SUPPLEMENT TO

Evolution of cross-resistance to medical triazoles in *Aspergillus fumigatus* through selection pressure of environmental fungicides

Jianhua Zhang, Joost van den Heuvel, Alfons J. M. Debets, Paul E. Verweij, Willem J. G. Melchers, Bas J Zwaan, Sijmen E. Schoustra

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Materials and Methods

Fungal isolates, medical triazoles and SI fungicides

All evolved fungal cultures used in this study are from a previous study that focused on the effect of variation in fungal reproduction on adaptation (the development of resistance to SI fungicides) [1]. In that study, we evolved ancestor *A. fumigatus* CBS 140053 in the laboratory over seven weeks. Six replicate lineages evolved in the presence of one of each of five SI fungicides and six lineages evolved without any SI fungicide as a reference (called control lineages). For one of the reproductive conditions, we weekly transferred a sample of asexual spores to fresh medium. The stored samples for each lineage at each of the seven time points are used in the present study. Thus, the total number of samples was 253 ((5 SI fungicides +control =6)*6 replicates* 7 time point + one ancestor = 253).

The five SI fungicides used for the selection were bromuconazole, tebuconazole, epoxiconazole, difenoconazole, and propiconazole with the concentration of 1mg/L. In the present study we used the medical triazoles itraconazole (ITR), posaconazole (POS), and voriconazole (VOR). All SI fungicides and medical triazoles were purchased from Sigma Company.

Susceptibility testing of evolved cultures to the medical triazoles

We assayed the level of resistance of all lineages that had evolved in the presence (or absence) of one of five different SI fungicides over seven weeks against the three medical triazoles. We performed resistance test following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method [2]. Briefly, resistance level was defined as the minimal inhibitory concentration (MIC) that results in 100%

growth inhibition of fungi. The relative MIC was defined as the individuals' MIC divided by the MIC of the triazole sensitive ancestor *A. fumigatus* CBS 140053. RPMI-1640- 2% G medium (Sigma) was supplemented with each of the medical triazoles in concentrations ranging from 0.016 to 16 mg/L in two-fold concentration steps and loaded into a 96-well plate. Spore suspensions used to initiate the assay were prepared from fungal colonies grown on solid medium using saline-Tween (0,8% HCl with 0.05% Tween 80 – Sigma- in water). We estimated the density of spore suspensions on a Coulter counter to standardize the concentration of spores to 1-2.5×10^5 CFU/mL prior to the MIC assay and then inoculated the standardized spore suspension into flat-bottomed 96-well microtiter plates containing a series with increasing amounts of fungicide and incubated at 37°C for 48 h. We performed readings from the bottom of the microtiter plates using a mirror and determined at which concentration we observed 100% inhibition [3]. There were six replicates for each assay. If at a concentration of 16 mg/L no inhibition was observed, we (arbitrarily) classified the MIC as 32 mg/L. We measured the MIC values for each time point for each lineage, allowing us to generate an evolutionary trajectory of relative MICs. In these trajectories, stepwise increases in MIC are indicative of the fixation of a mutation conferring and/or increasing resistance [4].

Population dynamics in the evolutionary lineages over time

We selected three lineages from the difenoconazole evolution treatment that showed the highest resistance increase (lineages D1, D3, and D6) for a more in-depth study of the evolutionary dynamics focusing on morphological changes of the fungal colonies. Around 100 spores from samples from different time points of each D1, D3, and D6 lineage were spread onto MEA (Malt Extract Agar) plates with 1mg/L of difenoconazole. The morphology of resulting fungal colonies was analyzed using the Tsview 7 program

(Fuzhou, Tucsen image Technology Co, Ltd, version 7) and the frequency of each type was recorded after two days of growth. Various different morphologies were observed, each of which was inoculated into a bottle with 1 ml/L difenoconazole, incubated at 37°C for a week to assess total spore production. To measure spore production, we harvested spores from each bottle by adding two mL of saline-Tween and beads into the bottle, which was then vortexed for five minutes. The spore production was assayed with a Coulter counter [5] after filtering out large pieces of hyphae using glass wool funnel filters. Further, the mycelial growth rate (MGR) of each morphological type was measured on MEA plates supplemented with 1 ml/L difenoconazole by measuring the radius of fungal coverage after 4 days of growth. To have an rough idea on the number of mutations present in the different morphotypes, we performed crosses of several evolved morphotypes with the ancestor and scored the number of segregating loci among the progeny by counting the number of distinct morphological classes. These crosses were done with an ancestral strain of the opposing mating type that we generated from other crosses [1].

