

1 **Antifungal susceptibility testing of *Malassezia* spp. with an**
2 **optimized colorimetric broth microdilution method**

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10 Running Head: Drug susceptibility testing in *Malassezia* spp

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26 **ABSTRACT**

27 *Malassezia* is a genus of lipid dependent yeasts. It is associated with common
28 skin diseases such as pityriasis versicolor or atopic dermatitis and can cause
29 systemic infections in immunocompromised individuals. Owing to slow growth
30 and lipid requirements of these fastidious yeasts, convenient and reliable
31 antifungal drug susceptibility testing assays for *Malassezia* spp. are not widely
32 available. We have therefore optimized a broth microdilution assay for the
33 testing of *Malassezia* that is based on the assays from CLSI and EUCAST for
34 *Candida* and other yeasts. The addition of ingredients such as lipids and
35 esculin provided a broth medium formulation, which enabled the growth of all
36 *Malassezia* spp. and could be read with the colorimetric indicator resazurin by
37 visual and by fluorescence reading. We have tested the susceptibility of 52
38 strains of 13 species of *Malassezia* against 11 commonly used antifungals.
39 MIC values determined by visual reading were in good agreement with MIC
40 values determined by fluorescence reading. The lowest MICs were found for
41 the azoles itraconazole, posaconazole and voriconazole with MIC₉₀ values of
42 0.03-1.0 µg/ml, 0.06–0.5 µg/ml and 0.03-2.0 µg/ml, respectively. All
43 *Malassezia* spp. were resistant to echinocandins and griseofulvin. Some
44 *Malassezia* spp. also showed high MIC values for ketoconazole, which is the
45 most widely recommended topical antifungal to treat *Malassezia* skin
46 infections. In summary, our assay enables the fast and reliable susceptibility
47 testing of *Malassezia* spp. against a large panel of different antifungals.

48

49 INTRODUCTION

50 *Malassezia* is a genus of lipid dependent yeasts currently comprising 14
51 species. With the exception of *M. pachydermatis*, *Malassezia* species are
52 lipid dependent. *M. sympodialis*, *M. slooffiae*, *M. furfur*, *M. globosa*, *M.*
53 *restricta*, *M. obtusa*, *M. japonica*, *M. dermatis*, and *M. yamatoensis* are
54 considered anthropophilic as they have been isolated from human skin (1).
55 *Malassezia* is the most common fungal genus of the healthy human skin
56 microbiome (2). Additionally, a pathogenic role is attributed to these yeasts in
57 common skin diseases such as pityriasis versicolor, atopic dermatitis and
58 seborrheic dermatitis, and can cause severe systemic infections in neonates
59 and immunocompromised individuals (3, 4).

60 In infectious diseases, antimicrobial susceptibility testing is a useful tool to
61 decide for the appropriate antimicrobial treatment, particularly if antimicrobial
62 susceptibility cannot be predicted based on the identity of the infectious agent
63 (5). Treatment of *Malassezia*-related infections has thus far relied on the
64 predicted susceptibility to common antifungals such as topical azoles.
65 However, the antifungal treatment of *Malassezia*-associated skin diseases is
66 clinically not efficacious in up to one third of patients (6). This may be
67 attributed to malcompliance but also the antifungal resistance of *Malassezia*
68 spp. Moreover, the knowledge on the susceptibility of different *Malassezia*
69 species and strains to particular antifungals is scarce (7-10). This
70 substantiates the need of antifungal susceptibility testing for *Malassezia* spp.
71 Existing assays for the determination of the MIC values of antifungals as
72 recommended by the CLSI-M27-A3 (11) and EUCAST (12) documents are
73 only applicable for fast-growing fungi such as *Candida* spp. These assays are

74 not suitable for *Malassezia* spp., because they slow growing and fastidious.
75 Therefore, some studies that have tested the antifungal susceptibility of
76 *Malassezia* spp. used assays modified from the CLSI and EUCAST guidelines.
77 These modifications comprised variations in growth medium composition and
78 the inoculum size (Table 1) (7-9). Turbidity was the preferred readout method
79 for most of these assays (7,8). However, drawbacks of turbidity reading are
80 limited reproducibility and the possibility to underestimate MIC values (13). To
81 overcome these limitations, we have developed a new antifungal susceptibility
82 assay based on a broth medium that allows growth as well as reliable and
83 convenient antifungal testing of all *Malassezia* species. It is compatible with
84 the fluorometric indicator resazurin thus enabling the rapid and effective
85 determination of MIC values by visual and by fluorescence reading.

86

87 **MATERIALS & METHODS**88 ***Malassezia* strains**

89 *Malassezia* reference strains were obtained from the Centraalbureau voor
90 Schimmelcultures, CBS (Utrecht, The Netherlands) (Table 2). Clinical strains
91 were obtained from patients seen at the Department of Dermatology at the
92 University Hospital of Zurich, Zurich, Switzerland. All strains were maintained
93 on modified Leeming Notman Agar (mLN) and identified as previously
94 described (14, 15).

