Online data supplement

Parasexual recombination enables *Aspergillus fumigatus* to persist in cystic fibrosis

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Extended methods

Selection of *A. fumigatus* isolates

The Radboud University Medical Center is a tertiary care hospital with beds for 950 patients. The medical microbiology laboratory receives specimens from patients admitted to the hospital as well as those seen as outpatients. All samples are registered in the laboratory information system (GLIMS 8 by MIPS, Ghent, Belgium). Routine identification of *A. fumigatus* isolates included typical macroscopic and microscopic characteristics as well as the ability to grow at 48°C. The mycology unit of the laboratory has the policy to routinely store fungal isolates of *A. fumigatus* in the department's fungal culture collection. From the isolates a conidia suspension was made in glycerol 10% and stored at -80°C. The laboratory information system, and subsequently the department's fungal culture collection, was searched for *A. fumigatus* isolates from three groups of patients and one environmental group as detailed below.

(i) *Chronically colonized CF-patients.* CF-patients with ≥20 *A. fumigatus* isolates cultured from respiratory samples were included. The medical records were assessed to verify the diagnosis of CF disease, which was based on typical clinical characteristics alongside a positive sweat test (chloride >60 mmol/l) and/or the presence of two known pathogenic cystic fibrosis transmembrane conductance regulator (CFTR) mutations. CF-patients follow a protocol of four systematically planned hospital visits per year while this is not the case for the other patients groups.

(ii) *Chronically colonized chronic lung disease patients.* The culture collection was searched for *A. fumigatus* isolates from patients with ≥ 8 isolates cultured from respiratory samples. The hospital patient information system was used to identify the patients underlying disease. These included chronic pulmonary aspergillosis (CPA), allergic bronchopulmonary aspergillosis

(ABPA), COPD and asthma patients with bronchiectasis who did not meet criteria for CPA or ABPA. A minimum of 30 days between the first and the last positive *A. fumigatus* culture was required to be allocated to this group. Furthermore, isolates from patients with proven aspergilloma were included. Patients with CF were excluded.

(iii) *Patients with acute invasive aspergillosis.* The culture collection was searched for *A. fumigatus* isolates of hematology patients and those cultured from tissue samples. The hospital records were used to identify those culture-positive patients that were diagnosed with invasive aspergillosis and the EORTC/MSG criteria were used to classify the *Aspergillus* disease (1).

(iv) *Environmental isolates*. The culture collection was searched for non-clinical *A. fumigatus* isolates from environmental origin. These included isolates recovered from the hospital indoor environment, through air sampling, as well as the outdoor environment (including cultures of soil, compost, seeds, leaves, air and water).

Conidial size measurement

Initial screening of the selected isolates was based on the size of the conidia as an indication for the presence of diploidy. The conidial size was measured with a Casy® TT cell counter (OLS OMNI Life Science, Germany), for validation of this method see Figure E1. For the conidial size measurement, the peak diameter was used for average cell size and for comparison between isolates. *A. fumigatus* conidia were recovered from the stored spore suspension, through inoculation of a loop of frozen spores on a Sabouraud dextrose agar slant with 0.2 mg/ml chloramphenicol (SAB) (Balis Laboratorium, Beneden Leeuwen, the Netherlands) and incubated at 37°C for 48 hours after which the conidia were harvested and suspended in NaCl tween 80 solution. In case of no sporulation of the isolate after 48 hours of incubation spores were

inoculated again on Takashio medium (Balis Laboratorium, the Netherlands). To validate the cell counter protocol multiple variables with a possible influence on the measured conidial size were investigated including incubation temperature, length of incubation and post-incubation storage at room temperature, prolonged storage of conidia suspensions at 4°C, passage of fungal cultures and type of culture medium.

For statistical analysis the R package 3.5.0 (Boston, MA, USA, http://www.rstudio.com/) was used to perform calculations. First, the Shapiro test was performed to test for normal distribution, and then the Kruskal-Wallis chi-squared test was performed to test whether the groups are similar or different populations. Finally, to test the significance of the pairwise comparisons of the groups the Pairwise Wilcoxon test was performed.