Statistical analyses

All statistical were performed in the IBM SPSS statistics version 22. To explain the observed variation in relative MICs of the evolved strains and controls against different SI fungicides and three medical triazoles, we first check whether our MIC data is normal distribution (electronic supplementary material Table 1A) and then we performed non-parametric statistical tests for the relative MIC data with type of SI fungicide and resistance to three medical triazoles (R) as fixed factors (model: Relative MIC ~ SI fungicides * R). Furthermore, because the effect of relative MIC depended on the SI

fungicide used for the selection and medical azole for testing (see results), we performed non-parametric—Kruskal-Wall 1 –way ANOVA-multiple comparison-tests to check whether the achieved level of resistance depended on the SI fungicide used for selection (model for each test SI fungicide separately: Relative MIC~ SI fungicide) and which medical triazoles were used to test (relative MIC~ medical triazoles test).

The analysis of the relative MIC are presented as mean values ± standard error of the mean (SEM). P-values of $\leqslant 0.05$ (*) were defined as significant. To test for a correlated response between an increase in resistance to the SI fungicide used for selection and cross-resistance to the medical triazoles, we constructed fitness trajectories in all four environments. From these trajectories, we recorded the timing of an increase in resistance (MIC) and we used linear regression asking whether there is a correlation in the timing of an increase in resistance to the SI fungicide and cross-resistance to the medical triazole.

Related references

1. Zhang J., Debets A.J., Verweij P.E., Melchers W.J., Zwaan B.J., Schoustra S.E. 2015 Asexual sporulation facilitates adaptation: The emergence of azole resistance in Aspergillus fumigatus. *Evolution* **69**(10), 2573-2586.
2. Kahlmeter G., Brown D., Goldstein F., MacGowan A., Mouton J., Odenholt I., Rodloff A., Soussy C.J., Steinbakk M., Soriano F. 2006 European Committee on Antimicrobial Susceptibility Testing (EUCAST) technical notes on antimicrobial susceptibility testing. *Clinical Microbiology and Infection* **12**(6), 501-503.
3. Camps S.M., Rijs A.J., Klaassen C.H., Meis J.F., O'Gorman C.M., Dyer P.S., Melchers W.J., Verweij P.E. 2012 Molecular Epidemiology of *Aspergillus fumigatus* Isolates Harboring the TR34/L98H Azole Resistance Mechanism. *Journal of clinical microbiology* **50**(8), 2674-2680. (doi:10.1128/JCM.00335-12).
4. Lenski R.E., Travisano M. 1994 Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proceedings of the National Academy of Sciences* **91**(15), 6808-6814.
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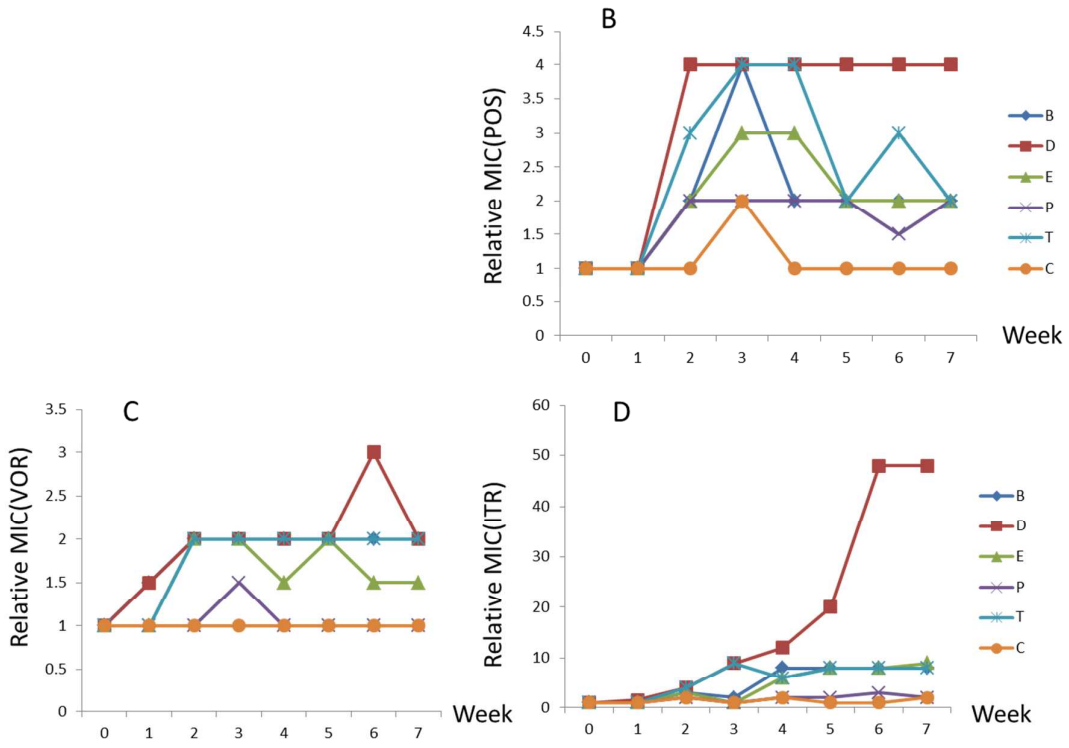
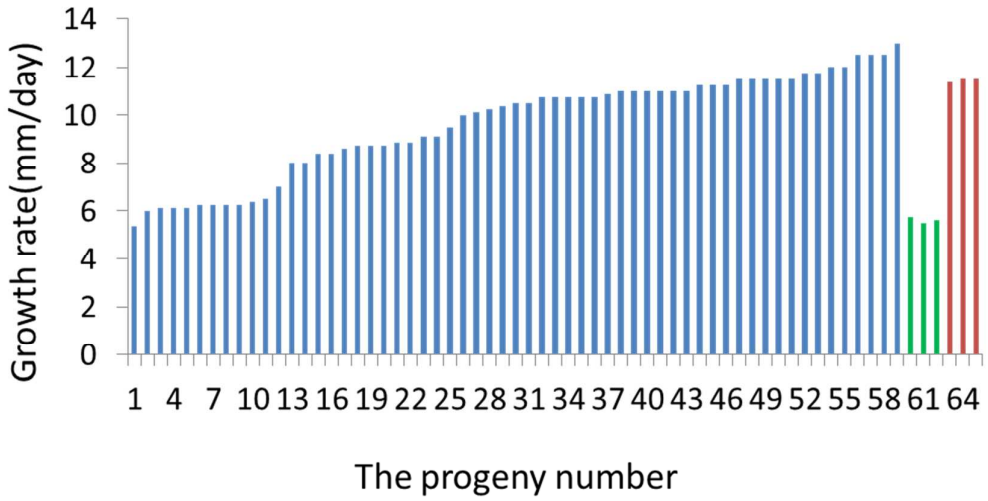
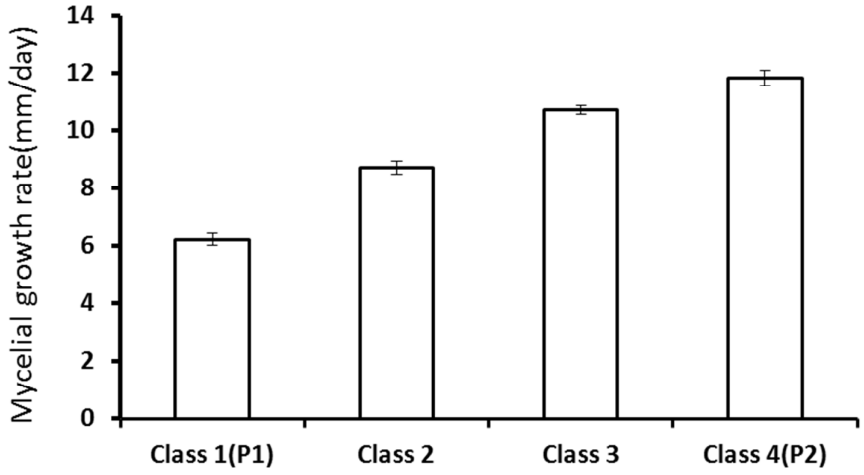
Supplementary Figure 1