95

96 **Optimizing broth medium formulation**

97 The basis of the broth medium was filtered RPMI 1640 with 0.165M 3-(N-
98 morpholino)propanesulfonic acid (MOPS) without sodium bicarbonate (Sigma-
99 Aldrich, Zurich, Switzerland). Sodium bicarbonate, glucose, Tween 60, oleic
100 acid, glycerin and esculin (Sigma-Aldrich) were selectively titrated and added
101 to the broth medium. Reference strains of *M. sympodialis*, *M. slooffiae*, *M.*
102 *furfur*, *M. globosa* and *M. pachydermatis* were inoculated in triplicate at the
103 stipulated optical density (OD) (Table 2) and incubated at 35°C for 48 h.
104 Growth was assessed by scoring the turbidity of the broth medium on an
105 arbitrary scale ranging from 0 (no growth) to 4 (highest growth). This led to the
106 formulation of three candidate media (Table 3). RPMI++ with esculin provided
107 the best conditions for growth and colorimetric readings for all tested
108 *Malassezia* strains. We have named this medium OptiMAL and used it for
109 antifungal susceptibility testing.

110

111 ***Malassezia* spp. inocula quantification and optimization**

112 *Malassezia* spp. inocula were quantified in suspension by OD₆₀₀
113 measurement (Cytation 3 plate reader, BioTek, Winooski, VT, USA). To
114 correlate OD₆₀₀ readings with the number of yeast cells, serial dilutions of the
115 suspensions were plated on mLN agar at 35°C and the CFUs were counted
116 after 4-7 days.

117 To determine the optimal inoculum range for *Malassezia* spp., five different
118 inocula of *M. furfur* CBS 1878 were tested (approximately 2,500, 5,000,
119 50,000, 100,000 and 200,000 CFU/ml) together with seven antifungals
120 (amphotericin B, terbinafine, ketoconazole, fluconazole, itraconazole,
121 posaconazole and voriconazole). Plates were read every 6 h for the first 24 h
122 and subsequently at 12 h intervals for up to 60 h as described below.

123

124 **Broth microdilution assay**

125 Antifungals tested in the assay were amphotericin B (concentration range,
126 0.06 – 8 µg/ml) , caspofungin (0.06-8 µg/ml), griseofulvin (0.06-8 µg/ml),
127 terbinafine (0.125-16 µg/ml), fluconazole (0.06-128 µg/ml), ketoconazole
128 (0.03-4 µg/ml), itraconazole (0.015-8 µg/ml), posaconazole (0.015-8 µg/ml),
129 voriconazole (0.003-8 µg/ml) (all from Sigma-Aldrich), micafungin and
130 anidulafungin (all from MedChemExpress Europe, Sollentuna, Sweden).
131 Serial 2-fold dilutions of 200X stocks of antifungals were prepared in dimethyl
132 sulfoxide (DMSO, Sigma-Aldrich) and stored at -80°C. 50 µl of inocula were
133 added to 50 µl of 2X concentrated antifungals to achieve a final cell density of
134 5.0x10³-5.0x10⁴ CFU/ml. For inoculum verification, 10 µl of a 1:10 diluted
135 inoculum were plated onto mLN agar and incubated for 4-7 days at 35°C. MIC

136 values from each well plate were deemed reliable only if counts on agar
137 plates were within 10-100 CFUs.

138 *Candida parapsilosis* ATCC22019 was used as quality control strain to assess
139 the accuracy of our antifungal dilution and reproducibility of results for
140 fluorescence and visual readings according to CLSI and EUCAST (11,12).

141

142 **Data analysis of antifungal susceptibility assay**

143 Plates were analyzed when the indicator resazurin (Sigma-Aldrich) changed
144 from blue to pink in the positive control. The color change was assessed by (i)
145 visual reading and (ii) fluorescence analysis at 530 nm/590 nm (Cytation 3
146 plate reader)). Growth was measured relative to the positive control. MIC
147 values for azoles and echinocandins were defined as 50% growth inhibition,
148 and for amphotericin B as complete growth inhibition in accordance with CLSI
149 standards (11). For terbinafine, the MIC was defined as the lowest drug
150 concentration completely inhibiting growth.

151 For each strain, at least duplicate testing was performed. If MIC values
152 differed by more than 2 dilution steps, experiments were repeated. In cases
153 where repeats gave MIC values on extreme ends of the dilution scale, an
154 inconclusive MIC result was recorded.

155 Heat maps of the final MIC values for each strain were plotted using the
156 minimum and maximum drug ranges tested, normalized across a scale of 0 to
157 1. ANOVA and a correlation analysis were applied by calculating Pearson's r.
158 Inferential statistics was calculated by StatPlus:mac 2016 (AnalystSoft Inc,
159 California, USA) and a p -value of <0.05 was considered significant.

