



Original Article

DNA Barcoding Coupled with High Resolution Melting Analysis Enables Rapid and Accurate Distinction of *Aspergillus* species

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Abstract

We describe a high-resolution melting (HRM) analysis method that is rapid, reproducible, and able to identify reference strains and further 40 clinical isolates of *Aspergillus fumigatus* (14), *A. lentulus* (3), *A. terreus* (7), *A. flavus* (8), *A. niger* (2), *A. welwitschiae* (4), and *A. tubingensis* (2). Asp1 and Asp2 primer sets were designed to amplify partial sequences of the *Aspergillus benA* (beta-tubulin) genes in a closed-, single-tube system. Human placenta DNA, further *Aspergillus* (3), *Candida* (9), *Fusarium* (6), and *Scedosporium* (2) nucleic acids from type strains and clinical isolates were also included in this study to evaluate cross reactivity with other relevant pathogens causing invasive fungal infections. The barcoding capacity of this method proved to be 100% providing distinctive binomial scores; 14, 34, 36, 35, 25, 15, 26 when tested among species, while the within-species distinction capacity of the assay proved to be 0% based on the aligned thermodynamic profiles of the Asp1, Asp2 melting clusters allowing accurate species delimitation of all tested clinical isolates. The identification limit of this HRM assay was also estimated on *Aspergillus* reference gDNA panels where it proved to be 10–10² genomic equivalents (GE) except the *A. fumigatus* panel where it was 10³ only. Furthermore, misidentification was not detected with human genomic DNA or with *Candida*, *Fusarium*, and *Scedosporium* strains. Our DNA barcoding assay introduced here provides results within a few hours, and it may possess further diagnostic utility when analyzing standard cultures supporting adequate therapeutic decisions.

Key words: molecular barcoding, high resolution melting, *Aspergillus*, species level identification, standard cultures.

Introduction

Invasive fungal infections (IFI) are associated with high lethality rates representing a serious health problem in immunocompromised patients. *Aspergilli* are among the most significant fungal etiological agents of life-threatening invasive infections especially in patients with neutropenia, hematologic malignancies (acute leukemia) and in patients undergoing hematopoietic stem cell transplantation.^{1,2} Even with underestimated, poor epidemiological data the burden of invasive aspergillosis (IA) is on the rise.³ This expansion is due to improved antimicrobial therapies and supportive care raising the number of severely immunocompromised patients thus putting them at higher risk of acquiring opportunistic fungal infections.⁴

Aspergillus fumigatus is the prominent agent of IA⁵; however, several other species have also been reported from various clinical samples, such as *A. terreus*, *A. flavus* and *A. niger*.^{6–12} Recently, IA cases due to rare *Aspergilli* such as *A. lentulus* have also been reported having low *in vitro* susceptibilities to a wide range of antifungals including amphotericin B, azoles, echinocandins.^{13,14} In clinical settings misidentification of *A. lentulus* with *A. fumigatus* has been increasingly reported by clinical laboratories.¹⁵ Infections due to *A. terreus* are also difficult to treat because of their refractoriness to certain antifungal drugs, often causing disseminated infections with increased lethality.¹⁶ The correct and prompt identification of *Aspergillus* species is of high importance because knowledge of species identity may influence adequate antifungal therapy given that different species have variable susceptibilities to multiple antifungal drugs.^{17–20}

Mortality among intensive care unit (ICU) patients with IA can be as high as 90% but the overall mortality rate for IA is about 50% if diagnosed timely and treated.^{21,22} Accurate diagnosis of IA raises challenges to clinical microbiology laboratories. Clinical signs and symptoms are non-specific and standard culture-based diagnostics are typically too insensitive or nonspecific.²³ Identification of unknown *Aspergillus* clinical isolates to species is a polyphasic approach including morphotyping, growth temperature regimes, investigation of drug susceptibility patterns, and molecular characterization.²⁴ Furthermore, clinical isolates are not necessarily morphologically uniform representing aberrant conidiophore formation, therefore mistaken identification of species by morphological characteristics have occurred in the past.¹⁴ Since culture techniques typically require specialized expertise for recovery and species determination, many laboratories can rely only on DNA based methodologies.^{25,26}

Surrogate-marker based molecular assays can provide better prognostic data. In routine clinical settings, the detection of *Aspergillus* galactomannan (GM) is based on

labor intensive, well standardized Platelia-*Aspergillus* enzyme immunoassay (EIA), which is considered to be the gold-standard possessing numerous attractive features. Nevertheless, as GM is a panfungal marker it is not suitable for the identification of *Aspergilli* to the species level.^{27–29} There is a dire need for the development of newer diagnostic techniques to identify causative agents to species rapidly, noninvasively, and at an early stage of the disease.

Molecular techniques in addition to morphological identification have been shown to offer high resolution of species within the genus.¹⁴ Recent, multiple studies prove that polymerase chain reaction (PCR)-based techniques appear to be promising in terms of speed, economy, and resolution power with available methodological recommendations to facilitate both manual and automated nucleic extraction technology.^{30–34}

Molecular barcoding relies on short, conserved genetic markers in the genome permitting the specific identification of the different species.^{35–37} Recently, fast, high throughput post-PCR due to high-resolution melting analysis has been developed and effectively used for this reason.³⁸ High resolution melting (HRM) analysis is able to determine accurately the relationship between temperature and the extent of denaturation of DNA in the presence of saturating, double stranded DNA intercalating dyes.³⁹ The denaturation of the different DNA fragments with increasing temperature defines the characteristic melting domains and the shape of the derivative melting curves represents the taxonomic signatures of the here-tested species.

This paper describes the development of an HRM based molecular barcoding assay tailored to prompt, accurate identification to the species level of clinically relevant *Aspergillus* isolates. Our sequence typing method targets two different regions of *Aspergillus benA* genes for the specific identification and discrimination of different clinical isolates of *Aspergilli* to the species level. Due to the fact that this HRM technique generates duplex, distinct peaks in case of different *Aspergillus* species, our method introduced here has a high resolving power with a short turnaround time reducing the risk of contamination and saving expenses. These features make our method advantageous for use as a first-pass diagnostic adjunct in microbiology laboratories.

Materials and methods

Collection and identification of fungal strains used in this study

Genomic DNA samples of clinically relevant *Aspergillus*, *Candida*, *Fusarium*, and *Scedosporium* strains were examined. The reference strains and the clinical isolates (Table 1) were maintained at the Department of Microbiology, University of Szeged on Sabouraud—chloramphenicol

Table 1. List of the reference and clinical strains examined by Asp1–Asp2 duplex HRM assay.