Evolution of cross-resistance, median values. Each line shows the median of six replicate lineages that had evolved in the presence (or absence) of one of five SI fungicides over seven weekly transfers. Resistance is given by the relative MIC-value of an evolved strain relative to the ancestor. B = bromuconazole, T= tebuconazole, E = epoxiconazole, D = difenoconazole, P=propiconazole, C= control, no SI fungicide present. Panel B: Cross-resistance of evolved lineages against the medical triazole POS. Panel C: Cross-resistance against the medical triazole VOR. Panel D: Cross-resistance against the medical triazole ITR.

Supplementary Figure 2

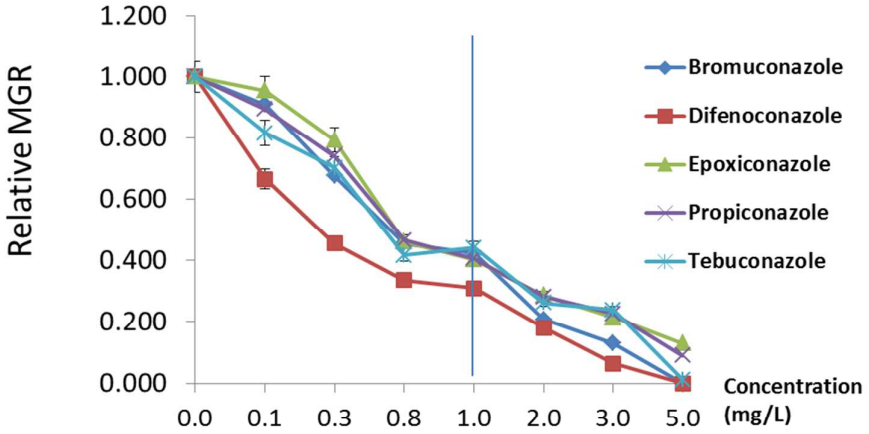




Segregation of MGR among the progeny from a sexual cross between the ancestral genotype (parental type 1; P1) and an evolved strain (D1large, parental type 2; P2) with high MIC and fitness. MGR of progenies was measured on the MEA containing 1 mg /L difenoconazole. The *top panel* shows all progeny separately and the two parental types for the cross (1-59 are progeny number; 60-62: parental type 1, ancestor of the evolution experiment (green); 63-65: parental type 2, evolved strainD1large (red). The

bottom panel shows how the progeny clusters in distinct phenotypic classes (evenly classify the group until ANOVA and following post-hoc test shows significant: using repeated ANOVAs increasing the number of distinct categories/classes in the data. The lowest number of categories giving a statistical significant effect of making categories was taken as the number of distinct classes among the progeny). Among the progeny we clearly observe segregation in 4 classes, two parental classes and two recombinant classes. This is indicative of two segregation loci, which in turn suggests that two mutations with an effect on MGR had fixed during experimental evolution.

Supplementary Figure 3



Relative MGR of ancestor *A. fumigatus* CBS 140053 on the MEA supplemented with a series of SI fungicides (0-5 mg/L). Dots represent the mean of three replicates of MGR relative to the strain growing on the MEA-free SI fungicide (relative MGR_0 =1.0), each line represents one of SI fungicide. Error bars indicate the standard error of the mean (SEM). The SI-fungicide concentration used for the selection experiment was 1.0 mg/L for all SI-fungicides. With this concentration, difenoconazole leads to a lower MGR than the other five SI-fungicides.

Supplementary Table 1A

P values from pairwise Kruskal-Wallis tests between SI fungicide MIC values at week seven tested on the medical azoles ITR, VOR and POS.

Pairwise comparison	ITR	VOR	POS
dif-bro	0.0209*	0.0917	0.0926
dif-teb	0.0209*	0.3173	0.019
dif-epo	0.1174	0.0433*	0.0748
dif-pro	0.0387*	0.0306*	0.057
dif-con	0.0019**	0.0013**	0.0017**
bro-teb	NA	0.138	0.3173
bro-epo	1	0.5751	0.5982
bro-pro	0.105	0.2012	0.3384
bro-con	9e-04**	0.019*	0.0013**
teb-epo	1	0.0555	1
teb-pro	0.105	0.0325*	0.5271
teb-con	9e-04**	9e-04**	9e-04**
epo-pro	0.3909	0.3865	0.6517
epo-con	0.0019**	0.0555	0.0062*
pro-con	0.0069*	0.3173	0.0209*

dif = difenoconazole, bro = bromuconazole, teb = Tebuconazole, epo = Epoxiconazole, pro = Propiconazole, con = Control: no SI-fungicides

*indicates significance at the level of $\alpha < 0.05$, while **indicates significance at this level corrected for multiple testing, i.e. 0.05/15 (since we perform 15 pairwise tests per medical azole).

Supplementary Table 1B

All (log2) MIC values (mean and median, 2nd and 3rd column resp.) in week seven of the six replicates test on the three medical azoles. In columns A and B treatments are indicated that are not significantly different in the pairwise Kruskal-Wallis test as shown in Supplementary Table 1A.

MIC-ITR					
SI fungicides	Log2 MIC values	Mean MIC	Median MIC	A	B
Difenoconazole	6,3,5,3,6,6	4,83	5.5	A	
Bromuconazole	3,3,3,3,3,3	3	3	A	
Tebuconazole	3,3,3,3,3,3	3	3	A	
Epoxiconazole	1,1,4,6,1,4	2.83	2.5	A	
Propiconazole	3,1,6,0,1,1	2	1	A	
Control	0,0,0,0,0,0	0	0		B
MIC-VOR					
SI fungicides	Log2 MIC values	Mean MIC	Median MIC	A	B
Difenoconazole	1,0,0,0,0,0	0.16	0	A	
Bromuconazole	-1,0,0,0,0-1	-0.33	0	A	B
Tebuconazole	0,0,0,0,0,0	0	0	A	
Epoxiconazole	-1,-1,0,0,-1,0	-0.5	-0.5	A	B
Propiconazole	-1,-1,1,-1,-1,-1	-0.67	-1	A	B
Control	-1,-1,-1,-1,-1,-1	-1	-1		B
MIC-POS					
SI fungicides	Log2 MIC values	Mean MIC	Median MIC	A	B
Difenoconazole	2,1,2,1,2,2	1.67	2	A	
Bromuconazole	1,2,1,1,1,1	1.17	1	A	
Tebuconazole	1,1,1,1,1,1	1	1	A	
Epoxiconazole	0,1,1,2,1,1	1	1	A	B
Propiconazole	1,1,2,0,1,0	0.83	1	A	B
Control	0,0,0,0,0,0	0	0		B