160

161 RESULTS

162 Growth of *Malassezia* spp. in OptiMAL broth medium

163 Serial dilutions of each broth medium component were tested to determine
164 optimal concentrations for the five *Malassezia* species *M. sympodialis*, *M.*
165 *slooffiae*, *M. furfur*, *M. globosa*, and *M. pachydermatis*. Optimal glycerol and
166 Tween 60 concentrations were 0.25-0.5% and 0.5%, respectively. We have
167 used a concentration of 0.05-2% oleic acid for OptiMAL, because most
168 *Malassezia* species preferred oleic acid to olive oil in assimilation assays.
169 Glucose concentrations of more than 2% did not improve growth. The optimal
170 pH range was between pH 6.0 to 6.5.

171 In the absence of sodium bicarbonate, RPMI and RPMI++ tended to color
172 bleach. Testing growth with sodium bicarbonate concentrations ranging from
173 0 to 32 mg/ml showed that *Malassezia* spp. were able to tolerate a sodium
174 bicarbonate concentration of up to 2 mg/ml before growth inhibition occurred.
175 A sodium bicarbonate concentration of 2 mg/ml was optimal for visual and
176 fluorescence readings, because it boosted color intensity and improved pH
177 buffering (Figure S1). The addition of 0.1 mg/ml of esculin increased the
178 fluorescence signal by 2-4 fold (Figure S1). These observations support the
179 use of sodium bicarbonate and esculin as useful broth medium additives,
180 which are compatible with resazurin for *Malassezia* spp. growth and broth
181 microdilution assays.

182

183 Optimal inoculum of *Malassezia* spp.

184 By testing an inoculum range and varying the incubation time until reading, we
185 observed that the MIC values of some antifungals strongly depended on the
186 inoculum and the incubation time while other antifungals were less affected.
187 For amphotericin B, growth curves were not influenced by inoculum nor
188 incubation time (Figure S2, left column). However, growth curves were less
189 reproducible with other antifungals, especially when an inoculum of $>100,000$
190 CFU/ml was used. To provide reproducible results for all antifungals, we
191 decided for a final inoculum of 5.0×10^3 - 5.0×10^4 CFU/ml for all tested
192 *Malassezia* strains.

193 Incubation times of 18-48 h gave comparable growth curves, and MIC values
194 determined at these time points did not differ by more than one to two dilution
195 steps for inocula of 5.0×10^3 - 5.0×10^4 CFU/ml (Figure S2, right column). Color
196 development after 6-12 h of incubation was insufficient for reliable MIC
197 readings, while after 60 h of incubation the fluorescence signal faded. We
198 therefore decided to read our plates after 24-48 h of incubation. Ketoconazole
199 was the only exception, where incubation periods of 24-36 h resulted in a loss
200 of growth curve sensitivity and high MIC readings.

201

202 **Agreement between visual and fluorescence reading**

203 We were interested in assessing if MIC values determined by visual reading
204 differed from MIC values determined by fluorescence reading. MIC values
205 determined by fluorescence reading appeared slightly higher than those
206 determined by visual reading, but the difference was not significant ($p=0.25$).
207 Overall, the MIC values determined by both readout methods strongly
208 correlated to each other (Figure 2, Pearson's correlation >0.857 for all drugs)

209 for all antifungals. For most antifungals, MIC values determined by both
210 methods were within two dilution steps in 96-100% of cases (Table 4). The
211 agreement of MIC values for ketoconazole was slightly lower, with MIC values
212 within two dilution steps in 88% of cases (Table 4).

213

214 **Susceptibility of *Malassezia* spp. to antifungals**

215 We assessed the antifungal susceptibility profiles of 52 *Malassezia* strains,
216 including 13 reference strains and 39 clinical strains (Table 2) to 11
217 antifungals commonly used in clinical routine. The lowest MIC values were
218 found for itraconazole, posaconazole and voriconazole for most strains tested
219 (Figure 2, Table 5). Among the azoles, the highest MIC values and the widest
220 MIC ranges were observed for fluconazole (Table 5, Table S1). Ketoconazole
221 showed low MICs for most strains, but very high MICs for a few strains
222 (Figure 2, Table 5). MICs for terbinafine and amphotericin B showed a wide
223 range; some strains showed low MICs while others had very high MIC values.
224 All strains had very high MIC values for the echinocandins anidulafungin,
225 caspofungin and micafungin as well as griseofulvin, suggesting an intrinsic
226 resistance of *Malassezia* spp. to these antifungals (Figure 2). Therefore, MIC
227 values for these antifungals were not shown in tables 3 and 4. Two species,
228 the anthropophilic *M. obtusa* and the zoophilic *M. caprae*, appeared resistant
229 to most antifungals (Figure 2, Table 5). Terbinafine was the only antifungal
230 effective against *M. caprae*.

231

232 **DISCUSSION**

233 RPMI 1640 media containing 0.165M MOPS buffer is the basis for the broth
234 microdilution methods recommended by CLSI and EUCAST(6, 7). This broth
235 is not optimal for the growth of fastidious fungi such as *Malassezia* spp.,
236 which require lipid supplementation. By adding multiple components including
237 lipids like oils and tweens, and esculin to our broth medium, we facilitated
238 growth of *Malassezia* spp. We have named this new broth medium OptiMAL.
239 OptiMAL may also be suitable for other yeast species such as *Candida*, that
240 utilize additives such as Tweens (16).

