2										
Metschnikowia caudata[f] (7)	EPZ	50%	5*	2								100%	0	0
		90%		5*	2									
	IZL	50%		1	1	1	2*	2				71.4%	2 (28.6%)	0
		90%						7*						
	KTZ	50%	7*									100%	0	0
		90%	4*	3										
	VCZ	50%	5*	2								100%	0	0
		90%		2	5*									
Total (120)	EPZ	50%	104 (86.7%)	10 (8.3%)	6 (5%)							97.5%	1 (0.8%)	2 (1.7%)
		90%	69 (57.5%)	27 (22.5%)	15 (12.5%)	6 (5%)	1 (0.8%)	1 (0.8%)	1 (0.8%)					
	IZL	50%	1 (0.8%)	4 (3.3%)	27 (22.5%)	50 (41.7%)	27 (22.5%)	7 (5.8%)	4 (3.3%)			91.7%	10 (8.3%)	0
		90%			3 (2.5%)	30 (25%)	30 (25%)	42 (35%)	11 (9.2%)	3 (2.5%)	1 (0.8%)			
	KTZ	50%	110 (91.7%)	9 (7.5%)	1 (0.8%)							99.2%	0	1 (0.8%)

	90%	93 (77.5%)	18 (15%)	6 (5%)	2 (1.7%)			1 (0.8%)					
VCZ	50%	110 (91.7%)	8 (6.7%)	2 (1.7%)							99.2%	0	1 (0.8%)
	90%	94 (78.3%)	12 (10%)	12 (10%)		1 (0.8%)				1 (0.8%)			

[a] EPZ, epoxiconazole; IZL, imazalil; KTZ, ketoconazole; VCZ, voriconazole.

[b] Percentage of growth inhibition relative to a positive control (i.e. test medium without azoles) set as end point for antifungal susceptibility testing.

[c] Number (and percentage, only for total results) of strains falling into each MIC value. The median MIC value for each yeast species, antifungal and end point combination is indicated by an asterisk.

[d] Percentage of essential agreement (i.e. discrepancy of no more than ±2 two-fold dilutions) between MIC values obtained for the different end points.

[e] Number (and percentage) of strains showing non-substantial differences (NSDs) or substantial differences (SDs) between MIC values obtained for the different end points.

[f] Azole susceptibility testing of *Metschnikowia caudata* strains was performed using yeast maltose (YM) broth instead of RPMI 1640 supplemented with 2% (w/v) of glucose (RPMI+2%G) as culture medium and after 96 h of incubation at 25ºC instead of 72 h (see details in the main text).

Table 3. Comparison of the results obtained for a selection of yeast strains from the *Metschnikowia* clade when tested for azole susceptibility in different culture media.

Species (no. of strains tested)	Antifungal[a]	≥50% inhibition end point					≥90% inhibition end point				
		MIC distribution[b]		%EA[c]	NSD[d]	SD[d]	MIC distribution[b]		%EA[c]	NSD[d]	SD[d]
		RPMI+2%G	YM broth				RPMI+2%G	YM broth			
Candida rancensis (6)	EPZ	≤0.016(1), 0.031(3), 0.063(2)	0.031(2), 0.063(3), 0.125(1)	100	0	0	≤0.016(1), 0.063(3), 0.125(2)	0.031(1), 0.063(1), 0.125(3), 0.25(1)	100	0	0
	IZL	0.25(1), 0.5(3), 1(2)	1(2), 2(2), 4(2)	100	0	0	0.5(4), 2(1), 4(1)	1(1), 2(2), 4(1), 8(1)	100	0	0
	KTZ	≤0.016(2), 0.031(4)	0.031(2), 0.063(2), 0.125(2)	100	0	0	0.031(4), 0.063(1), 0.125(1)	0.031(1), 0.063(2), 0.125(3)	100	0	0
	VCZ	≤0.016(2), 0.031(4)	0.031(2), 0.063(2), 0.125(2)	100	0	0	≤0.016(1), 0.031(2), 0.063(3)	0.031(1), 0.063(3), 0.125(1), 0.25(1)	100	0	0
Metschnikowia gruessii (6)	EPZ	≤0.016(6)	≤0.016(5), 0.031(1)	100	0	0	≤0.016(6)	≤0.016(3), 0.031(2), 0.063(1)	100	0	0
	IZL	0.031(1), 0.063(4), 0.125(1)	0.063(1), 0.125(1), 0.25(1), 0.5(2), 1(1)	50	3 (50%)	0	0.125(5), 0.25(1)	0.125(1), 0.25(1), 0.5(1), 1(2), 2(1)	50	3 (50%)	0
	KTZ	≤0.016(6)	≤0.016(5), 0.031(1)	100	0	0	≤0.016(6)	≤0.016(4), 0.031(2)	100	0	0
	VCZ	≤0.016(6)	≤0.016(5), 0.031(1)	100	0	0	≤0.016(6)	≤0.016(3), 0.031(2), 0.063(1)	100	0	0
Metschnikowia koreensis (6)	EPZ	≤0.016(6)	≤0.016(1), 0.031(5)	100	0	0	≤0.016(2), 0.031(3), 0.063(1)	≤0.016(1), 0.063(5)	100	0	0