Scanning Electron Microscopy

The samples were mounted on a SEM stubs by carbon adhesive glue (EMS Washington USA) and subsequently coated with 12nm Tungsten (Leica MED 020). Samples were analyzed at 2 KV, 6 pA, in a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands).

Nuclear staining

Conidia were harvested using wet cotton swabs and placed in Milli-Q containing 0.01% Tween 20 at a final concentration of 4×10^5 conidia/mL. Conidia were fixed in 4% formaldehyde, 0.2% Triton X-100 in PBS from 30 minutes and incubated for 10 min with 1.5 µg/mL DAPI Vectashield® antifade mounting medium (Vector Laboratories, Maraval Life Sciences) for one

hour, and mounted on a microscope slide for viewing by using the AxioPhot 1 fluorescence microscope (Zeiss).

Benomyl susceptibility testing

All isolates with a conidial size ≥ 2.90 µm, and from all other size ranges (2.20 µm – 2.89 µm) ten isolates per decimal group were selected and analyzed for ploidy level by benomyl testing. Standard Minimal medium (MM) was used and supplemented with three different concentrations of benomyl (0.66 p.p.m, 0.76 p.p.m and 1 p.p.m) to prepare the agar plates for benomyl susceptibility testing. Besides higher benomyl susceptibility, diploid colonies also show sectoring on benomyl medium. Sectoring is due to the haploidization of a diploid, leading to a sector with lower susceptibility to benomyl, and can be seen as one or more vigorous separate sectors originating from the primary growth. To see proper sectoring of the diploids on the benomyl medium, the conidia suspension was serially diluted three times in tenfold till 10^{-2} and spotted on the benomyl plates in triplicate and incubated for 48 hours at 37°C.

Fluorescence-activated cell sorting

The nuclear content is doubled in diploid isolates compared to haploid isolates, which can be determined by staining the nuclear content with propidium iodide (PI) and measured by using Fluorescence-Activated Cell Sorting (FACS) technology. The same selection of isolates for benomyl susceptibility testing were also subjected to FACS analysis as previously described by Veselská *et al*. (2) with minor adaptations. A volume of conidia suspension corresponding to approximately 10^7 cells/mL was collected. The suspension was transferred over a 40 µm cell strainer to eliminate hyphal fragments. One mL of the cell suspension was centrifuged for 10

minutes at 14,000 rpm. After discarding the supernatant, 500 µl of methanol: glacial acetic acid (MA+) buffer was added and the cells were resuspended by pipetting. The samples were incubated during 15 min with constant stirring (300 rpm) at 4°C. After centrifugation for 5 min at 14,000 rpm the samples were washed with the detergent Triton X-100 (0.1%). Again after centrifugation for 5 min at 14,000 rpm the supernatant was discarded and 950 µl of Tris-MgCl2 buffer with 0.1 mg/mL RNAse A was added, and incubated with constant stirring at 300 rpm at 37°C for 15 min. Samples were centrifuged for 5 min at 14,000 rpm and after discarding the supernatant, 500 µl of propidium iodide (PI) (50 µg/mL) was added to stain nuclei at room temperature for 30 minutes in the dark. All samples were analyzed by the Macs Quant® Analyzer (Miltenyi Biotec) fluorescence activated cell sorting (FACS) flow cytometer using a reference haploid *A. fumigatus* isolate and a previously constructed diploid *A. fumigatus* isolate (Laboratory of Genetics, Wageningen University & Research, the Netherlands, not published) as references to set two independent gates for comparison of the samples subjected to analysis. The same haploid and diploid reference isolate were included in each independent sample run.

Haploidization of diploids

As a proof of principle, a diploid isolate should be able to haploidize and reduce in cell size while this is not possible for a haploid isolate. Using the sectors presenting on benomyl agar plate two diploid isolates were haploidized. For each isolate ten sectors were selected that were derived from diploid colonies on the benomyl agar plate and subcultured on a MM agar plate. From the subcultured sectors the conidial size was measured by using the Casy® TT cell counter and compared to their conidial size measured on a SAB agar slant without any prior benomyl sector selection.