DNA serial ID.	Strain Designation Number	Organism	Origin
Reference	FGSC A1156	<i>Aspergillus terreus</i>	-
1	SZMC 2414	<i>Aspergillus terreus</i>	India
2	SZMC 2424	<i>Aspergillus terreus</i>	India
3	SZMC 2460	<i>Aspergillus terreus</i>	India
4	SZMC 22546	<i>Aspergillus terreus</i>	India
5	SZMC 22547	<i>Aspergillus terreus</i>	India
6	SZMC 22548	<i>Aspergillus terreus</i>	India
7	SZMC 22549	<i>Aspergillus terreus</i>	India
Reference	CBS 101355 / AF 293	<i>Aspergillus fumigatus</i>	-
8	SZMC 2504	<i>Aspergillus fumigatus</i>	India
9	SZMC 2490	<i>Aspergillus fumigatus</i>	India
10	SZMC 2486	<i>Aspergillus fumigatus</i>	India
11	SZMC 22550	<i>Aspergillus fumigatus</i>	India
12	SZMC 22551	<i>Aspergillus fumigatus</i>	India
13	SZMC 22552	<i>Aspergillus fumigatus</i>	India
14	SZMC 22553	<i>Aspergillus fumigatus</i>	India
15	SZMC 22554	<i>Aspergillus fumigatus</i>	India
16	SZMC 3111	<i>Aspergillus fumigatus</i>	India
17	SZMC 3109	<i>Aspergillus fumigatus</i>	India
18	SZMC 3100	<i>Aspergillus fumigatus</i>	India
19	SZMC 3117	<i>Aspergillus fumigatus</i>	India
20	SZMC 3106	<i>Aspergillus fumigatus</i>	India
21	SZMC 3104	<i>Aspergillus fumigatus</i>	India
Reference	CBS 117885	<i>Aspergillus lentulus</i>	-
22	SZMC 3118	<i>Aspergillus lentulus</i>	India
23	SZMC 3123	<i>Aspergillus lentulus</i>	India
24	SZMC 20911	<i>Asprgillus lentulus</i>	Hungary
Reference	NRRL 11611	<i>Aspergillus flavus</i>	-
25	SZMC 22583	<i>Aspergillus flavus</i>	India
26	SZMC 22582	<i>Aspergillus flavus</i>	India
27	SZMC 22581	<i>Aspergillus flavus</i>	India
28	SZMC 22580	<i>Aspergillus flavus</i>	India
29	SZMC 22579	<i>Aspergillus flavus</i>	India
30	SZMC 22578	<i>Aspergillus flavus</i>	India
31	SZMC 22577	<i>Aspergillus flavus</i>	India
32	SZMC 22575	<i>Aspergillus flavus</i>	-
Reference	CBS 113.46	<i>Aspergillus niger</i>	-
33	SZMC 3119	<i>Aspergillus niger</i>	India/keratitis
34	SZMC 3108	<i>Aspergillus niger</i>	India/keratitis
35	SZMC 2402	<i>Aspergillus welwitschiae</i>	Hungary
36	SZMC 23407	<i>Aspergillus welwitschiae</i>	Hungary
37	SZMC 23406	<i>Aspergillus welwitschiae</i>	Hungary
38	SZMC 23408	<i>Aspergillus welwitschiae</i>	-
Reference	CBS 134.48	<i>Aspergillus tubingensis</i>	-
39	SZMC 2758	<i>Aspergillus tubingensis</i>	Hungary/ear

Table 1. continued

40	SZMC 3127	<i>Aspergillus tubingensis</i>	Netherland
41	SZMC 2040	<i>Aspergillus udagawae</i>	environmental isolate
42	SZMC 2041	<i>Aspergillus udagawae</i>	environmental isolate
43	SZMC 21694	<i>Aspergillus viridinutans</i>	environmental isolate
Reference	ATCC 22019	<i>Candida parapsilosis</i>	-
44	SZMC 23640	<i>Candida parapsilosis</i>	-
45	SZMC 8114	<i>Candida parapsilosis</i>	Germany
46	SZMC 1593	<i>Candida parapsilosis</i>	Hungary
Reference	ATCC 10231	<i>Candida albicans</i>	-
47	SZMC 22801	<i>Candida albicans</i>	-
48	SZMC 22800	<i>Candida albicans</i>	-
49	SZMC 1523	<i>Candida albicans</i>	Hungary
50	SZMC 1422	<i>Candida albicans</i>	-
51	SZMC 11493	<i>Fusarium napiforme</i>	India/keratitis
52	SZMC 11496	<i>Fusarium delphinoides</i>	India/keratitis
53	SZMC 11487	<i>Fusarium verticilloides</i>	India/keratitis
54	SZMC 11504	<i>Fusarium oxysporum</i>	India/keratitis
55	SZMC 11525	<i>Fusarium solani</i>	India/keratitis
56	SZMC 11481	<i>Fusarium incarnatum</i>	India/keratitis
57	CBS 116910	<i>Scedosporium aurantiacum</i>	ankle abscess
58	CBS 136046	<i>Scedosporium aurantiacum</i>	lung invasive

Note: The six *Aspergillus* (FGSC A1156, Af293, CBS 117885, NRRL 11611, CBS 113.46, CBS 134.48) and the two *Candida* reference strains (ATCC 22019, ATCC 10231) are highlighted in black. Clinical isolates are from the Szeged Microbiology Collection (SZMC). Reference strains are from: (FGSC); Fungal Genetics Stock Center, (CBS); Centraalbureau voor Schimmelfcultures, Fungal and Yeast Collection; (NRRL); Northern regional Research Laboratories, (ATCC); American Type Culture Collection.

slant agar and periodically subcultured. The species level identifications of the different clinical isolates were carried out by conventional morphological methods and the results were confirmed by sequence analysis of part of the calmodulin (*Aspergilli*), ribosomal RNA (*Candida* and *Scedosporium* species), and TEF1- α (*Fusaria*) genes.

DNA extraction

All fungal DNA extraction steps were performed in a class II laminar-flow cabinet to avoid environmental contamination.

- Aspergillus* reference strains and clinical isolates were cultivated on standard minimal nitrate medium.⁴⁰ *Aspergillus* genomic DNA extraction was carried out at the University of Debrecen and at the University of Szeged. DNA was isolated from liquid cultures grown in minimal medium at 37°C (*A. fumigatus*, *A. niger*), 25°C (*A. terreus*, *A. lentulus*, *A. flavus*, *A. tubingensis*) and 30°C (*A. welwitschiae*) at 220 rpm for 18 h. The mycelium was disrupted by Roche MagNa Lyser (Roche Diagnostics, Risch-Rotkreuz, Switzerland), and genomic DNA

was isolated using the Genomic DNA Purification Kit (Thermo Scientific, Maryland, USA) according to the manufacturer's instructions.

- Candida*, *Fusarium*, and *Scedosporium* cultures used for the molecular barcoding were grown on yeast peptone D-glucose (YPD) broth for 2 days, and DNA was extracted from the strains using the Masterpure™ Yeast DNA Purification Kit (Epicentre Biotechnol., Madison, USA) according to the manufacturer's instructions.⁴¹

Asp1-Asp2 HRM assay design

Annotated *Aspergillus benA* genes were extracted from public databases to make multiple alignments using Clustal Omega. When designing primers, three main criteria were considered: (i) the amplicon length beyond 200 bp was considered to be maleficent, (ii) the length of forward and reverse primers should be beyond 18 bp to enhance specificity and proper hybridization to the target regions, (iii) amplicons should cover enough mismatches to enable proper discrimination among the tested strains.