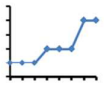
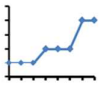
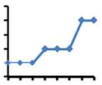
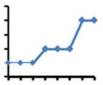
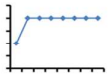
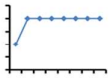
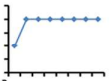
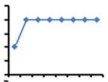
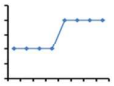
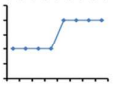
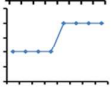
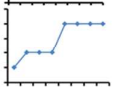
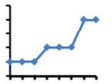
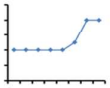
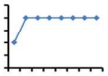
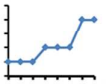
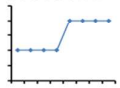
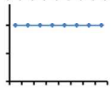
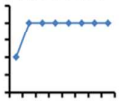
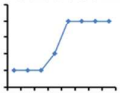
Supplementary Table 2A

The number of individual cultures out of a total of 42 cultures per treatment that evolved cross-resistance to different medical triazoles at a level of clinical resistance (MIC above resistance breakpoint, Figure 1 in main text) after selection on a SI fungicide.

	Number of resistant cultures		
Stress (Triazole fungicide)	POS MIC > 0.25mg/L	VOR MIC > 2mg/L	ITR MIC > 2mg/L
Bromuconazole	0	0	26
Difenoconazole	0	6	25
Epoxiconazole	0	0	17
Propiconazole	0	1	8
Tebuconazole	0	1	21
Total	0	8	97

Supplementary Table 2B

Examples of step-wise trajectory patterns of evolved lineages to one agricultural SI fungicide (SI) and three medical triazoles (POS, VOR, ITR).

Evolutionary trajectory pattern	Pattern of AFCs	Pattern of POS	Pattern of VOR	Pattern of ITR
D1, D3, D6, E4, P3				
D4, B6, T4, E1				
B5				
T1				
P2				

Supplementary Table 2C

Linear regression of analysis of the correlation in timing of MIC increase between the trajectory of (SI) fungicide and MIC trajectory of three medical triazoles (POS, VOR, ITR).

Regression	**D1(POS)**	**D1(VOR)**	**D1(ITR)**
D1(D)	R=0.796, $F_{1,22}$ = 37.983, P <0.01**	R=0.873, $F_{1,22}$ = 70.328, P <0.01**	R=0.876, $F_{1,22}$ = 72.849, P <0.01**
Regression	**D4(POS)**	**D4(VOR)**	**D4(ITR)**
D4(D)	R=0.664, $F_{1,22}$ = 17.320, P <0.01**	R=1.00, $F_{1,22}$ = -,P <0.01**	R=0.646, $F_{1,22}$ = 15.775, P <0.01**
Regression	**D5(POS)**	**D5(VOR)**	**D5(ITR)**
D5(D)	R=0.624, $F_{1,22}$ = 14.007, P <0.01**	R=0.701, $F_{1,22}$ =21.218,P <0.01**	R=0.796, $F_{1,22}$ = 38.060, P <0.01**
Regression	**D6(POS)**	**D6(VOR)**	**D6(ITR)**
D6(D)	R=0.864, $F_{1,22}$ = 64.809, P <0.01**	R=0.805, $F_{1,22}$ =40.525,P <0.01**	R=0.836, $F_{1,22}$ = 51.118, P <0.01**
Regression	**T1(POS)**	**T1(VOR)**	**T1(ITR)**
T1(T)	R=0.525, $F_{1,22}$ = 8.361, P <0.01**	R=0.659, $F_{1,22}$ =16.844 P <0.01**	R=0.650, $F_{1,22}$ = 16.058, P <0.01**
Regression	**T4(POS)**	**T4(VOR)**	**T4(ITR)**
T4(T)	R=0.974, $F_{1,22}$ = 412.5, P <0.01**	R=0.808, $F_{1,22}$ =41.365 P <0.01**	R=0.934, $F_{1,22}$ = 149.904, P <0.01**
Regression	**B5(POS)**	**B5(VOR)**	**B5(ITR)**
B5(B)	R=0.444, $F_{1,22}$ = 5.392, P <0.05*	R=1.00, $F_{1,22}$ =-, P <0.01**	R=0.965, $F_{1,22}$ = 297, P <0.01**
Regression	**B6(POS)**	**B6(VOR)**	**B6(ITR)**
B6(B)	R=1.00, $F_{1,22}$ = -, P <0.01**	R=0.378, $F_{1,22}$ =3.667, P =0.069	R=0.608, $F_{1,22}$ = 12.891, P <0.01**
Regression	**E1(POS)**	**E1(VOR)**	**E1(ITR)**
E1(E)	R=0.618, $F_{1,22}$ = 13.606, P <0.01**	R=0.714, $F_{1,22}$ =22.846, P <0.01**	R=0.799, $F_{1,22}$ = 38.717, P <0.01**
Regression	**E4(POS)**	**E4(VOR)**	**E4(ITR)**
E4(E)	R=0.836, $F_{1,22}$ = 50.869, P <0.01**	R=0.832, $F_{1,22}$ =49.417, P <0.01**	R=0.863, $F_{1,22}$ = 64.095, P <0.01**
Regression	**P3(POS)**	**P3(VOR)**	**P3(ITR)**
P3(P)	R=0.825, $F_{1,22}$ = 47.028, P<0.01**	R=0.920, $F_{1,22}$ = 121.352, P<0.01**	R=0.74, $F_{1,22}$ = 26.666, P<0.01**
Regression	**P2(POS)**	**P2(VOR)**	**P2(ITR)**
P2(P)	R=0.079, $F_{1,22}$ =0.138, P=0.714	--	R=0.176 $F_{1,22}$ = 0.7, P=0.412