Whole genome sequencing

To understand the genetic adaptation of the isolates that changed in ploidy over time whole genome sequencing analysis was performed. Genomic DNA was extracted of four *A. fumigatus* isolates from one CF-patient, representing two haploid and two diploid isolates. Isolates were grown on SAB agar slants and the conidia were harvested with wet cotton swabs and placed in extraction buffer (200 mM Tris-CI pH 8.0. 0.5 M NaCI, 0.01 M EDTA, 1% SDS) (3) in a 1.5 mL tube containing 400–600 μm acid-washed glass beads (4 mm diameter). Conidial cells were disrupted by three rounds of snap-freezing in liquid nitrogen and homogenization with a MagnaLyser (Roche) for 30 sec at 7,000 rpm. DNA was extracted using phenol/chloroform extraction and additionally purified using the QIAamp DNA Blood Mini kit (Qiagen, Germany). Sequencing was performed on the BGISEQ-500 platform, with a minimum of 1,5 Gb clean data per sample, by the Beijng Genome Insititute (BGI, Shenzhen, China).

Mapping and variant calling

Raw FASTQ files were trimmed and filtered by TRIMMOMATIC (v 0.27, LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, MINLENGTH:70 (4). The resulting trimmed reads were aligned using bwa mem (v 0-7-15, default parameters, (5) using the *Aspergillus fumigatus* Af293 (assembly ASM265v1, https://www.ncbi.nlm.nih.gov /genome/18/genome_assembly_ id=22576). Reads with mapping quality lower than 20 were filtered using samtools (v 0.1.19,) (6). We then removed duplicates using picard tools (v.1.109, http://picard.sourceforge.net) and performed realignment around indels with GATK (v .3.7-0,) (7).

The resulting BAM files were combined in mpileup format (samtools, mpileup, default parameters, v 0.1.19, (6) after which SNPs and indels were separately called using varscan (v 2.3.9, using mpileup2snps and mpileup2indel resp., --output-vcf 1, --min-var-freq 0.05 (8)). The

resulting vcf files were inspected for genetic differences between any pairwise comparison, using vcfR (v 1.8.0,) (9), for which we used a minimum allele frequency difference of 0.6 and a minimum coverage of 10 to filter SNPs and indels. Every resulting variant was then visually inspected in all samples using IGV (v 2.4.14,) (10). For larger structural variation (including large indels) we used unfiltered BAM files and filtered out all reads with soft and hard clipped reads, as well as indel and deletion calls. Furthermore we filtered on flags 67, 131, 115, 179, 81, 161, 97, 145, 65, 129, 113 and 177 which potentially indicate large indels or deletions or chromosomal rearrangements. We then quantified coverage of all these reads in 100 bp windows using TIDDIT (v 2.2.6) (11). The resulting coverage distribution was divided to the total coverage of the initial BAM files which therefore yielded the frequencies of 'alternate call' mapped reads to those with 'normal' mapping. These frequencies were then compared between all pairwise samples, ordered upon frequency and visually inspected using IGV without any *a priori* cutoffs.

Casy TT® Cell counter validation

The following variables with a possible influence on the measured conidial size were assessed; I incubation temperature, II length of incubation and post-incubation storage at room temperature, III prolonged storage of conidia suspensions at 4°C, IV passage of fungal isolates and V type of culture medium. All evaluations were performed on seven *A. fumigatus* isolates in biological triplicates.

Incubation temperature

A. fumigatus isolates were incubated for 48 hours at 28°C, 37°C and 48°C on SAB. After incubation conidial size was evaluated conform standard protocol.

Length of incubation

A. fumigatus isolates were incubated for 48, 96 and 192 hours at 37°C on SAB. After incubation conidial size was evaluated conform standard protocol. To measure the influence of storage of the cultures at room temperature before preparing a conidia suspension *A. fumigatus* isolates were incubated for 48 hours at 37°C on SAB. After incubation SAB tubes were stored at room temperature for 48 and 144 hours and after storage period conidial size was evaluated conform standard protocol.