	IZL	0.125(5), 0.25(1)	0.25(2), 0.5(1), 1(3)	50	3 (50%)	0	0.25(2), 0.5(4)	0.25(1), 0.5(1), 1(4)	100	0	0
	KTZ	≤0.016(4), 0.031(2)	≤0.016(2), 0.031(4)	100	0	0	≤0.016(4), 0.031(2)	≤0.016(2), 0.031(4)	100	0	0
	VCZ	≤0.016(5), 0.031(1)	≤0.016(1), 0.031(5)	100	0	0	≤0.016(5), 0.031(1)	0.031(2), 0.063(4)	100	0	0
Metschnikowia proteae (6)	EPZ	≤0.016(6)	≤0.016(6)	100	0	0	≤0.016(6)	≤0.016(6)	100	0	0
	IZL	0.063(1), 0.125(4), 0.25(1)	0.25(5), 0.5(1)	100	0	0	0.125(2), 0.25(4)	0.25(1), 0.5(5)	100	0	0
	KTZ	≤0.016(6)	≤0.016(6)	100	0	0	≤0.016(5), 0.031(1)	≤0.016(5), 0.031(1)	100	0	0
	VCZ	≤0.016(6)	≤0.016(5), 0.031(1)	100	0	0	≤0.016(6)	≤0.016(5), 0.031(1)	100	0	0
Metschnikowia reukaufii (6)	EPZ	≤0.016(6)	≤0.016(6)	100	0	0	≤0.016(2), 0.031(2), 0.063(1), 1(1)	≤0.016(1), 0.031(4), 0.063(1)	83.3	0	1 (16.7%)
	IZL	0.063(1), 0.125(2), 0.25(3)	0.125(6)	100	0	0	0.25(2), 0.5(2), 1(1), 2(2)	0.125(4), 0.25(1), 0.5(1)	83.3	1 (16.7%)	0
	KTZ	≤0.016(6)	≤0.016(6)	100	0	0	≤0.016(5), 2(1)	≤0.016(6)	83.3	0	1 (16.7%)
	VCZ	≤0.016(6)	≤0.016(4), 0.031(2)	100	0	0	≤0.016(5), 8(1)	≤0.016(1), 0.031(5)	83.3	0	1 (16.7%)
TOTAL (30)	EPZ	≤0.016(25), 0.031(3), 0.063(2)	≤0.016(18), 0.031(8), 0.063(3), 0.125(1)	100	0	0	≤0.016(17), 0.031(5), 0.063(5), 0.125(2), 1(1)	≤0.016(11), 0.031(7), 0.063(8), 0.125(3), 0.25(1)	96.7	0	1 (3.3%)
	IZL	0.031(1), 0.063(6), 0.125(12), 0.25(6), 0.5(3), 1(2)	0.063(1), 0.125(7), 0.25(8), 0.5(4), 1(6), 2(2), 4(2)	80	6 (20%)	0	0.125(7), 0.25(9), 0.5(10), 1(1), 2(2), 4(1)	0.125(5), 0.25(4), 0.5(8), 1(8), 2(3), 4(1), 8(1)	86.7	4 (13.3%)	0

	KTZ	≤0.016(24), 0.031(6)	≤0.016(19), 0.031(7), 0.063(2), 0.125(2)	100	0	0	≤0.016(20), 0.031(7), 0.063(1), 0.125(1), 2(1)	≤0.016(17), 0.031(8), 0.063(2), 0.125(3)	96.7	0	1 (3.3%)
	VCZ	≤0.016(25), 0.031(5)	≤0.016(15), 0.031(11), 0.063(2), 0.125(2)	100	0	0	≤0.016(23), 0.031(3), 0.063(3), 8(1)	≤0.016(9), 0.031(11), 0.063(8), 0.125(1), 0.25(1)	96.7	0	1 (3.3%)

[a] EPZ, epoxiconazole; IZL, imazalil; KTZ, ketoconazole; VCZ, voriconazole.