241 Resazurin (also known as Alamar Blue®) is used as an indicator in the
242 colorimetric YeastOne Sensititre® assay (Thermoscientific, Zug, Switzerland)
243 for routine antifungal susceptibility testing of yeasts and molds such as
244 *Candida* spp. and *Aspergillus* spp. (17,18). It changes from blue to pink as a
245 result of growth (19) and allows for the visual and spectrophotometric
246 determination of MIC values. Until now, it has not been used in *Malassezia*
247 spp. broth media because it is not always color stable during prolonged
248 incubation and therefore needs to be buffered (19, 20). The careful buffering
249 of OptiMAL with sodium bicarbonate and esculin allowed for the concurrent
250 use of resazurin as a color indicator. The use of esculin in OptiMAL improved
251 growth of all *Malassezia* species by up to 4 fold (Figure S2) and was a key
252 ingredient to improve the accuracy and reproducibility of our assay. Studies
253 on fungal growth usually omit sodium bicarbonate as it causes growth
254 inhibition of many fungal species (21). We observed that *Malassezia* spp.
255 grew well in OptiMAL containing 2 mg/ml sodium bicarbonate. The
256 bicarbonate served as a complementary buffer to MOPS, which boosted the

257 intensity of resazurin color change and enabled higher sensitivity of
258 fluorescence detection.

259 The lack of standardized guidelines for drug susceptibility testing in
260 *Malassezia* spp. has led some studies (8,9) to use small inocula in
261 accordance with the guidelines specified by the CLSI-M27-A3 document for
262 yeasts (11). This is because overtly high inocula are associated with
263 inaccurate MIC values (22). However, the EUCAST guidelines for yeasts use
264 a 100-fold higher inoculum than CLSI. To test the relevance of these
265 differences between the CLSI and the EUCAST guidelines for *Malassezia*
266 spp., we performed inoculum titration studies. We decided on 5.0×10^3 -
267 5.0×10^4 CFU/ml as the optimal inoculum for *Malassezia* spp. broth
268 microdilution assays because this gave reproducible MIC values within two
269 dilution steps for repeats of the same *Malassezia* strain. Ketoconazole was
270 particularly sensitive to variations in inoculum and incubation time.

271 Visual readings for changes in color and turbidity are the most common form
272 of MIC determination. Quantitative readings can be performed by measuring
273 absorbance or fluorescence, with the latter being more sensitive (23). In
274 general, visual determination of color changes may be preferable over
275 absorbance or fluorescence reading in laboratory routine, because it does not
276 require additional technical equipment, is faster and therefore cheaper (13).
277 Few studies have investigated the agreement of MIC values determined by
278 visual and fluorescence readings. For example, a comparison of MIC values
279 of azoles for 88 *A. fumigatus* isolates determined by absorbance and visual
280 turbidity reading were in good agreement (24). However, neither absorbance
281 nor turbidity reading were an option in our assay, because (i) some very slow

282 growing species such as *M. furfur* would have required much higher inocula
283 than recommended to visualize turbidity; (ii) the clumpiness of some species
284 such as *M. globosa* and *M. restricta* hampered reliable accurate absorbance
285 or turbidity reading; (iii) our assay is based on resazurin, but turbidity reading
286 is not recommended when resazurin is used (25). We therefore decided to
287 use visual color change and fluorescence to determine MIC values. Overall,
288 our MIC values determined by visual reading and fluorescence were in high
289 agreement. Slightly higher MIC values were obtained when read by
290 fluorescence, presumably owing to the higher sensitivity of fluorescence
291 reading. These differences were generally within one to two dilution steps,
292 which is acceptable by CLSI and EUCAST standards. Our findings
293 substantiate the concordance of visual and fluorescence reading for MIC
294 values in resazurin-based assays.

295 Despite using an optimized growth medium and standardized inoculum,
296 repeated testing (at least duplicates) showed that the azoles and to a lesser
297 extent terbinafine might be prone to considerable test-to-test variation. In
298 particular, ketoconazole testing required more repeats. This may be attributed
299 to (i) the antagonization of medium components (10), and/or (ii) the
300 susceptibility of ketoconazole to variations in inoculum and incubation time as
301 described above.

302 We have applied this optimized broth microdilution method to test the
303 antifungal susceptibility of 52 *Malassezia* strains against 11 antifungals. Our
304 assay included the echinocandin class as well as griseofulvin. The data
305 showed that *Malassezia* is resistant to these antifungals what is in line with
306 the literature (3,26). The azoles itraconazole, posaconazole and voriconazole

307 appeared as the most effective antifungals against *Malassezia* spp. High MIC
308 values for these azoles were rare, which is in line with previous studies
309 (10,27). Most of our *Malassezia* strains were susceptible to concentrations of
310 fluconazole and ketoconazole as previously described (7-9, 28). However,
311 some strains were resistant to ketoconazole. This could be of clinical
312 significance, because ketoconazole is the first-line topical antifungal in the
313 treatment of *Malassezia*-associated skin diseases such as pityriasis versicolor
314 or seborrhoeic dermatitis (29). Terbinafine, a widely used allylamine, is active
315 against dermatophytes and molds and can be applied topically or systemically
316 (30). It is not recommended as first line treatment of *Malassezia* -associated
317 diseases (29). The relatively low MIC values of terbinafine against many of
318 our *Malassezia* strains suggest that terbinafine may be a treatment alternative
319 for *Malassezia* infections that do not respond to azoles.