Storage of conidia suspensions at 4°C

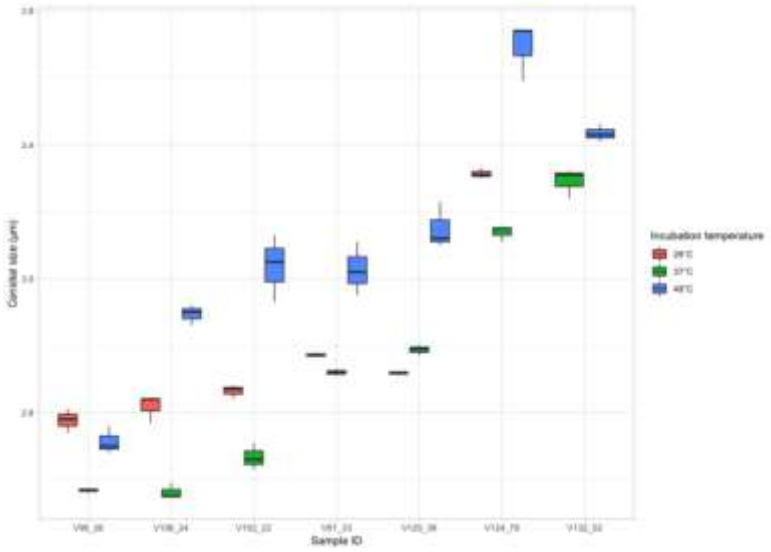
A. fumigatus isolates were incubated for 48 hours at 37°C on SAB. After incubation conidia suspension in NaCl tween 80 solution were made and stored at 4°C for 24 and 48 hours, 1 week, 1, 2 and 3 months. After storage conidial size was evaluated conform standard protocol.

Passage of fungal isolates

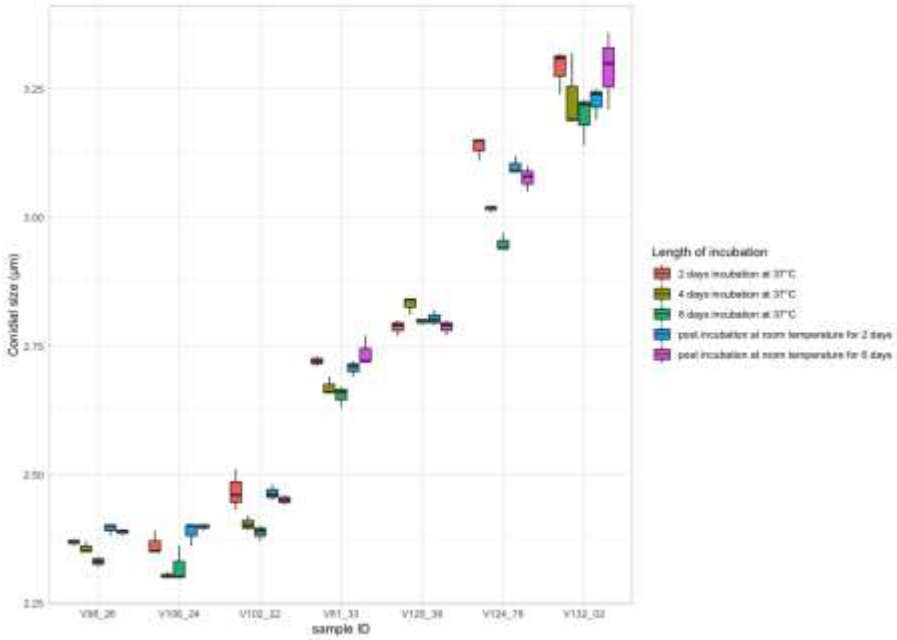
A. fumigatus isolates were incubated for 48 hours at 37°C on SAB. After incubation the samples were subcultured on SAB and incubated again for 48 hours at 37°C. After incubation conidial size was evaluated conform standard protocol. *Culture medium*

A. fumigatus isolates were incubated for 48 hours at 37°C on SAB, Takashio and malt extract medium (Merck, Germany). After incubation conidial size was evaluated conform standard protocol. Seven *A. fumigatus* isolates were randomly selected based on representing different sizes in the whole size spectrum (2.34, 2.38, 2.46, 2.73, 2.81, 3.17 and 3.49 µm) for validation of the CasyTT cell counter on incubation temperature, length of incubation, post-incubation storage at room temperature, storage of conidia suspensions at 4°C and type of culture medium. All isolates showed sporulation for all growth conditions except isolate V132-02 that did grew but did not sporulate at 28°C and was therefore excluded from the analysis. Incubation temperature