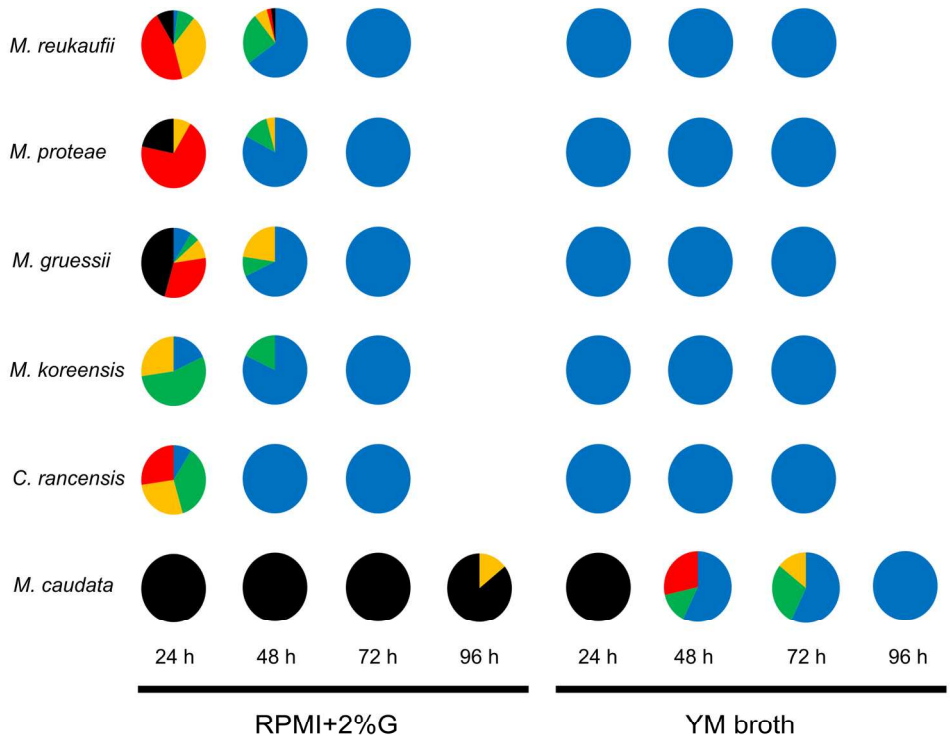
[b] For each species and combination of test conditions (test medium, antifungal compound, and inhibition end point), the number of strains displaying each MIC value (in mg/l) is given. RPMI+2%G, RPMI 1640 supplemented with glucose and buffered with 3-(N-morpholino) propanesulfonic acid (see main text); YM broth, yeast malt broth.

[c] Percentage of essential agreement (i.e. discrepancy of no more than ±2 two-fold dilutions) between MIC values obtained in different culture media.

[d] Number (and percentage) of strains showing non-substantial differences (NSDs) or substantial differences (SDs) between MIC values obtained in different culture media.

FIGURE LEGENDS

Figure 1. Pie charts showing the percentages of isolates of the tested species which displayed enough growth for reliable azole susceptibility determination by the EUCAST method (i.e. absorbance at 530 nm >0.2 in the positive control well) at each reading time (24, 48 and 72 h for most species, and also 96 h for *Metschnikowia caudata*, see Results) in 100% (blue sectors), ≥75% but <100% (green), ≥50% but <75% (orange), <50% but >0% (red) and 0% (black) of the test plates. Total numbers of isolates (*N*) and tests (*n*, mean ± S.D.) per species are as follows: i) experiments using RPMI-1640 medium supplemented with 2% (w/v) glucose (RPMI+2%G): *Metschnikowia reukaufii*, *N* = 46, *n* = 423 (9.2 ± 1.6); *M. proteae*, *N* = 23, *n* = 193 (8.4 ± 0.7); *M. gruessii*, *N* = 22, *n* = 181 (8.2 ± 0.8); *M. koreensis*, *N* = 11, *n* = 102 (9.3 ± 1.1); *Candida rancensis*, *N* = 11, *n* = 108 (9.8 ± 1.8); and *M. caudata*, *N* = 7, *n* = 56 (8 ± 0); ii) experiments using yeast malt (YM) broth: *M. reukaufii*, *N* = 6, *n* = 48 (8 ± 0); *M. proteae*, *N* = 6, *n* = 51 (8.5 ± 1.1); *M. gruessii*, *N* = 6, *n* = 61 (10.2 ± 1.7); *M. koreensis*, *N* = 6, *n* = 66 (11 ± 3.2); *C. rancensis*, *N* = 6, *n* = 52 (8.7 ± 1.5); and *M. caudata*, *N* = 7, *n* = 88 (12.6 ± 0.5).


M. reukaufii
M. proteae
M. gruessii
M. koreensis
C. rancensis
M. caudata
24 h
48 h
72 h
96 h
24 h
48 h
72 h
96 h
RPMI+2%G
YM broth

Tables

Table S1. Yeast strains included in the present study.