320 In summary, we have developed an optimized broth microdilution assay
321 compatible with the colorimetric indicator resazurin for the fast and efficient
322 profiling of antifungal susceptibility in *Malassezia* spp. Determination of MIC
323 values by visual reading of color change versus fluorescence reading is
324 comparably reliable. Among the 52 *Malassezia* strains tested, azoles such as
325 voriconazole, itraconazole and posaconazole were the most effective
326 antifungals. Terbinafine might be a treatment alternative for *Malassezia*
327 infections.

328

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332

333 The authors declare no conflicts of interest.

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- 428

429 **TABLE 1. Synopsis of studies investigating broth microdilution assays for the antifungal susceptibility testing of *Malassezia* spp.**

Reference	Species (strains)	Antifungals	Broth medium composition	Inocula (CFU/ml)	Incubation (days)	Incubation temperature (°C)	Reading method
Our study	52 strains <i>M. sympodialis</i> <i>M. slooffiae</i> <i>M. furfur</i> <i>M. globosa</i> <i>M. restricta</i> <i>M. obtusa</i> <i>M. dermatis</i> <i>M. japonica</i> <i>M. yamatoensis</i> <i>M. pachydermatis</i> <i>M. nana</i> <i>M. caprae</i> <i>M. cuniculi</i>	Amphotericin B Terbinafine Ketoconazole Fluconazole Posaconazole Itraconazole Voriconazole Caspofungin Micafungin Anidulafungin Griseofulvin	RPMI 1640 medium buffered with 0.165M MOPS 0.2% sodium bicarbonate 0.5% glycerol 0.5% Tween 60 2% oleic acid 1% esculin	5.0×10^3 - 5.0×10^4	2 or 3	35	Visual (color) & Fluorescence
Rojas 2014 (7)	<i>M. furfur</i> (39) <i>M. sympodialis</i> (20) <i>M. globosa</i> (14)	Fluconazole Ketoconazole Voriconazole Itraconazole Amphotericin B Miconazole	RPMI 1640 1.8% glucose, 1% peptone, 0.5% ox bile, 0.5% malt extract, 1% glycerol, 0.5% Tween 40, 0.05% Tween 80	$0.5-2.5 \times 10^3$	3 or 4	32	Turbidity
Velegraki 2004 (8)	53 strains <i>M. furfur</i> <i>M. pachydermatis</i> <i>M. sympodialis</i> <i>M. slooffiae</i> <i>M. globosa</i> <i>M. restricta</i> <i>M. dermatis</i>	Amphotericin B Itraconazole Fluconazole Voriconazole Ketoconazole Terbinafine Posaconazole	RPMI 1640 20 g glucose, 4 g ox bile 1 ml glycerol, 0.5 g glycerol monostearate 0.4 ml Tween 20	$2.0-3.5 \times 10^4$ $3.0-4.0 \times 10^3$	<i>M. furfur</i> (2) <i>M. pachydermatis</i> (2) <i>M. sympodialis</i> (3) <i>M. slooffiae</i> (3) <i>M. globosa</i> (3) <i>M. restricta</i> (3) <i>M. dermatis</i> (3)	32	Turbidity
Miranda 2007 (9)	<i>M. furfur</i> (74) <i>M. sympodialis</i> (11) <i>M. obtusa</i> (8) <i>M. globosa</i> (2)	Fluconazole Ketoconazole Itraconazole Voriconazole	Modified Leeming–Notman medium containing 0.1% glucose, 0.1% peptone, 0.8% bile salts, 0.2% yeast extract, 0.1% glycerol, 0.5% Tween 60, 3% olive oil	$2.5 \pm 1.0 \times 10^3$	<i>M. furfur</i> (3) <i>M. sympodialis</i> (3) <i>M. globosa</i> (5) <i>M. obtusa</i> (5)	32	Turbidity
Gupta 2000 ^a (10)	55 strains <i>M. furfur</i> <i>M. sympodialis</i> <i>M. slooffiae</i> <i>M. pachydermatis</i> <i>M. globosa</i> <i>M. obtusa</i> <i>M. restricta</i>	Ketoconazole Voriconazole Itraconazole Terbinafine	Diagnostic Sensitivity Testing agar and glycerol	1.0×10^4	7	35	Colony growth on agar plates