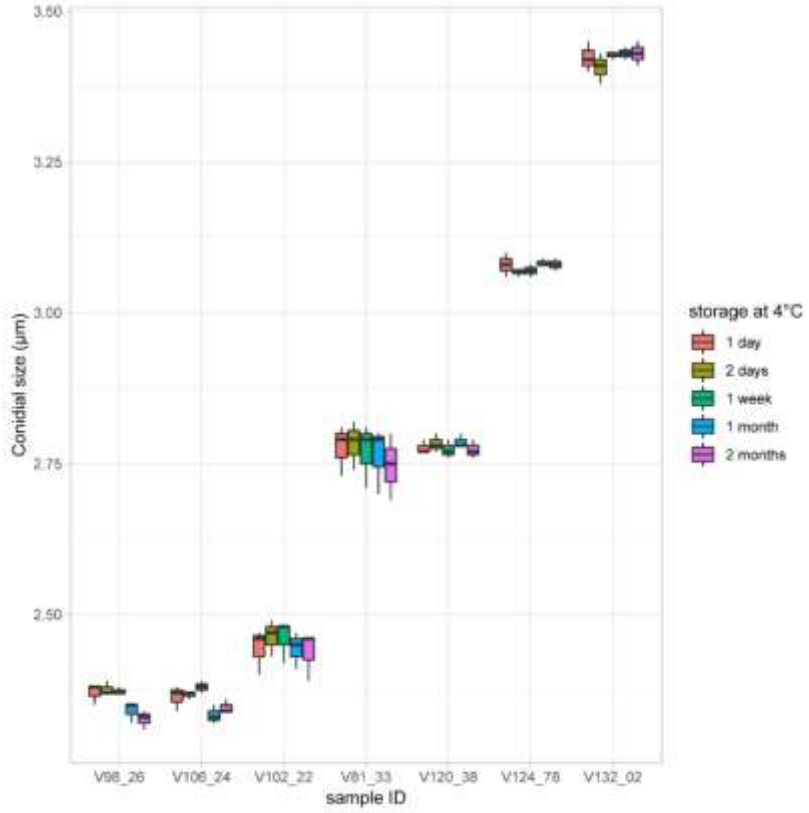
changed mean conidial size considerably, up to 0,57 µm difference for isolate V102-22 (37°C versus 48°C) and significantly for all isolates tested (Figure E1a). The influence on mean conidial size of length of incubation (Figure E1b), post-incubation storage (Figure E1b) and culture medium (Figure E1e) was smaller, but still significant. The temperature, length of incubation, storage time and medium that rendered the largest or smallest conidia was not consistent and differed per isolate. The storage of conidia suspension at 4°C and passage of fungal isolates (Figure E1d) did however not change conidial size significantly, even after two months of storage at 4°C (Figure E1c). By using a standard protocol of SAB medium and preparing the conidia suspension after 48 hours of incubation at 37°C, directly followed by a CasyTT measurement on the same day, biological errors in the size measurement are expected to be reduced to a minimum and proved to be a robust protocol for high throughput conidial size screening. It should be noted though that in the screening of the large cohort a total of 69 isolates did not sporulate on SAB medium, from those 49 isolates did sporulate on a Takashio culture medium and therefore will slightly diverge with their conidial size measurement.



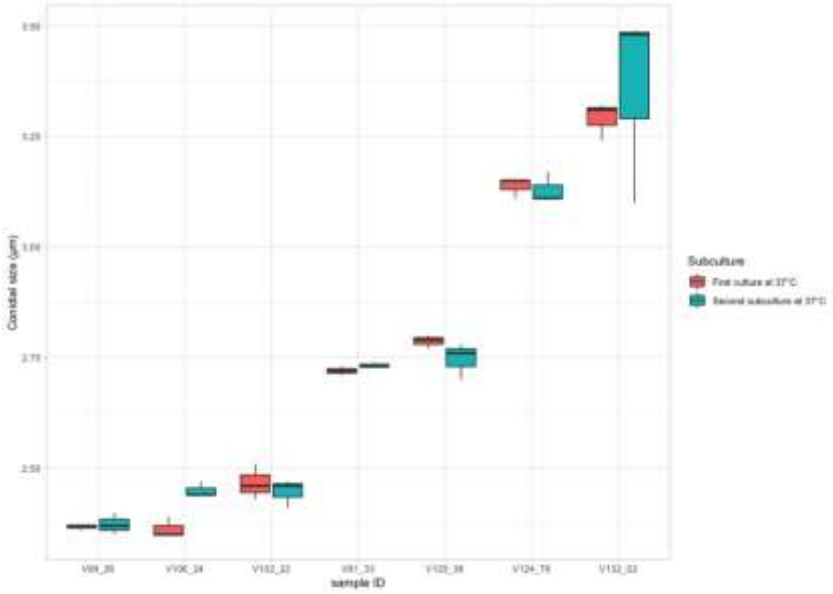
A Effect of different incubation temperatures on conidial size.