Strain	Yeast species	Country of origin	Habitat	Host species (family)	Year	Reference[a]
SA16	*Candida rancensis*	South Africa	Floral nectar	*Dierama trichorhizum* (Iridaceae)	2008	
SA20	*Candida rancensis*	South Africa	Floral nectar	*Graderia scabra* (Orobanchaceae)	2008	
SA25	*Candida rancensis*	South Africa	Floral nectar	*Kniphofia sp.* (Xanthorrhoeaceae)	2008	
SA26	*Candida rancensis*	South Africa	Floral nectar	*Burchellia bubalina* (Rubiaceae)	2008	
SA34	*Candida rancensis*	South Africa	Floral nectar	*Watsonia lepida* (Iridaceae)	2008	
SA35-1	*Candida rancensis*	South Africa	Floral nectar	*Watsonia pillansii* (Iridaceae)	2008	
SA40	*Candida rancensis*	South Africa	Floral nectar	*Haemanthus humilis* (Amaryllidaceae)	2008	
SA45-1	*Candida rancensis*	South Africa	Floral nectar	*Protea caffra* (Proteaceae)	2008	
SA71-2	*Candida rancensis*	South Africa	Floral nectar	*Adhatoda andromeda* (Acanthaceae)	2008	
SA73-1	*Candida rancensis*	South Africa	Floral nectar	*Adhatoda andromeda* (Acanthaceae)	2008	
NRRL Y-48759^{T}	*Candida rancensis*	USA	Floral nectar	*Mimulus aurantiacus* (Phrymaceae)	1984	
EBD-CdVSA08-1^{T}	*Metschnikowia caudata*	South Africa	Floral nectar	*Protea dracomontana* (Proteaceae)	2010	de Vega *et al.* 2014
EBD-B8Y1	*Metschnikowia caudata*	South Africa	Floral nectar	*Apis mellifera* (Apidae)	2010	de Vega *et al.* 2014
EBD-CdVSA21-2	*Metschnikowia caudata*	South Africa	Floral nectar	*Protea roupelliae* (Proteaceae)	2010	de Vega *et al.* 2014
EBD-CdVSA23-1	*Metschnikowia caudata*	South Africa	Floral nectar	*Protea roupelliae* (Proteaceae)	2010	de Vega *et al.* 2014
EBD-CdVSA57-2^{AT}	*Metschnikowia caudata*	South Africa	Floral nectar	*Protea subvestita* (Proteaceae)	2010	de Vega *et al.* 2014
EBD-SA53	*Metschnikowia caudata*	South Africa	Floral nectar	*Protea roupelliae* (Proteaceae)	2008	de Vega *et al.* 2014
EBD-SA54	*Metschnikowia caudata*	South Africa	Floral nectar	*Protea roupelliae* (Proteaceae)	2008	de Vega *et al.* 2014
CBS 7657^{T}	*Metschnikowia gruessii*	Portugal	Floral nectar	*Hebe salicifolia* (Plantaginaceae)	1992	
6D1	*Metschnikowia gruessii*	Spain	Floral nectar	*Digitalis obscura* (Plantaginaceae)	2009	Pozo *et al.* 2012

6D10	*Metschnikowia gruessii*	Spain	Floral nectar	*Digitalis obscura* (Plantaginaceae)	2009	Pozo *et al.* 2012
6D12	*Metschnikowia gruessii*	Spain	Floral nectar	*Digitalis obscura* (Plantaginaceae)	2009	Pozo *et al.* 2012
6E10	*Metschnikowia gruessii*	Spain	Floral nectar	*Gladiolus illyricus* (Iridaceae)	2008	Pozo *et al.* 2011
6E5	*Metschnikowia gruessii*	Spain	Floral nectar	*Lonicera implexa* (Caprifoliaceae)	2008	Pozo *et al.* 2011
6E6	*Metschnikowia gruessii*	Spain	Floral nectar	*Teucrium pseudochamaepytis* (Lamiaceae)	2008	Pozo *et al.* 2011
6E8	*Metschnikowia gruessii*	Spain	Floral nectar	*Teucrium pseudochamaepytis* (Lamiaceae)	2008	Pozo *et al.* 2011
6E9	*Metschnikowia gruessii*	Spain	Floral nectar	*Jasminum fruticans* (Oleaceae)	2008	Pozo *et al.* 2011
6F11	*Metschnikowia gruessii*	Spain	Floral nectar	*Antirrhinum australe* (Plantaginaceae)	2008	Pozo *et al.* 2011
6F12	*Metschnikowia gruessii*	Spain	Floral nectar	*Antirrhinum australe* (Plantaginaceae)	2008	Pozo *et al.* 2011
6F5	*Metschnikowia gruessii*	Spain	Floral nectar	*Digitalis obscura* (Plantaginaceae)	2008	Pozo *et al.* 2011
6F6	*Metschnikowia gruessii*	Spain	Floral nectar	*Digitalis obscura* (Plantaginaceae)	2008	Pozo *et al.* 2011
6F7	*Metschnikowia gruessii*	Spain	Floral nectar	*Digitalis obscura* (Plantaginaceae)	2008	Pozo *et al.* 2011
6F8	*Metschnikowia gruessii*	Spain	Floral nectar	*Phlomis lychnitis* (Lamiaceae)	2008	Pozo *et al.* 2011
6F9	*Metschnikowia gruessii*	Spain	Floral nectar	*Phlomis lychnitis* (Lamiaceae)	2008	Pozo *et al.* 2011
6G2	*Metschnikowia gruessii*	Spain	Floral nectar	*Prunella grandiflora* (Lamiaceae)	2008	Pozo *et al.* 2011
6G3	*Metschnikowia gruessii*	Spain	Floral nectar	*Prunella grandiflora* (Lamiaceae)	2008	Pozo *et al.* 2011
6G4	*Metschnikowia gruessii*	Spain	Floral nectar	*Prunella grandiflora* (Lamiaceae)	2008	Pozo *et al.* 2011
6G5	*Metschnikowia gruessii*	Spain	Floral nectar	*Atropa baetica* (Solanaceae)	2008	Pozo *et al.* 2011
6G6	*Metschnikowia gruessii*	Spain	Floral nectar	*Atropa baetica* (Solanaceae)	2008	Pozo *et al.* 2011
6G7	*Metschnikowia gruessii*	Spain	Floral nectar	*Atropa baetica* (Solanaceae)	2008	Pozo *et al.* 2011
CBS 8854^{T}	*Metschnikowia koreensis*	Korea	Flower	*Lilium* sp. (Liliaceae)	1999	
SA1-3	*Metschnikowia koreensis*	South Africa	Floral nectar	*Gladiolus longicollis* (Iridaceae)	2008	
SA21	*Metschnikowia koreensis*	South Africa	Floral nectar	*Ruellia cordata* (Acanthaceae)	2008	
SA41-1	*Metschnikowia koreensis*	South Africa	Floral nectar	*Stachys aethiopica* (Lamiaceae)	2008	
SA44-1	*Metschnikowia koreensis*	South Africa	Floral nectar	*Ajuga ophrydis* (Lamiaceae)	2008	