Verification of the amplicons

Before applying the Asp1 and Asp2 primer sets (Fig. 1) on *Aspergillus* clinical isolates we pretested them on the genomic DNA of *A. fumigatus* (Af293), *A. lentulus* (CBS 117885), *A. terreus* (FGSC A1156), *A. flavus* (NRRL 11611), *A. niger* (CBS 113.46), *A. tubingensis* (CBS 134.48) reference strains. The yielded Asp1 and Asp2 *benA* amplicons were electrophoresed on 1% TAE-agarose gel stained with ethidium-bromide. PCR products were purified using post-reaction clean-up columns (Sigma-Aldrich, Missouri, USA). For capillary sequencing BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Scientific, Maryland, USA) was used. Cycle sequencing PCR was performed according to manufacturers' protocol. Capillary sequencing was performed on ABI Prism 3100-Avant

Genetic Analyzer instrument (Applied Biosystems) in both directions using the Asp1 and Asp2 forward and reverse primers. Figure 1 shows the thermal stability of the Asp1 and Asp2 amplicons along with their guanine and cytosine (GC) content and melting temperatures (T_m). Sequencing data were then analyzed comparing to the databases (<http://blast.ncbi.nlm.nih.gov/BLAST.cgi>) to clarify any discrepancy.

Setting the optimal HRM-real time PCR conditions

In order to monitor the accumulation of the amplified products through real-time PCR reactions, to determine characteristic melting-curve profiles and the representative melting temperatures (T_m) of the different strains, the real-time

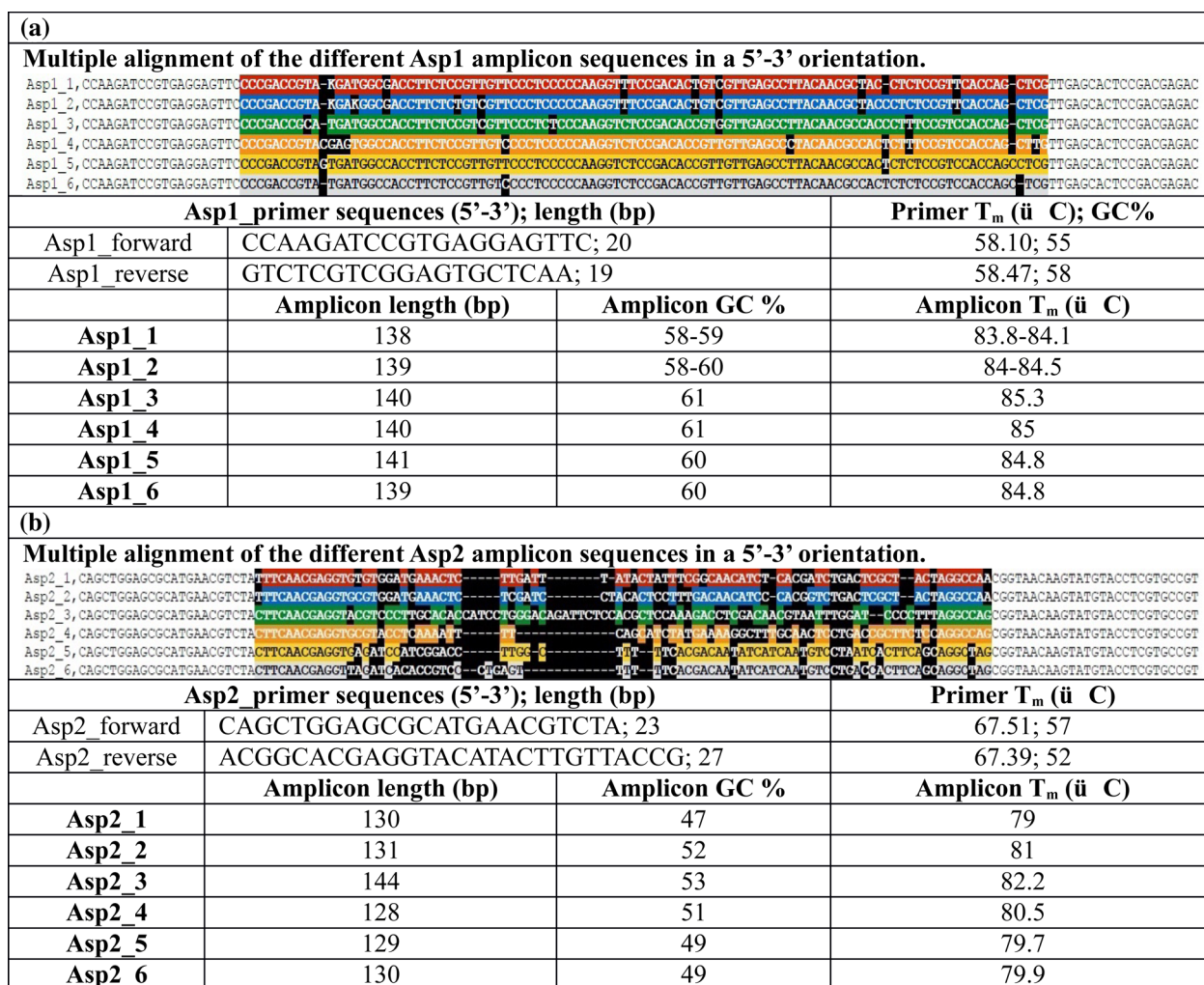


Figure 1. Multiple alignment of the amplicons of the Asp1 (Asp1.1 - Asp1.6) and Asp2 (Asp2.1 - Asp2.6) melting domains in a 5'-3' orientation representing the interspecies variations. Asp1 (a) and Asp2 (b) forward and reverse primer sequences are highlighted in grey. Numbers and colors specify the origin of the different target DNA molecules: 1.red (*A. fumigatus* Af293); 2.blue (*A. lentulus* CBS 117885); 3.green (*A. terreus* FGSC A1156); 4.orange (*A. flavus* NRRL 11611); 5.yellow (*A. niger* CBS 11.346); 6.grey (*A. tubingensis* CBS 134.48). Amplicon and primer length, guanine and cytosine (GC) content and melting temperatures (T_m) are also shown. Amplicon mismatches are depicted by black color. The sequence differences of the amplicons will bring different melting temperature (T_m) values for the Asp1 and Asp 2 amplicons. This Figure is reproduced in color in the online version of *Medical Mycology*.

PCR amplification reactions of the target molecules were conducted in a LightCycler 96 thermal cycler (Roche Diagnostics, Risch-Rotkreuz, Switzerland) instrument using the High Resolution Master Mix 480 (Roche Applied Science, Penzberg, Germany) that contained saturating double-stranded DNA binding Light Cycle 480 ResoLight dye. Figure 1 shows the forward and reverse Asp1 and Asp2 primer sequences.

- **Annealing temperature optimization.** Temperature gradient assay was performed from 55 to 72°C for assessing the performance of the primer pair during amplification with a temperature gradient program using the LightCycler 96 Instrument.
- **MgCl₂ concentration optimization.** The MgCl₂ optimization was performed by adding different amounts of MgCl₂ in the range 1 to 3.5 mM.
- **Primer concentration optimization.** The primer optimization assay was performed using 0.2, 0.5, 0.8 µM of the primer sets Asp1 and Asp2.