430 ^a This assay was performed using agar based medium

431 **TABLE 2. *Malassezia* species and proposed inocula for antifungal susceptibility**
 432 **testing**
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#	Species	Host	Number of strains tested	Reference strain	OD ₆₀₀ for inoculum ^a
1	<i>M. sympodialis</i>	Human	24	CBS 7222	0.1
2	<i>M. slooffiae</i>	Human	10	CBS 7956	0.05
3	<i>M. furfur</i>	Human	5	CBS 1878	0.005
4	<i>M. globosa</i>	Human	3	CBS 7966	0.2-1
5	<i>M. restricta</i>	Human	2	CBS 7877	0.2-1
6	<i>M. obtusa</i>	Human	2	CBS 7876	0.2-1
7	<i>M. dermatis</i>	Human	1	CBS 9169	0.1
8	<i>M. japonica</i>	Human	1	CBS 9432	0.2
9	<i>M. yamatoensis</i>	Human	1	CBS 9725	0.02
10	<i>M. nana</i>	Animal	1	CBS 9557	0.1
11	<i>M. pachydermatis</i>	Animal	1	CBS 1879	0.01
12	<i>M. cuniculi</i>	Animal	1	CBS 11721	0.1
13	<i>M. caprae</i>	Animal	1	CBS 10434	0.1

434 ^aOD₆₀₀ values for broth microdilution testing as determined by plating 10 µl of a 1:10
 435 dilution to achieve inocula of 10⁴-10⁵ CFU/ml. For the susceptibility assay, 50 µl of
 436 inoculum was added to 50 µl of 2X concentration of antifungals to achieve a final cell
 437 density of 5.0x10³-5.0x10⁴ CFU/ml.
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439

440 **TABLE 3. Broth media for *Malassezia* spp.**

Component (% of volume)	RPMI+ ^a	RPMI ++	RPMI++/esculin (OptiMAL)
MOPS	0.165M	0.165M	0.165M
Sodium bicarbonate	0.2	0.2	0.2
Glucose	2	2	2
Glycerol	0.1	1	0.5
Glycerol monostearate	0.05	-	-
Tween 20	0.04	-	-
Tween 60	-	0.5	0.5
Olive oil	-	-	-
Oleic acid	-	2	2
Esculin	-	-	0.1
Resazurin	12.5 µg/ml	12.5 µg/ml	12.5 µg/ml
pH	6.32	6.25	6.26

441 ^a Adapted from Velegriaki et al. 2004(8).

442 **TABLE 4. Agreement of MIC values between visual and fluorescence reading**

Agreement level	Agreement in percentage						
	Amphotericin B	Terbinafine	Ketoconazole	Fluconazole	Itraconazole	Posaconazole	Voriconazole
MIC identical	75	73	63	67	67	73	75
MIC within 1 dilution	96	94	84	88	88	94	96
MIC within 2 dilutions	98	96	88	96	98	100	98

443

444

TABLE 5. MIC ranges and mean MIC of antifungals as determined by visual reading

Species (no. of strains)	MIC range (Mean)						
	Amphotericin B	Terbinafine	Ketoconazole	Fluconazole	Itraconazole	Posaconazole	Voriconazole
<i>M. sympodialis</i> (24)	0.125-4 (1.27)	≤0.125-16 (1.15)	≤0.03->4 (1.03)	0.25->128 (7.09)	≤0.015->8 (0.37)	≤0.015->8 (0.36)	≤0.003->8 (0.38)
<i>M. slooffiae</i> (10)	0.5->8 (2.85)	0.25-4 (1.48)	≤0.03-0.125 (0.07)	0.5-4 (1.95)	≤0.015-0.06 (0.04)	≤0.015-0.125 (0.03)	≤0.003-0.03 (0.02)
<i>M. furfur</i> (5)	0.125-4 (1.18)	2->16 (9.2)	≤0.03-2 (0.52)	4->128 (42.4)	0.06-1 (0.37)	0.03- 0.5 (0.24)	0.03-2 (0.51)
<i>M. globosa</i> (3)	0.25-1	4	>4	4	≤0.015->8	≤0.015-0.03	≤0.003-0.03
<i>M. restricta</i> (2)	≤0.06->0.125	2->16	≤0.03-0.06	1-4	0.03	≤0.015-0.125	≤0.003-0.015
<i>M. obtusa</i> (2)	1->8	>16	>4	>128	>8	>8	>8
<i>M. dermatitis</i> (1) ^a	0.5	0.25	≤0.03	0.5	0.03	≤0.015	≤0.003
<i>M. japonica</i> (1) ^a	0.125	≤0.125	>4	2	≤0.015	≤0.015	0.015
<i>M. yamatoensis</i> (1) ^a	0.5	≤0.125	0.06	4	0.06	0.03	0.03
<i>M. pachydermatis</i> (1) ^a	≤0.06	2	0.06	32	0.03	0.03	0.06
<i>M. caprae</i> (1) ^a	2	0.25	>4	>128	>8	>8	>8
<i>M. nana</i> (1) ^a	0.125	1	0.06	4	0.06	1	0.03
<i>M. cuniculi</i> (1) ^a	≤0.06	1	≤0.03	4	≤0.015	≤0.015	0.03