SA60	*Metschnikowia koreensis*	South Africa	Floral nectar	*Glumicalyx goseloides* (Scrophulariaceae)	2008	
SA66	*Metschnikowia koreensis*	South Africa	Floral nectar	*Silene bellidioides* (Caryophyllaceae)	2008	
SA70	*Metschnikowia koreensis*	South Africa	Floral nectar	*Disa crassicornis* (Orchidaceae)	2008	
SA71-1	*Metschnikowia koreensis*	South Africa	Floral nectar	*Adhatoda andromeda* (Acanthaceae)	2008	
SA8	*Metschnikowia koreensis*	South Africa	Floral nectar	*Gladiolus appendiculatus* (Iridaceae)	2008	
SA9	*Metschnikowia koreensis*	South Africa	Floral nectar	*Dierama luteo-albidum* (Iridaceae)	2008	
CdVSA78_2	*Metschnikowia proteae*	South Africa	Floral nectar	*Protea simplex* (Proteaceae)	2010	de Vega *et al.* 2012
EBDCdVSA 34_1	*Metschnikowia proteae*	South Africa	Floral nectar	*Protea caffra* (Proteaceae)	2010	de Vega *et al.* 2012
EBDCdVSA 35_1	*Metschnikowia proteae*	South Africa	Floral nectar	*Protea caffra* (Proteaceae)	2010	de Vega *et al.* 2012
EBDCdVSA 36_1	*Metschnikowia proteae*	South Africa	Floral nectar	*Protea caffra* (Proteaceae)	2010	de Vega *et al.* 2012
EBDCdVSA 37_1	*Metschnikowia proteae*	South Africa	Floral nectar	*Protea caffra* (Proteaceae)	2010	de Vega *et al.* 2012
EBDCdVSA 39_1	*Metschnikowia proteae*	South Africa	Floral nectar	*Protea caffra* (Proteaceae)	2010	de Vega *et al.* 2012
EBDCdVSA 46_1	*Metschnikowia proteae*	South Africa	Floral nectar	*Protea caffra* (Proteaceae)	2010	de Vega *et al.* 2012
EBDSA45_2	*Metschnikowia proteae*	South Africa	Floral nectar	*Protea caffra* (Proteaceae)	2008	de Vega *et al.* 2012
EBDT1Y1^{T}	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Trichostetha fascicularis* (Scarabaeidae: Cetoniinae)	2008	de Vega *et al.* 2012
EBDC2Y2^{AT}	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Cyrtothyrea marginalis* (Scarabaeidae: Cetoniinae)	2008	de Vega *et al.* 2012
EBDA10Y1	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Atrichelaphinis tigrina* (Scarabaeidae: Cetoniinae)	2008	de Vega *et al.* 2012
EBDA7Y1	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Atrichelaphinis tigrina* (Scarabaeidae: Cetoniinae)	2008	de Vega *et al.* 2012
EBDC1Y3	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Cyrtothyrea marginalis* (Scarabaeidae: Cetoniinae)	2008	de Vega *et al.* 2012
EBDC3Y1	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Cyrtothyrea marginalis* (Scarabaeidae: Cetoniinae)	2008	de Vega *et al.* 2012
EBDC4Y1	*Metschnikowia proteae*	South Africa	Insect	*Cyrtothyrea marginalis* (Scarabaeidae:	2008	de Vega *et al.* 2012