The 20 µl reactions consisted of 10 µl 2× LightCycler 480 High Resolution Melting Master (Roche Applied Science), 0.5–0.5 µl (0.2 µM) Asp1 and Asp2 primer sets in a 1:1 ratio, 2.4 µl MgCl₂ (3 mM) and 6.6 µl template DNA (20 ng/PCR reaction). There were two negative controls without DNA (non-template control - NTC). The thermocycling reactions (PCR) were conducted in a LightCycler 480 Multiwell Plate 96, white (Roche Diagnostics, Risch-Rotkreuz, Switzerland) using an initial denaturing step of 95°C for 10 minutes followed by 55 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 15 s and extension at 72°C for 10 s. All fluorescent data were collected in the ResoLightDye channel (470/514 nm) of the PCR instrument at the end of the cycles. Following the completion of real-time PCR, the products were denatured at 95°C for 60 s (4.4°C/s) and then renatured at 40°C for 60 s to randomly form DNA duplexes. HRM analysis was performed by increasing the temperatures from 65 to 95°C (0.04°C/s) recording changes in fluorescence with changes in temperature (dF/dT) and plotting against changes in temperature. The HRM profiles were then analyzed using the LightCycler®96 Software Version 1.1 (Roche Diagnostics, Risch-Rotkreuz, Switzerland).

Taxonomy footprints

To typify the fungal footprints of the Asp1-Asp2 HRM assay on the major, clinically relevant *Aspergilli* the primers were used with the genomic DNA samples of the *A. fumigatus* Af293, *A. lentulus* CBS 117885, *A. terreus* FGSC A1156, *A. flavus* NRRL 11611, *A. niger* CBS 113.46, *A. tubingensis* CBS 134.48 reference strains and with the

gDNA of the *A. welwitschiae* SZMC 2402 clinical isolate depicted by ID35 in Table 1. Approximately 20 ng of genomic DNA was used for every PCR reaction. To determine the differences in thermal stability of the resulting amplicons and representing the characteristic duplex T_m peaks (LightCycler®96 HRM analysis Software, Roche Diagnostics) and the descriptive melting curve profiles of the different strains samples were analyzed in duplicates.

Limit of detection

For measuring the analytical sensitivity of the Asp1-Asp2 HRM assay we artificially contaminated (spiked) PCR grade water samples with fungal gDNA, and we made seven reference panels (*A. fumigatus* Af293 panel.1, *A. lentulus* CBS 117885 panel.2, *A. terreus* FGSC A1156 panel.3, *A. flavus* NRRL 11611 panel.4, *A. niger* CBS 113.46 panel.5, *A. welwitschiae* ID-35 panel.6 and *A. tubingensis* CBS 134.48 panel.7). Serial dilution was made in a 5 log range with 30 ng, 3 ng, 300 pg, 30 pg, 3 pg fungal gDNA in 6.6 µl nuclease free water (S1). Triplicate PCR reactions were performed. Threshold cycle (C_q) data were estimated. The correlation between C_q and genomic load was determined by linear regression plotting C_q values against the log of genome number. Standard curves were built where the linear ranges of these plots determined the linear dynamic ranges. Efficiency was calculated according to the following formula, $E = (10 - 1 / \text{slope})$. Efficiency was converted to percentage efficiency by using the formula, $E\% = (E - 1) \times 100$.^{42,43}

Limit of reliable identification

For measuring the limit of reliable identification of the Asp1-Asp2 HRM assay we estimated the lowest template DNA concentration by which the joint appearance of the Asp1 and Asp2 melting domains are still observable. We also compared the overlaying melting peaks of the melting domains of the different *Aspergillus* reference panels to test the different template DNA concentrations (30 ng – 300 fg) providing reliable HRM patterns.

Cross reactions and discriminatory power

- Possible cross reactions of the Asp1-Asp2 HRM assay were tested with approximately 5–25 ng human genomic DNA samples of human placenta (Sigma Aldrich, Missouri, USA) and with gDNA samples of two *Candida* type strains (*Candida albicans* ATCC 10231, *C. parapsilosis* ATCC 22019), further seven *Candida* (ID44–50), three

Aspergillus (ID41–43), six *Fusarium* (ID51–53), two *Scedosporium* (ID57–58) isolates (Table 1).

- The discriminatory power of the Asp1-Asp2 HRM assay was tested on three *Aspergillus* gDNA panels (*Aspergillus* panel_1, _2, _3) composed of 5–15 ng gDNA extracted from the pure cultures of the *Aspergillus* clinical strains (ID1-ID40) (Table 1) on three different days (plate_1, _2, _3). Duplicate PCR reactions were performed in case of every sample with the Asp1-Asp2 primer sets of the duplex HRM assay. Following thermocycling reactions downstream HRM analysis was performed in a closed tube manner in case of every single sample of the three *Aspergillus* clinical panels (S2). Upon completion of the HRM analyses Asp1 and Asp2 T_m data of the 40 different clinical strains were subtracted and assigned to six *Aspergillus* species; *A. fumigatus*, *A. lentulus*, *A. terreus*, *A. flavus*, *A. niger*, *A. welwitschiae*, and *A. tubingensis*.

In-house quality assessment of Asp1-Asp2 duplex HRM assay

The aim was to estimate the precision of the Asp1-Asp2 HRM assay and to confirm that results generated are consistent over time.

- **Determining the repeatability.** To estimate the intra-assay consistency of the Asp1-Asp2 HRM assay coefficient of variation (% C.V.) was calculated for Asp1- T_m and Asp2- T_m triplicate melting temperatures in case of every sample of the six different *Aspergillus* reference gDNA panels and on the *A. welwitschiae* ID35 clinical gDNA panel. For this, standard deviation (\pm SD) of triplicates was taken, dividing that numbers by the means of the triplicate values and multiplying them by 100 (S1). Finally, the grand mean of the sample coefficient of variations (average % C.V.-s) of the triplicates was taken. In case the intra-assay % C.V. is less than 10% the method has high precision.
- **Determining the reproducibility.** Inter-assay consistency (plate-to-plate variation) of the Asp1-Asp2 duplex HRM assay was estimated between the three *Aspergillus* clinical panels containing the DNA samples of 40 different *Aspergillus* clinical strains. Duplicate PCR reactions were performed on every sample on three different days (plate_1, plate_2, plate_3). Asp1- T_m and Asp2- T_m means were calculated (S2). Plate T_m duplicate means of adherent clinical strains of the different species were assembled and overall mean was calculated. Plate coefficient of variations (% C.V.) was calculated (Table 2). Finally, grand mean of the sample coefficient of variations (average % C.V.-s) was taken. Inter-assay % C.V. values less than 15% are generally acceptable.