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^a No range, only the reference strain was tested for these spe

446 **Figure Legends:**

447 **FIG 1** (A) MIC values of antifungals (except griseofulvin and echinocandins) for 52
448 *Malassezia* strains determined by visual reading (x-axis) versus fluorescence reading (y-
449 axis). Black circles represent points at which both data readings are at 100% agreement.
450 The number of MIC values per data point in the graph is indicated by the color bar.
451 Agreement between visual and fluorescence reading was determined by Pearson correlation.
452 (B) Agreement between MIC values determined by visual reading and fluorescence reading
453 on a representative antifungal resistance assay plate testing *M. sympodialis*. Each row
454 represents a particular antifungal, with increasing concentrations from left to right. (+)
455 indicates the positive control without antifungal, (-) indicates the negative control with broth
456 medium without *M. sympodialis*. Pink wells indicate growth of *M. sympodialis*, blue wells
457 indicate no growth. The lower graph represents the fluorescence reading of the color change.
458

459 **FIG 2** MIC values of 11 antifungals for 52 *Malassezia* strains as determined by visual
460 reading. MIC values were normalized from 0 to 1, with 1 being the highest antifungal
461 concentration tested. The first row of each *Malassezia* species represents the susceptibility
462 profile of the reference strain. Grey plots indicate inconclusive MIC values.