			(Coleoptera)	Cetoniinae)		
EBDM1Y1	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Heterochelus* sp. (Scarabaeidae: Hopliinae)	2008	de Vega *et al.* 2012
EBDM2Y1	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Heterochelus* sp. (Scarabaeidae: Hopliinae)	2008	de Vega *et al.* 2012
EBDM3Y1	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Heterochelus* sp. (Scarabaeidae: Hopliinae)	2008	de Vega *et al.* 2012
EBDM6Y1	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Heterochelus* sp. (Scarabaeidae: Hopliinae)	2008	de Vega *et al.* 2012
EBDM7Y1	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Heterochelus* sp. (Scarabaeidae: Hopliinae)	2008	de Vega *et al.* 2012
EBDT2Y1	*Metschnikowia proteae*	South Africa	Insect (Coleoptera)	*Trichostetha fascicularis* (Scarabaeidae: Cetoniinae)	2008	de Vega *et al.* 2012
EBDF1Y1	*Metschnikowia proteae*	South Africa	Insect (Diptera)	Drosophilidae sp.	2008	de Vega *et al.* 2012
EBDF2Y1	*Metschnikowia proteae*	South Africa	Insect (Diptera)	Drosophilidae sp.	2008	de Vega *et al.* 2012
ST12.14/017	*Metschnikowia reukauffi*	Belgium	Floral nectar	*Symphytum officinale* (Boraginaceae)	2013	Lenaerts *et al.* 2015
ST12.14/020	*Metschnikowia reukauffi*	Belgium	Floral nectar	*Symphytum officinale* (Boraginaceae)	2013	Lenaerts *et al.* 2015
7.3K/FT9 A	*Metschnikowia reukaufii*	Belgium	Floral nectar	*Pulmonaria officinalis* (Boraginaceae)	2012	Jacquemyn *et al.* 2013
7.8L/FT6	*Metschnikowia reukaufii*	Belgium	Floral nectar	*Pulmonaria officinalis* (Boraginaceae)	2012	Jacquemyn *et al.* 2013
7.9L/FT9 A	*Metschnikowia reukaufii*	Belgium	Floral nectar	*Pulmonaria officinalis* (Boraginaceae)	2012	Jacquemyn *et al.* 2013
ST12.14/023	*Metschnikowia reukaufii*	Belgium	Floral nectar	*Symphytum officinale* (Boraginaceae)	2013	Lenaerts *et al.* 2015
ST12.14/029	*Metschnikowia reukaufii*	Belgium	Floral nectar	*Symphytum officinale* (Boraginaceae)	2013	Lenaerts *et al.* 2015
ST12.14/030	*Metschnikowia reukaufii*	Belgium	Floral nectar	*Symphytum officinale* (Boraginaceae)	2013	Lenaerts *et al.* 2015
ST12.14/496	*Metschnikowia reukaufii*	Belgium	Floral nectar	*Symphytum officinale* (Boraginaceae)	2013	Lenaerts *et al.* 2015
6.3.2-Y2	*Metschnikowia reukaufii*	Belgium	Floral nectar	*Pulmonaria officinalis* (Boraginaceae)	2012	Jacquemyn *et al.* 2013
CECT 10671^{T}	*Metschnikowia reukaufii*	Canada	Flower	*Epilobium angustifolium* (Onagraceae)	1968	
CdV Mar10.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Nonea vesicaria* (Boraginaceae)	2013	

CdV Mar11.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Nonea vesicaria* (Boraginaceae)	2013	
CdV Mar13.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Teucrium pseudochamaepitys* (Lamiaceae)	2013	
CdV Mar16.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Lamium album* (Lamiaceae)	2013	
CdV Mar17.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Lavandula multifida* (Lamiaceae)	2013	
CdV Mar21.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Lavandula multifida* (Lamiaceae)	2013	
CdV Mar31.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Gladiolus italicus-communis* (Iridaceae)	2013	
CdV Mar33.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Gladiolus italicus-communis* (Iridaceae)	2013	
CdV Mar34.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Gladiolus italicus-communis* (Iridaceae)	2013	
CdV Mar4.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Linaria sp.* (Plantaginaceae)	2013	
CdV Mar8.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Echium plantagineum* (Boraginaceae)	2013	
CdV Mar9.1	*Metschnikowia reukaufii*	Morocco	Floral nectar	*Nonea vesicaria* (Boraginaceae)	2013	
SA33	*Metschnikowia reukaufii*	South Africa	Floral nectar	*Gladiolus parvulus* (Iridaceae)	2008	
SA44-2	*Metschnikowia reukaufii*	South Africa	Floral nectar	*Ajuga ophrydis* (Lamiaceae)	2008	
SA72-1	*Metschnikowia reukaufii*	South Africa	Floral nectar	*Adhatoda andromeda* (Acanthaceae)	2008	
1A2	*Metschnikowia reukaufii*	Spain	Floral nectar	*Helleborus foetidus* (Ranunculaceae)	2009	
1B11	*Metschnikowia reukaufii*	Spain	Floral nectar	*Aquilegia vulgaris* (Ranunculaceae)	2008	Pozo *et al.* 2011
1B4	*Metschnikowia reukaufii*	Spain	Floral nectar	*Anthyllis vulneraria* (Fabaceae)	2008	Pozo *et al.* 2011
1B5	*Metschnikowia reukaufii*	Spain	Floral nectar	*Tetragonolobus maritimus* (Fabaceae)	2008	Pozo *et al.* 2011
1B7	*Metschnikowia reukaufii*	Spain	Floral nectar	*Aquilegia vulgaris* (Ranunculaceae)	2008	Pozo *et al.* 2011
1C5	*Metschnikowia reukaufii*	Spain	Floral nectar	*Tetragonolobus maritimus* (Fabaceae)	2008	Pozo *et al.* 2011
1D6	*Metschnikowia reukaufii*	Spain	Floral nectar	*Tetragonolobus maritimus* (Fabaceae)	2008	Pozo *et al.* 2011
2C12	*Metschnikowia reukaufii*	Spain	Floral nectar	*Iris foetidissima* (Iridaceae)	2008	Pozo *et al.* 2011
2C2	*Metschnikowia reukaufii*	Spain	Floral nectar	*Vicia onobrychioides* (Fabaceae)	2008	Pozo *et al.* 2011
2D10	*Metschnikowia reukaufii*	Spain	Floral nectar	*Prunella grandiflora* (Lamiaceae)	2008	Pozo *et al.* 2011