Results

In silico assessment of the discriminatory capacity

Using the DNA sequence data of the GENE database of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/gene>) we designed primers based on *Aspergillus benA* sequences (Fig. 1a). First we ascertained in advance that the Asp1-Asp2 HRM assay shows sufficient diversity for the species level identification and discrimination of relevant *Aspergillus* species by testing our Asp1 and Asp2 primer sets with the gDNA isolates of the reference *A. fumigatus* (Af293), *A. lentulus* (CBS 117885), *A. terreus* (FGSC A1156), *A. flavus* (NRRL 11611), *A. niger* (CBS 113.46), and *A. tubingensis* (CBS 134.48) strains. After capillary sequencing we successfully re-identified the resulted amplicon sequences. The approximate Asp1 and Asp2 domain lengths proved to be about 136 ± 8 bp in length. Melting temperature (T_m) data were calculated to the Asp1 and Asp2 melting domains (range; 79–85.3°C). The mean melting temperature (T_m) values of the resulted amplicons proved to be $82.27^\circ\text{C} \pm 1.91$, suggesting that the Asp1-Asp2 HRM assay shows sufficient diversity among clinically relevant *Aspergillus* species allowing their identification (Fig. 1b).

Optimal reaction conditions

Optimal reaction conditions were determined as described in the materials section. 0.2 μM primer concentration, 3 mM MgCl_2 and annealing at 62°C proved to be optimal.

Footprints of the assay on different species

Asp1-Asp2 HRM assay was applied on panels containing approximately 20 ng of the genomic DNA of seven *Aspergillus* strains. Following real-time PCR amplifications HRM analyses were performed. We were able to source unambiguously the six clinically relevant *A. fumigatus* (Af293), *A. lentulus* (CBS 117885), *A. terreus* (FGSC A1156), *A. flavus* (NRRL 11611), *A. niger* (CBS 113.46), *A. tubingensis* (CBS 134.48) reference strains and the *A. welwitschiae* (ID35) clinical isolate on the basis of their characteristic thermodynamic patterns and the relative distribution of their double (Asp1- T_{m2} and Asp2- T_{m1}) melting peaks. Representative normalized peaks of the Asp1 and Asp2 melting domains are shown in Figure 2a. The melting curves were normalized to eliminate differences in background fluorescence and are shown in the form of a temperature-shifted curve along the temperature axis (Fig. 2b).

Table 2. Calculating inter-assay coefficient of variation of the Asp1-Asp2 duplex HRM assay.

	<i>Aspergillus</i> panel_1 / plate_1		<i>Aspergillus</i> panel_2 / plate_2		<i>Aspergillus</i> panel_3 / palte_3	
	Mean of Asp1 T _m (°C) ±(SD)	Mean of Asp2 T _m (°C) ±(SD)	Mean of Asp1 T _m (°C) ±(SD)	Mean of Asp2 T _m (°C) ±(SD)	Mean of Asp1 T _m (°C) ±(SD)	Mean of Asp2 T _m (°C) ±(SD)
<i>A. fumigatus</i> (ID8-21)	86.69±0.12	82.08±0.12	86.69±0.15	82.06±0.12	86.71±0.11	82.11±0.11
Overall mean	84.39°C		84.38°C		84.41°C	
(±SD) of T _m means	±(2.36)		±(2.36)		±(2.34)	
Plate C.V. %	2.80		2.80		2.78	
<i>A. lentulus</i> (ID22-24)	86.82±0.13	84.27±0.17	86.88±0.11	84.18±0.17	86.93±0.13	84.28±0.14
Overall mean	85.56°C		85.53°C		85.61°C	
(±SD) of T _m means	±(1.42)		±(1.49)		±(1.46)	
Plate C.V. %	1.61		1.71		1.7	
<i>A. terreus</i> (ID1-7)	87.94±0.19	84.50±0.36	87.97±0.17	84.54±0.34	88.00±0.17	84.54±0.36
Overall mean	86.30°C		86.25°C		86.27°C	
(±SD) of T _m means	±(1.73)		±(1.80)		±(1.82)	
Palte C.V. %	2.00		2.09		2.11	
<i>A. flavus</i> (ID25-32)	87.35±0.18	84.15±0.10	87.33±0.15	84.03±0.07	87.42±0.16	84.12±0.05
Overall mean	85.75°C		85.68°C		85.77°C	
(±SD) of T _m means	±(1.66)		±(1.71)		±(1.71)	
Plate C.V. %	1.94		1.99		1.99	
<i>A. niger</i> (ID33, 34)	87.54±0.04	83.12±0.04	87.47±0.04	83.01±0.05	87.53±0.04	83.07±0.07
Overall mean	85.35°C		85.24°C		85.3°C	
(±SD) of T _m means	±(2.34)		±(2.38)		±(2.38)	
Plate C.V. %	2.74		2.79		2.79	
<i>A. welwitsciae</i> (ID 35-38)	87.38±0.09	82.38±0.98	87.43±0.11	82.37±0.23	87.46±0.05	82.45±0.07
Overall mean	84.87°C		84.90°C		85.0°C	
(±SD) of T _m means	±(2.67)		±(2.70)		±(2.68)	
Plate C.V. %	3.15		3.19		3.15	
<i>A. tubingensis</i> (ID39, 40)	87.70±0.04	83.09±0.03	87.74±0.11	83.08±0.06	87.78±0.03	83.08±0.05
Overall mean	85.40°C		85.41°C		85.43°C	
(±SD) of T _m means	±(2.47)		±(2.49)		±(2.51)	
Plate C.V. %	2.89		2.92		2.94	
Grand C.V. %	2.48%					

Note: Asp1-T_m and Asp2-T_m duplicate means of adherent clinical strains of the different species were assembled in case of every plate and overall mean was calculated. Plate coefficient of variation was calculated for the different species. Finally, grand mean of the sample coefficient of variations (average % C.V.-s) was calculated.

Limit of detection and the limit of reliable identification

The detection limit of the Asp1-Asp2 HRM assay was determined on seven *Aspergillus* gDNA panels (S1) containing serially diluted genomic DNA samples in a 5-log range (30 ng to 3 pg). In each case, triplicate PCR reactions were performed to subtract threshold cycle (C_q) values and to analyze overlaying Asp1 and Asp2 melting peaks of the different samples. To study the correlation between the C_q-s

of the qPCR and the genomic load standard curves were obtained by plotting C_q values against the log of genome number (GE). Linear dynamic ranges (Fig. 3a), PCR efficiency (E) and correlation coefficient (R²) were also estimated using the standard curve data (Fig. 3b) of the different *Aspergillus* panels. Along with measuring the analytical sensitivity we also estimated the lowest concentration of template DNA where reliable identification was attainable with our assay on these gDNA panels. The lowest amount