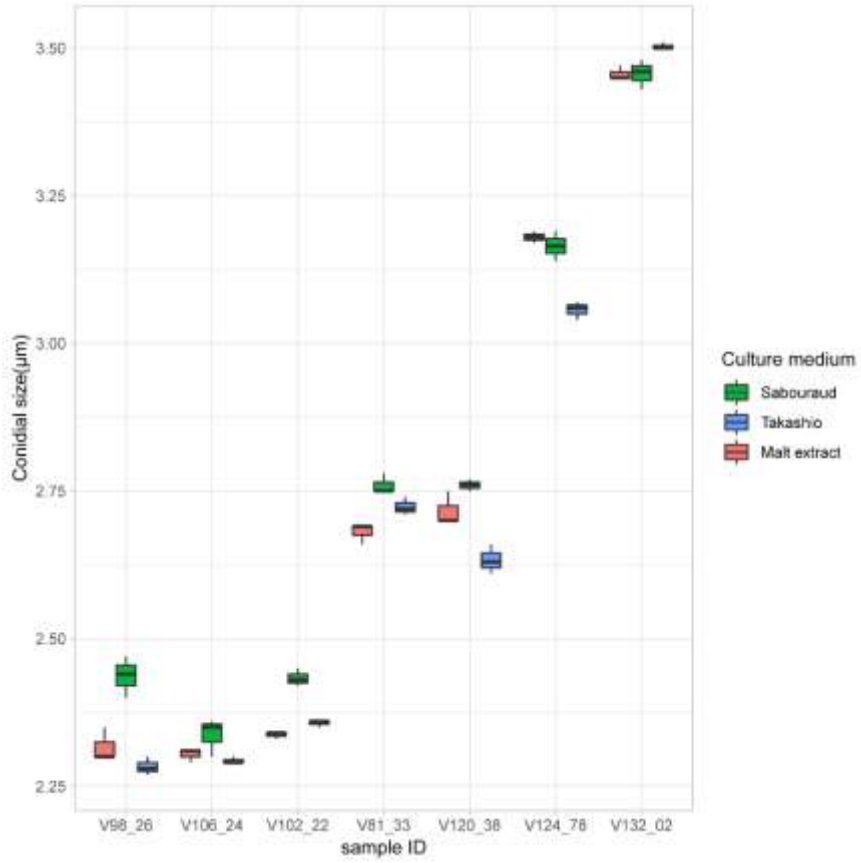
B Effect of different lengths of incubation at 37°C or different lengths of subsequent storage of cultures at room temperature on conidial size.