Supplementary Table 3A

One–way ANOVA for variation in mycelial growth rate among evolved stains and their ancestor, followed by Fishers least significant difference post-hoc test (LSD) to test for a specific difference between the evolved isolates and the ancestor.

Evolved types	**One-way ANOVA followed by post-hoc LSD test to test for differences in mycelial growth rate between evolved type and ancestor**
D1 lineage	
D1-middle	$F_{2,6}$= 1463.769, P <0.01, post-hoc LSD test, $P_{ancestor\text{-}middle}$ <0.01**
D1-large	$F_{2,6}$= 1463.769, P <0.01, post-hoc LSD test, $P_{ancestor\text{-}large}$ <0.01**
D3 lineage	
D3-dense	$F_{2,6}$= 10299, P<0.01, post-hoc LSD test, $P_{ancestor\ –dense}$ <0.01**
D3-fluffy	$F_{2,6}$= 10299, P<0.01, post-hoc LSD test, $P_{ancestor\ -fluffy}$ <0.01**
D6 lineage	
D6-large	$F_{3,8}$= 1771.202, P<0.01, post-hoc LSD test, $P_{ancestor\text{-}large}$ <0.01**
D6-fluffy	$F_{3,8}$= 1771.202, P<0.01, post-hoc LSD test, $P_{ancestor\text{-}fluffy}$ <0.01**
D6-white	$F_{3,8}$= 1771.202, P<0.01, post-hoc LSD test, $P_{ancestor\text{-}white}$ =0.103

Supplementary Table 3B

One–way ANOVA for variation in spore production among evolved stains and their ancestor, followed by Fishers least significant difference post-hoc test (LSD) to test for a specific difference between the evolved isolates and the ancestor.

Evolved types	**One-way ANOVA followed by post-hoc LSD test to test for differences in spore production between evolved type and ancestor**
D1 lineage	
D1-middle	$F_{2,6}$= 69.095, P <0.01, post-hoc LSD test, $P_{ancestor-middle}$ = 0.891
D1-large	$F_{2,6}$= 69.095, P <0.01, post-hoc LSD test, $P_{ancestor-large}$ <0.01**
D3 lineage	
D3-dense	$F_{2,6}$= 586.204, P <0.01, post-hoc LSD test, $P_{ancestor-dense}$ <0.01**
D3-fluffy	$F_{2,6}$= 586.204, P <0.01, post-hoc LSD test, $P_{ancestor-fluffy}$ <0.05*
D6 lineage	
D6-large	$F_{3,8}$= 771.498, P<0.01, post-hoc LSD test, $P_{ancestor-large}$ <0.05*
D6-fluffy	$F_{3,8}$= 771.498, P<0.01, post-hoc LSD test, $P_{ancestor-fluffy}$ <0.01**
D6-white	$F_{3,8}$= 771.498, P<0.01, post-hoc LSD test, $P_{ancestor-white}$ <0.01**

Supplementary Table 4A

Descriptive statistics of the genomics. From left to right are listed, the number of raw reads, the number of filtered and aligned reads, and the average coverage.