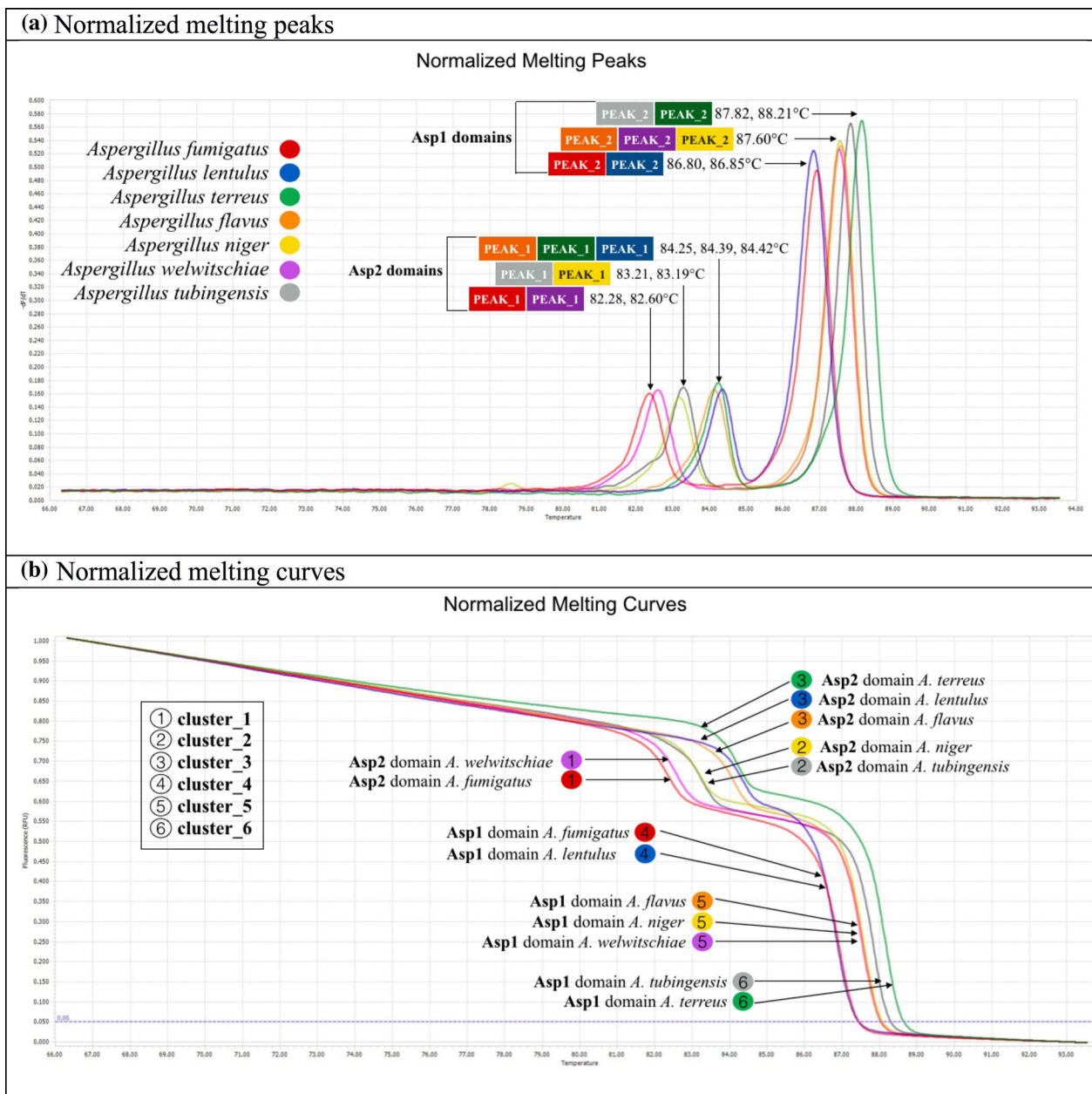


Figure 2. Independence of the template DNA belonging to the different *Aspergillus* species and the HRM melting profiles. (a). Distribution of the double melting peaks barcoding the genomic DNA of the different *Aspergillus* species. The negative derivative of the fluorescence (F) over temperature (T); $-dF/dT$ curve displays representative plots and the representative T_m values for *Aspergillus fumigatus* Af293 (T_{m1} ; 82.28°C, T_{m2} ; 86.80°C), *A. lentulus* CBS 117885 (T_{m1} ; 84.28°C, T_{m2} ; 86.85°C), *A. terreus* IH2624 (T_{m1} ; 84.39°C, T_{m2} ; 88.21°C), *A. flavus* NRRL 11611 (T_{m1} ; 84.25°C, T_{m2} ; 87.60°C) and *A. niger* CBS 11346 (T_{m1} ; 83.19°C, T_{m2} ; 87.60°C) and *A. welwitschiae* ID 35 (T_{m1} ; 82.6°C, T_{m2} ; 87.60°C) and *A. tubingensis* CBS 134.48 (T_{m1} ; 83.21°C, T_{m2} ; 87.82°C). (b). Representation of the normalized melting curves of Asp1-Asp2 HRM assay of the Asp1 and Asp2 melting domains. Overlaying melting curves formed six discrete clusters. Asp2 primers form the first three melting clusters; melting curves of *A. fumigatus* (red) and *A. welwitschiae* (pink) show no distinct difference in cluster_1, cluster_2 represents *A. niger* (yellow) and *A. tubingensis* (grey), while cluster_3 represents *A. lentulus* (blue), *A. flavus* (orange) and *A. terreus* (green). Asp1 primers form further three melting clusters; cluster_4 represents *A. fumigatus* (red) and *A. lentulus* (blue), melting curves of *A. flavus* (orange), *A. niger* (yellow) and *A. welwitschiae* (pink) show no distinct difference in cluster_5, while both *A. tubingensis* (grey) and *A. terreus* (green) fall into cluster_6. This Figure is reproduced in color in the online version of *Medical Mycology*.

of the template DNA where reliable HRM curves were obtainable with conclusive double peaks of the Asp1 and Asp2 melting domains proved to be 3 pg (10^2 GE) on all gDNA panels (Figure 4b, 4g) but the *A. fumigatus* Af293 and the

A. tubingensis CBS 134.48 gDNA panels (Fig. 4a). In the case of the *A. fumigatus* Af293 panel the Asp1-Asp2 duplex HRM assay provided unreliable melting curves in the presence of 3 pg gDNA representing only a single,