2D11	*Metschnikowia reukaufii*	Spain	Floral nectar	*Prunella grandiflora* (Lamiaceae)	2008	Pozo *et al.* 2011
2D6	*Metschnikowia reukaufii*	Spain	Floral nectar	*Vicia villosa* (Fabaceae)	2008	Pozo *et al.* 2011
2D7	*Metschnikowia reukaufii*	Spain	Floral nectar	*Vicia villosa* (Fabaceae)	2008	Pozo *et al.* 2011
2E2	*Metschnikowia reukaufii*	Spain	Floral nectar	*Aquilegia pyrenaica cazorlensis* (Ranunculaceae)	2008	Pozo *et al.* 2011
2E3	*Metschnikowia reukaufii*	Spain	Floral nectar	*Aquilegia pyrenaica cazorlensis* (Ranunculaceae)	2008	Pozo *et al.* 2011
2E4	*Metschnikowia reukaufii*	Spain	Floral nectar	*Aquilegia pyrenaica cazorlensis* (Ranunculaceae)	2008	Pozo *et al.* 2011
3A3	*Metschnikowia reukaufii*	Spain	Floral nectar	*Helleborus foetidus* (Ranunculaceae)	2008	Pozo *et al.* 2011
3B10	*Metschnikowia reukaufii*	Spain	Floral nectar	*Helleborus foetidus* (Ranunculaceae)	2008	Pozo *et al.* 2011
3C12	*Metschnikowia reukaufii*	Spain	Floral nectar	*Helleborus foetidus* (Ranunculaceae)	2008	Pozo *et al.* 2011
5C4	*Metschnikowia reukaufii*	Spain	Floral nectar	*Helleborus foetidus* (Ranunculaceae)	2008	Pozo *et al.* 2011

^{T}, type strain; ^{AT}, allotype strain.

[a] When relevant, references for field-collected strains are provided (see details in the References section of the paper).

Table S2. Distribution of minimum inhibitory concentrations (MICs, in mg/l) obtained for quality control strains.[a]

Strain	Test medium[b]	Antifungal (*n*)[c]	≥50% inhibition end point			≥90% inhibition end point		
			24 h	48 h	72 h	24 h	48 h	72 h
Candida krusei ATCC 6258	RPMI+2%G	EPZ (23)	ND(65.2%), ≤0.016(13%), 0.031(17.4%), 0.125(4.3%)	0.031(8.7%), 0.063(60.9%), 0.125(30.4%)	0.063(4.3%), 0.125(73.9%), 0.25(21.7%)	ND(65.2%), 0.125(17.4%), 0.25 (17.4%)	0.25(21.7%), 0.5(78.3%)	0.5(30.4%), 1(69.6%)
		IZL (22)	ND(54.5%), 0.063(4.5%), 0.125(4.5%), 0.25(13.6%), 0.5(22.7%)	0.5(22.7%), 1(63.6%), 2(13.6%)	1(4.5%), 2(86.4%), 4(9.1%)	ND(54.5%), 1(13.6%), 2(31.8%)	4(100%)	8(100%)
		KTZ (22)	ND(63.6%), ≤0.016(4.5%), 0.031(4.5%), 0.125(18.2%), 0.25(9.1%)	0.031(9.1%), 0.063(22.7%), 0.125(59.1%), 0.25(9.1%)	0.063(18.2%), 0.125(59.1%), 0.25(22.7%)	ND(63.6%), 0.25(13.6%), 0.5(22.7%)	0.5(90.9%), 1(9.1%)	0.5(22.7%), 1(77.3%)
		VCZ (22)	ND(45.5%), 0.031(4.5%), 0.063(27.3%), 0.125(22.7%)	0.125(72.7%), 0.25(27.3%)	0.125(9.1%), 0.25(86.4%), 0.5(4.5%)	ND(45.5%), 0.25(54.5%)	0.25(4.5%), 0.5(95.5%)	0.5(86.4%), 1(13.6%)
	YM broth	EPZ (9)	0.125(33.3%), 0.25(66.7%)	1(100%)	1(55.6%), 2(44.4%)	0.5(100%)	1(77.8%), 2(22.2%)	1(33.3%), 2(66.7%)
		IZL (10)	1(10%), 2(30%), 4(60%)	4(40%), 8(60%)	8(70%), >8(30%)	2(10%), 4(80%), 8(10%)	4(10%), 8(90%)	8(40%), >8(60%)
		KTZ (9)	0.25(33.3%), 1(66.7%)	0.5(33.3%), 2(66.7%)	0.5(22.2%), 1(11.1%), 2(55.6%), 4(11.1%)	0.5(33.3%), 1(55.6%), 2(11.1%)	0.5(22.2%), 1(11.1%), 2(66.7%)	0.5(11.1%), 1(11.1%), 2(11.1%), 4(66.7%)
		VCZ (9)	0.5(100%)	1(88.9%), 2(11.1%)	1(33.3%), 2(66.7%)	0.5(11.1%), 1(88.9%)	1(55.6%), 2(44.4%)	2(100%)