Morphotype	Number of raw reads	Number of aligned and filtered reads	Average coverage
Anc	13,311,662	10,985,616	46.85
Con 2	13,306,314	10,816,857	46.10
Con 4	13,313,044	10,854,658	46.27
Con 5	13,313,956	10,854,183	46.29
D1-large	13,315,436	10,906,594	46.48
D3-dense	13,314,526	10,845,651	46.22
D3-fluffy	13,315,838	10,830,523	46.14
D6-fluffy	13,273,570	10,378,705	44.26
D6-white	13,316,674	10,767,166	45.86

Supplementary Table 4B

SNP-calling between the ancestor and evolved isolates with different morphotypes. From left to right columns indicate, the position on reference genome (clinical strain AF293, chromosome number / nucleotide position), the gene as annotated for reference genome, the snp information (change in nucleotide, position in amino acid sequence and amino acid change, if applicable), and information per strain (nucleotide / amino acid and coverage, ancestor gives the reference state, difference compared to reference strain are indicated, else a "." is given). For positions that are variable both nucleotides, amino acids, and coverages are given. For variable sites pariwise F_{ST} values are given between brackets. For completely divergent loci these are 1.

Ref chromosome (Clinical reference strainAF293)	**Gene**	**Snp**	**anc**	**Con2**	**Con4**	**Con5**	**D1-large**	**D3-dense**	**D3-fluffy**	**D6-fluffy**	**D6-white**
4 / 1784849	cytochrome P450 14-alpha sterol demethylase [Aspergillus fumigatus] Sequence ID: gb|AKE50937.1|	C -> T 138 G->S	C G 54	. . 41	. . 44	. . 60	T S 53	. . 45	. . 42	. . 59	. . 47
2 / 986005	HMG-CoA reductase [Aspergillus fumigatus Z5] Sequence ID: gb|KMK57733.1|	C -> T 320 P -> L	C P 40	. . 39	. . 54	T L 52	T L 48	T L 40	. . 38	. . 34	. . 48
2 / 3435913	Putative amidohydrolase ytcJ [Aspergillus fumigatus Z5] Sequence ID: gb|KMK61702.1|Or conserved hypothetical protein [Aspergillus fumigatus Af293] Sequence ID: ref|XP_755663.1|	C -> T 374 P -> S	C P 40	C / T P / S 41 / 4 (0.05)	. . 34	. . 34	. . 36	. . 51	C / T P / S 7 / 21 (0.60)	. . 40	. . 41
3 / 568217	alpha,alpha trehalose glucohydrolase TreA/Ath1 [Aspergillus fumigatus var. RP-2014] Sequence ID: gb|KEY75594.1|	C -> T 109 S -> L	C S 45	. . 37	. . 39	. . 52	. . 43	. . 46	C / T S / L 11 / 49 (0.69)	. . 54	. . 45
2 / 1515289 – 1515290	Conserved hypothetical protein [Aspergillus fumigatus Af293] Sequence ID: ref|XP_749671.1|	TG -> (-) Frameshift and stop codon*	TG H 47 46	. . . 47 47	. . . 45 46	(-) Frame-shift	. . . 35 36	. . . 52 52	. . . 37 37	. . . 35 35	. . . 35 36
5 / 2390677	DUF1479 domain protein [Aspergillus fumigatus Af293] Sequence ID: ref|XP_753711.1|	G -> A 276 A -> T	G A 50	. . 45	. . 39	. . 44	. . 50	. . 40	G / A A / T 19 / 34 (0.47)	. . 47	. . 47

Ref chromosome (Clinical reference strainAF293)	Gene	Snp	anc	Con2	Con4	Con5	D1-large	D3-dense	D3-fluffy	D6-fluffy	D6-white
2 / 3310760	PtaB protein [Aspergillus fumigatus Af293] Sequence ID: ref|XP_755622.1|	C -> T 264 Q -> STOP	C Q 35	. . 39	. . 37	. . 43	. . 38	. . 29	. . 30	. . 34	T STOP 31
7/ 1837636	hypothetical protein AFUB_092560 [Aspergillus fumigatus A1163] Sequence ID: gb|EDP48538.1|	C -> T 167 G -> D	C G 58	. . 44	. . 38	. . 53	. . 66	. . 42	. . 60	. . 58	T D 48
1 / 3895800	U3 small nucleolar RNA-associated protein Utp11 [Aspergillus fumigatus Af293] Sequence ID: ref|XP_752827.1|	C ->G 199 D -> E	C D 39	C /G D / E 23 / 24 (0.34)	. . 44	. . 39	. . 37	. . 40	. . 39	. . 54	. . 38
1 / 3893227	phosphoribosyl-AMP cyclohydrolase [Aspergillus fumigatus Af293] Sequence ID: ref|XP_752826.1|	C -> A 325 W-> C	C W 39	C /A W / C 16 / 15 (0.32)	. . 40	. . 40	. . 48	. . 38	. . 45	. . 37	. . 41
2 / 399978	mitochondrial outer membrane translocase receptor (TOM70), putative [Aspergillus fumigatus Af293] Sequence ID: ref|XP_749301.1|	C -> T 107 Q -> STOP	C Q 43	. . 55	. . 49	. . 37	. . 34	. . 56	. . 51	C / T Q / STOP 25 / 13 (0.21)	. . 46
1 / 3887699	Mn superoxide dismutase MnSOD [Aspergillus fumigatus Af293] Sequence ID: ref|XP_752824.1|	C -> A 129 G -> V	C G 60	. . 36	. . 39	. . 46	. . 53	. . 52	. . 42	C / A G / V 40 / 6 (0.07)	. . 58

*From amino acid 551 **HRTVIISP to QNCYHFA(STOP)**

Supplementary Table 4C

Whole-genome sequence analysis of evolved strains. Substitution, genes and gene functions associated with the mutations found in the evolved strains from three lineages via whole-genome sequence and putative function of each gene.