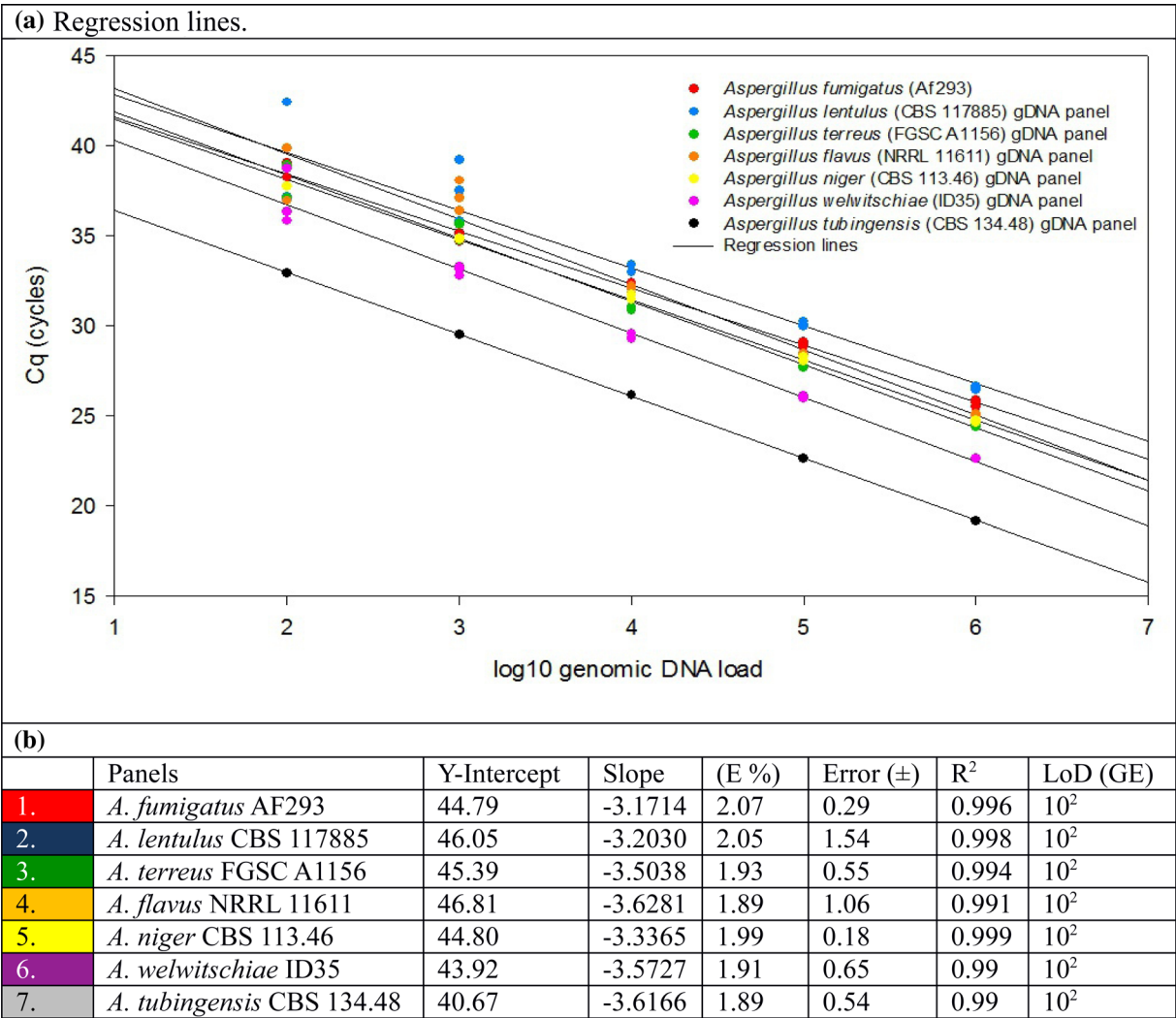


Figure 3. (a). Regression lines of Asp1-Asp2 HRM assay on *Aspergillus* gDNA panels. The limit of detection (LoD) of the combined Asp1-Asp2 HRM assay was evaluated on seven various fungal gDNA panels of the reference *Aspergillus fumigatus* (Af293), *A. lentulus* (CBS 117885), *A. terreus* (FGSC A1156), *A. flavus* (NRRL 11611), *A. niger* (CBS 113.46) and *A. tubingensis* (CBS 134.48) type strains and on the gDNA of the *A. welwitschiae* ID35 clinical isolate. In the case of the seventh (*A. tubingensis* CBS 134.48) panel the limit of reliable detection proved to be 10 GE (data not shown). (b). Representation of slope, reaction efficiency (E %), error (±), coefficient of correlation (R²), and limit of detection (LoD). This Figure is reproduced in color in the online version of *Medical Mycology*.

inconclusive melting domain of this sample (Fig. 4a). When testing *A. tubingensis* CBS 134.48 gDNA panel, the reliable LoD proved to be 300 fg (10 GE) (Fig. 4g).

Assay cross reactivity

The cross-reactivity of the Asp1-Asp2 HRM assay was examined with human genomic DNA (5–25 ng), *Aspergilli* isolates (ID41–43), *Candida* type strains (*Candida albicans* ATCC 10231, *C. parapsilosis* ATCC 22019), and *Candida* (ID44–50), *Fusarium* (ID51–56) and *Scedosporium* (ID57–58) clinical isolates (Table 1). No cross-amplification was detected with the human genomic DNA and no misidentification was observed when analyzing the above mentioned strains. Moderate false-positivity was observed when

testing *Candida* gDNA samples generating cycle threshold (Cq) values greater than Cq > 38, displaying inconclusive HRM curve shapes and peaks below <81.00 °C (data not shown). Two *Aspergillus* isolates provided single peaks (*A. viridinutans* at 85.38–85.41°C; *A. udagawae* at 86.12–86.17°C). Lack of double peak formation was also detected when testing the *Fusarium* isolates (*F. napiforme* at 87.61–87.68°C; *F. delphinoides* at 87.60°C; *F. verticilloides* at 87.81–87.90°C; *F. oxysporum* at 87.30–87.56°C; *F. solani* at 87.44–87.57°C; *F. incarnatum* at 86.31–86.44°C). Both *Scedosporium aurantiacum* isolates formed amorphous single peaks at 84.11–84.22°C. The appearance of the single peak formation underlines the significance of the collective consideration of the Asp1 and Asp2 double peaks.