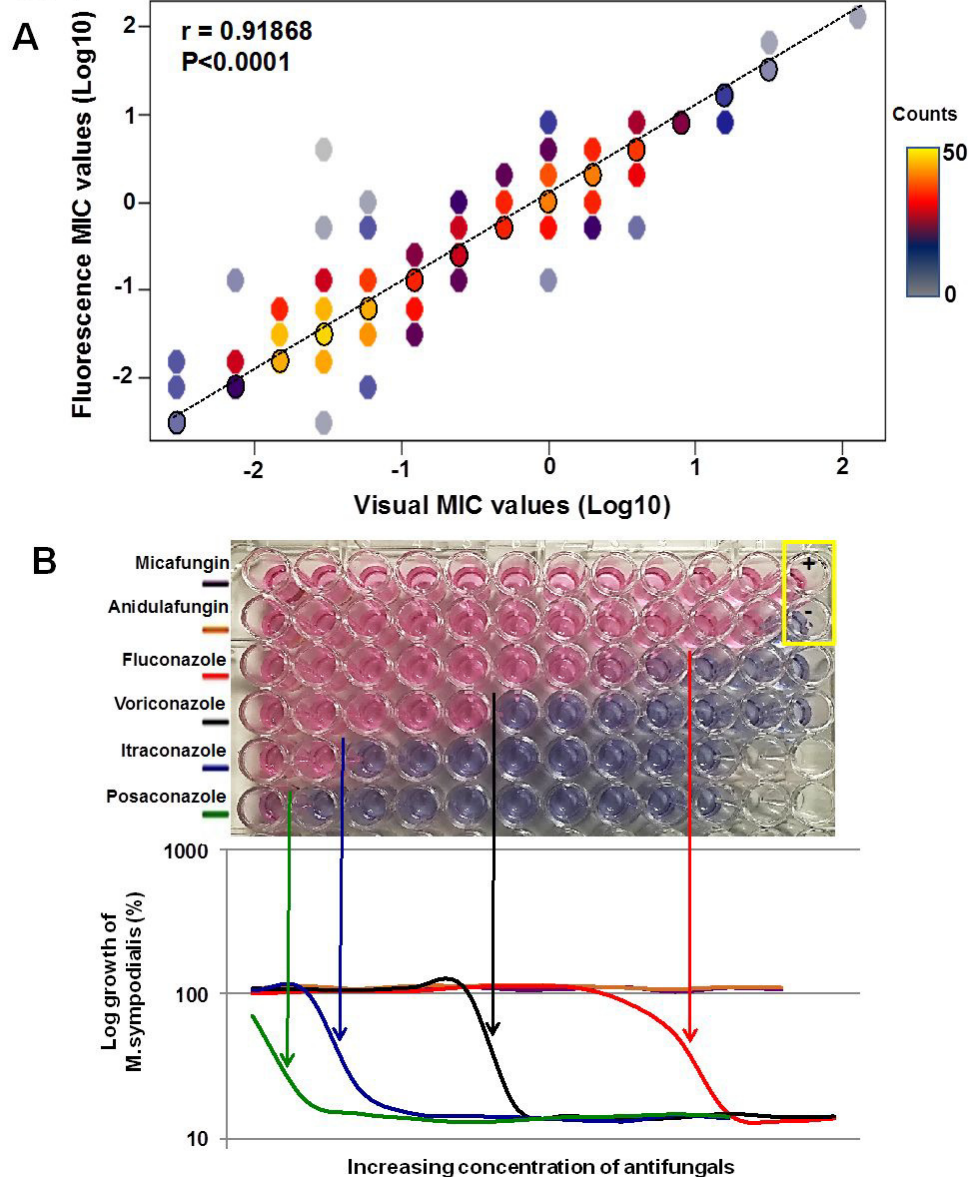
FIG 1

FIG 1 (A) MIC values of antifungals (except griseofulvin and echinocandins) for 52 *Malassezia* strains determined by visual reading (x-axis) versus fluorescence reading (y-axis). Black circles represent points at which both data readings are at 100% agreement. The number of MIC values per data point in the graph is indicated by the color bar. Agreement between visual and fluorescence reading was determined by Pearson correlation. (B) Agreement between MIC values determined by visual reading and fluorescence reading on a representative antifungal resistance assay plate testing *M. sympodialis*. Each row on the plate represents a particular antifungal, with increasing concentrations from left to right. (+) indicates the positive control without antifungal, (-) indicates the negative control with broth medium without *M. sympodialis*. Pink wells indicate growth of *M. sympodialis*, blue wells indicate no growth. The lower graph represents the fluorescence reading of the color change.

FIG 2

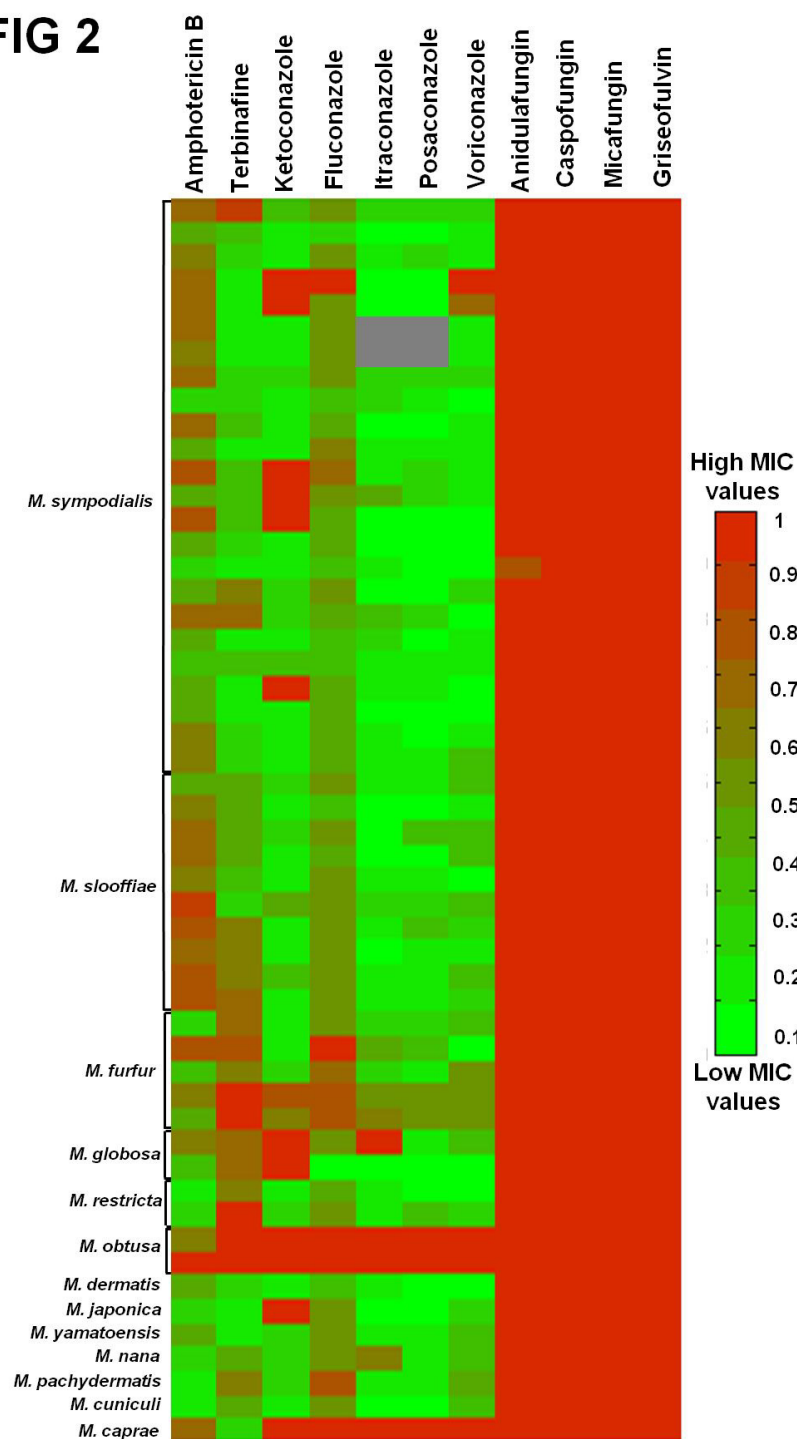


FIG 2 MIC values of 11 antifungals for 52 *Malassezia* strains as determined by visual reading. MIC values were normalized from 0 to 1, with 1 being the highest antifungal concentration tested. The first row of each *Malassezia* species represents the susceptibility profile of the reference strain. Grey plots indicate inconclusive MIC values.