Candida parapsilosis ATCC 22019	RPMI+2%G	EPZ (33)	ND(45.5%), ≤0.016(9.1%), 0.031(45.5%)	0.063(66.7%), 0.125(33.3%)	0.063(3%), 0.125(93.9%), 0.25(3%)	ND(45.5%), ≤0.016(3%), 0.125(48.5%), 0.25(3%)	0.25(93.9%), 0.5(6.1%)	0.25(24.2%), 0.5(75.8%)
		IZL (30)	ND(36.7%), 0.125(20%), 0.25(43.3%)	0.25(13.3%), 0.5(86.7%)	0.5(36.7%), 1(63.3%)	ND(36.7%), 1(56.7%), 2(6.7%)	2(30%), 4(70%)	4(86.7%), 8(13.3%)
		KTZ (33)	ND(36.4%), ≤0.016(60.6%), 0.031(3%)	≤0.016(33.3%), 0.031(66.7%)	≤0.016(3%), 0.031(90.9%), 0.063(6.1%)	ND(36.4%), 0.031(3%), 0.063(60.6%)	0.063(9.1%), 0.125(90.9%)	0.125(90.9%), 0.25(9.1%)
		VCZ (33)	ND(30.3%), ≤0.016(69.7%)	≤0.016(33.3%), 0.031(66.7%)	0.031(100%)	ND(30.3%), 0.031(63.6%), 0.063(6.1%)	0.031(6.1%), 0.063(93.9%)	0.063(90.1%), 0.125(9.1%)
	YM broth	EPZ (8)	0.031(25%), 0.063(75%)	0.125(87.5%), 0.25(12.5%)	0.25(25%), 0.5(75%)	0.25(100%)	1(100%)	2(75%), 4(25%)
		IZL (6)	0.25(33.3%), 0.5(33.3%), 1(33.3%)	1(33.3%), 2(16.7%), 4(50%)	2(33.3%), 8(66.7%)	1(16.7%), 2(50%), 4(33.3%)	4(33.3%), 8(66.7%)	8(50%), >8(50%)
		KTZ (7)	≤0.016(100%)	≤0.016(42.9%), 0.063(57.1%)	0.031(42.9%), 0.125(57.1%)	≤0.016(14.3%), 0.031(28.6%), 0.063(57.1%)	0.031(14.3%), 0.063(28.6%), 0.125(42.9%), 0.25(14.3%)	0.063(14.3%), 0.125(14.3%), 0.25(42.9%), 0.5(14.3%), 1(14.3%)
		VCZ (8)	0.031(100%)	0.063(100%)	0.063(12.5%), 0.125(87.5%)	0.063(37.5%), 0.125(62.5%)	0.125(12.5%), 0.25(87.5%)	0.25(25%), 0.5(75%)

[a] For each quality control strain and combination of test conditions (test medium, antifungal compound, incubation time –24, 48 and 72 h–, and inhibition end point), the percentage of tests in which each MIC value was obtained is given within parentheses. In some cases, the actual MIC value could not be determined (ND) due to scarce growth (i.e. absorbance at 530 nm ≤0.2; Arendrup *et al.* 2012).

[b] RPMI+2%G, RPMI 1640 supplemented with glucose and buffered with 3-(N-morpholino) propanesulfonic acid (see main text); YM broth, yeast malt broth.

[c] EPZ, epoxiconazole; IZL, imazalil; KTZ, ketoconazole; VCZ, voriconazole. The number of tests performed (*n*) in each case is shown within parentheses.