Evolved strain	Substitution (SNP/del)	Gene	Gene function/Putative function
D1-large	G138S (SNP)	CYP51	-A key enzyme in the ergosterol synthesis
	P320L (SNP)	HMG-CoA reductase	-A key enzyme in mevalonate biosynthesis confers a rise in the ergosterol content
D3-fluffy	S109L (SNP)	TreA / Ath1	-Required for the phenotype of growth on trehalose, i.e. trehalose utilization
	A276T (SNP)	DUF1479 domain protein	-Unknown
D3-dense	P320L (SNP)	HMG-CoA reductase	-A key enzyme in mevalonate biosynthesis confers a rise in the ergosterol content
D6-fluffy	Q107STOP (SNP)	TOM70	-Tom70 is located in the outer membrane. It is in the protein influx pathway of the mitochondria
	G129V (SNP)	MnSOD	-MnSOD2 is a mitochondrial superoxide dismutase.
	VK 751--(DEL)	Mdm31	-Mdm31 is an inner membrane protein of the mitochondria, it is in the protein influx pathway of the mitochondria
D6-white	Q264STOP (SNP)	PtaB	-Predicted to be related to white phenotype and less sporulation
	G167D (SNP)	Hypothetical protein	-Associate to the increased MIC
Control 5	P320L (SNP)	HMG-CoA reductase	-A key enzyme in mevalonate biosynthesis confers a rise in the ergosterol content
	HRTVIISP551-558 QNCYHFASTOP (DEL)	Conserved hypothetical protein	-Unknown

Note: Con5 contains an additional deletion (TG) that caused a frame shift (adding stop codon) in a conserved hypothetical gene (see table in supplementary Table 4B).

Supplementary Table 5A

Tracing-back the mutations from the different time points of experimental evolution.

Supplementary Table 5A on the next page shows the number of colonies harboring specific fixed mutations as a fraction of the number of colonies tested during week 2, 4, and 6 of the evolutionary experiment in the lineages D1, D3, and D6. The reason for performing this trace-back experiment was dual. First we wanted to establish whether the re-sequencing yielded SNPs that can be found in a PCR based method which could detect SNPs that were 100% divergent, meaning that the SNPs identified form the re-sequencing experiments are biologically real and not sequencing artefacts/mistakes. This is a proof of principle that the pipeline is successful as a SNP discovery tool. The second reason is to establish which mutation came first, and which second in situations where multiple phenotypes were found. The numbers listed in Supplementary Table 5A are considered adequate to achieve the goals of our trace back experiment. Mutations were detected via PCR (primers are listed in Supplementary Table 5B). Results showed that D1-middle, D1-large, and D3-dense contained the P320L SNP in gene HMG-CoA reductase. D6–white harboured the Q264STOP SNP in gene Ptab from the moment its distinctive phenotype appeared, which suggests that this mutation is causally related to the white phenotype. In contrast the mutation G167D in a hypothetical protein appeared later on during evolution, which indicates it might be related to the observed change in the level of resistance. NA indicates either that the type of phenotype was not observed, or that the PCR did not yield interpretable results.

Lineage	Phenotype	Gene	Week 2	Week 4	Week 6
D1	Middle	HMG-CoA	NA	(7/7)	NA
D1	Large	HMG-CoA	NA	NA	(2/2)
D3	Dense	HMG-CoA	NA	(12/12)	(5/5)
D6	White	Ptab	(7/7)	(7/7)	(6/6)
D6	White	Hypothetical protein	(0/4)	(0/6)	(5/6)

Supplementary Table 5B

The sequence of the primers used to trace-back the mutation of the three candidate genes during experimental evolution.

Primers	**Sequence (5'-3')**
Ptab ptotein-PF	AGTGTAACACCTCAGCCGC
Ptab protein-PR	TTGATTCTGAGTCTGCTGGGC
Hypothetical protein -PF	CAGCGAAACTTCAGGCACAC
Hypothetical protein -PR	TATCTTGCGCTCGGTCACAG
HMG-COA reductase -PF	CTTCTCGGGCGTTTTTGCAT
HMG-COA reductase -PR	TGGTGCTTGACGAAGCTTGA