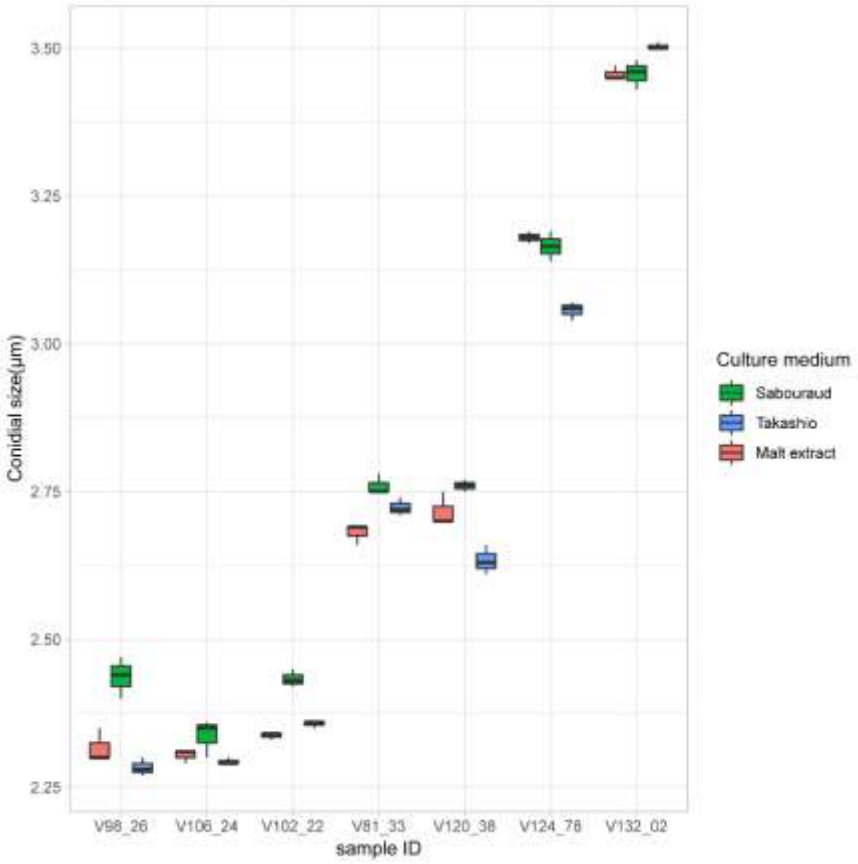
C Effect of prolonged storage of fungal suspensions at 4°C on conidial size.



D Effect of subculture on conidial size.



E Effect of different types of culture medium (Malt extract, Sabouraud and Takashio medium) on conidial size.



E Effect of different types of culture medium (Malt extract, Sabouraud and Takashio medium) on conidial size.

Figure. E1.

Casy TT® cell counter validation with measurement of seven *A. fumigatus* strains of different conidial sizes. Different incubation temperatures (A), different lengths of incubation at 37°C or different lengths of storage of cultures at room temperature (B), prolonged storage at 4°C (C), subculture (D) and different types of culture medium (E) were assessed. Storage of conidia suspensions at 4°C or subculturing isolates does not significantly influence the conidial size measurement. The other tested parameters can influence conidial size measurement, therefore screening of all isolates in this study was performed by using one standard protocol.

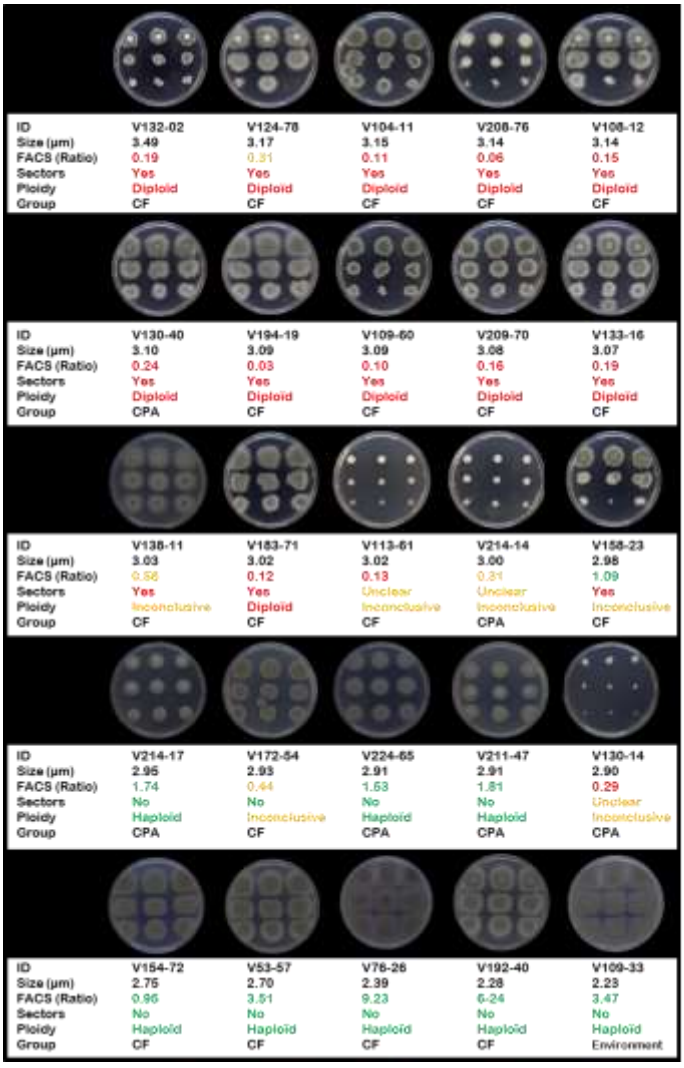


Figure. E2.

Confirmation of ploidy by benomyl susceptibility and FACS analysis. *A. fumigatus* isolates are grown on benomyl agar plates to depict the morphological differences. The twenty *A. fumigatus* isolates with the largest conidial size and five control isolates are shown. In the accompanying table below the photos conidial size measurement is indicated, FACS ratio, presence of sectoring, the final grouping of ploidy level and the group of origin of the *A. fumigatus* isolate (CF= Cystic Fibrosis, CPA = Chronic Pulmonary Aspergillosis, Red color: diploid, Orange color: inconclusive ploidy, Green color: Haploid)

Table E1. Minimal inhibitory concentration values of antifungal compounds and microsatellite analysis of four *A. fumigatus* isolates from a CF patient selected for whole genome sequencing analysis. Although the final isolate, V230-14, had one locus difference in the microsatellite analysis (44), the sequencing data showed that this isolate was highly similar to the three preceding isolates (V109-58, V183-71 and V209-70) and considered to belong to the same lineage. This final isolate also showed a voriconazole and isavuconazole specific antifungal resistance.

ID	Collection date	Conidial size (µm)	Ploidy	Azole susceptibility (mg/l)								Microsatellite analysis								
				Itra	Vori	Posa	Isav	Anid	Casp	Mica	Amph	2A	2B	2C	3A	3B	3C	4A	4B	4C
V109-58	22/12/2010	2.4	haploid	0.125	0.5	0.031	1	0.016	0.25	0.002	0.25	20	19	16	51	13	8	8	26	12
V183-71	23/07/2015	3.0	diploid	0.25	1	0.031	1	0.016	0.25	0.004	0.25	20	19	16	52	13	8	8	26	12
V209-70	18/10/2016	3.1	diploid	0.25	0.5	0.031	1	0.016	0.25	0.004	0.25	20	19	16	52	13	8	8	26	12
V230-14	05/09/2017	2.4	haploid	1	**>16**	0.125	**>16**	0.016	0.25	0.004	0.25	20	19	16	**44**	13	8	8	26	12

Collection date = Day/Month/Year

Itra = Itraconazole, Vori = Voriconazole, Posa = Posaconazole, Isav = Isavuconazole, Anid = Anidulafungin, Casp = Caspofungin, Mica = Micafungin, Amph = AmphotericinB

Table E2. Alignment statistics per whole genome sequence sample. Samtools flagstat and Qualimap bamqc were used to analyze the four selected *A. fumigatus* isolates.

	V109-58 haploid	V183-71 diploid	V209-70 diploid	V230-14 haploid
Raw reads	25,918,416	26,161,730	22,037,390	22,104,630
Trimmed reads	24,967,898	25,328,354	21,108,154	21,210,884
Raw Mapped reads	24,437,690	24,775,298	20,660,101	20,740,023
Flagstat				
Clean filtered reads	22,267,341	22,701,089	19,200,624	19,091,619
Properly paired	21,875,141	22,378,701	18,835,288	18,804,201
Mate on different chromosome	217,197	188,720	194,526	189,389
Qualimap				
Mean Cov (+/- sd)	75.44 (45.39)	76.91 (45.03)	65.05 (37.26)	64.68 (39.67)
Mapping quality	58.57	58.57	58.58	58.56
Insert size (P25/median/P75)	201 / 228 / 257	187/212/238	210 / 238 / 268	214 / 242 / 274
Mismatches	9,273,907	9,533,647	7,941,556	8,053,536
Insertions	189,230	193,761	160,086	161,356
Deletions	201,019	205,539	170,275	172,024
Clipped reads	313,394	330,650	262,189	269,582
Percentage reference covered (>0)	93.89%	93.85%	93.87%	93.89%

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