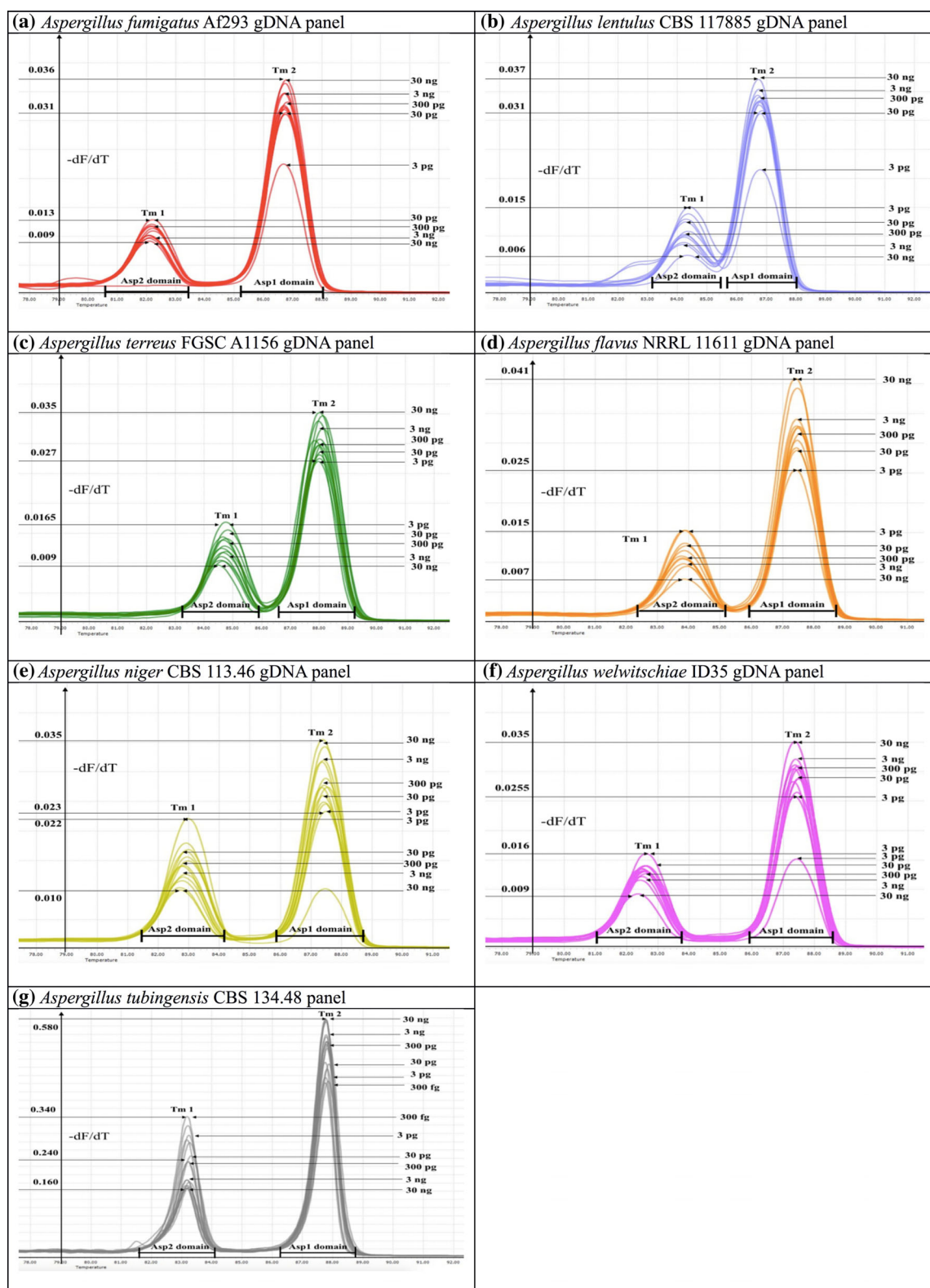


Figure 4. Overlaying melting peaks of the Asp1 and Asp2 melting domains of the different *Aspergillus benA* genes on different genomic DNA panels and the limit of the reliable identification. The identification capacity of the Asp1-Asp2 assay proved to be reliable providing double melting peaks on Asp1 (T_{m2}) and Asp2 (T_{m1}) melting domains in the presence of 30 ng – 3 pg template concentrations on all panels (b: *A. lentulus*, c: *A. terreus*, d: *A. flavus*, e: *A. niger*, f: *A. welwitschiae*, g: *A. tubingensis*) but the *A. fumigatus* Af293 (a). In the case of this latter mentioned panel in the presence of 3 pg template *A. fumigatus* Af293 gDNA the representative melting peak (T_{m1}) of the Asp2 melting domain did not appear. In the case of the *A. tubingensis* CBS 134.48 panel (g) reliable double peak formation was also detected in the presence of 300 fg genomic DNA. This Figure is reproduced in color in the online version of *Medical Mycology*.

Maximum barcoding power was achieved by allocating six melting peak windows and by the consideration of the melting peak distances

- Estimating the barcoding accuracy of the Asp1-Asp2 HRM assay melting temperature data from the previously introduced *Aspergillus* reference DNA panels (*A. fumigatus* Af293, *A. lentulus* CBS 117885, *A. terreus* FGSC A1156, *A. flavus* NRRL 11611, *A. niger* CBS 113.46, *A. tubingensis* CBS 134.48) were taken and grouped into 12 data groups according to their distribution (Asp1 melting domain T_m data: group 1–6; Asp2 melting domain T_m data: group 7–12) and according to the origin of the template DNA (*A. fumigatus* Af293: gr.1, gr.7; *A. lentulus* CBS 117885: gr.2, gr.8; *A. terreus* FGSC A1156: gr.3, gr.9; *A. flavus* NRRL 11611: gr.4, gr.10; *A. niger* CBS 113.46: gr.5, gr.11; *A. tubingensis* CBS 134.48: gr.6, gr.12) (Figure 5).
- Furthermore, three *Aspergillus* clinical panels (panel.1, .2, .3) were constructed containing samples of 5–15 ng gDNA extracted from the pure cultures of the 40 available *Aspergillus* clinical strains (ID1–ID40). Upon completion of HRM analyses Asp1- T_m and Asp2- T_m data were taken and assigned to seven *Aspergillus* species as also shown in Figure 5; *A. fumigatus* (ID8–21) *A. lentulus* (ID22–24), *A. terreus* (ID1–7), *A. flavus* (ID25–32), *A. niger* (ID33–34), *A. welwitschiae* (ID35–38), and *A. tubingensis* (ID39–40).
- Whisker plots were made where group1 to group12 represent the distribution of the melting domain T_m values measured on the different *Aspergillus* reference panels while group 13–group 26 show the distribution of the Asp1 and Asp2 T_m values of the 40 *Aspergillus* clinical panels (Fig. 5). For every data set the median, minimum, maximum T_m values along with the 25th and 75th percentile are shown. According to the relative distribution of the melting peaks of the different datasets we created six melting clusters; cluster.1 (81.00–82.72°C), cluster.2 (82.73–83.61°C), cluster.3 (83.62–85.60°C), cluster.4 (85.61–87.10°C), cluster.5 (87.11–87.64°C), cluster.6 (87.65–89.00°C) for the accurate identification of the different *Aspergillus* strains to the species level (Table 3).
- To enhance the discrimination between *A. terreus* (score 36) and *A. flavus* (score 35), *A. lentulus* (score 34) and *A. flavus* (score 35), *A. fumigatus* (score 14) and *A. welwitschiae* (score 15) and the *A. niger* (score 25) and *A. welwitschiae* (score 15), finally *A. niger* (score 25) and *A. tubingensis* (score 26) sharing one clusters in common and adjacent clusters we also suggest considering the peak distances as an adjunct parameter when barcoding the species (Fig. 6a). Species specific Asp1 and Asp2 peak T_m values were used for measuring the median

of the T_m difference data (Fig. 6b–6c). Mann–Whitney statistics was used to compare the Asp1–Asp2 peak T_m difference data sets. It was estimated that the difference in species specific data sets (35 vs. 36, 34 vs. 35, 14 vs. 15, 15 vs. 25, and 25 vs. 26) is greater ($P = <.001$) than would be expected by chance representing a statistically significant difference between the species (*A. flavus* vs. *A. terreus* and *A. lentulus*, *A. fumigatus* vs. *A. welwitschiae*, finally *A. niger* vs. *A. welwitschiae* and *A. tubingensis*) tested.

Asp1–Asp2 HRM assay passed the in-house quality assessment

To assess the repeatability of the Asp1–Asp2 HRM assay we applied on the six *Aspergillus* reference panels (*A. fumigatus* Af293, *A. fumigatus* Af293, *A. lentulus* CBS 117885, *A. terreus* FGSC A1156, *A. flavus* NRRL 11611, *A. niger* CBS 113.46, *A. tubingensis* CBS 134.48) and on the *A. welwitschiae* ID35 panel. For measuring the precision of the assay average coefficient of variation was calculated for the triplicate Asp1 and Asp2 mean T_m values where intra-assay % C. V. proved to be 0.09% accounting for a very high accuracy of the assay (S1). Plate-to-plate consistency was also assessed for the assay on three *Aspergillus* clinical panels (panel.1, .2, .3) on three different days composing of 5–15 ng genomic DNA of the 40 different clinical isolates (S2). Inter-run precision (reproducibility) was measured between three *Aspergillus* clinical panels and sample coefficient of variations (average % C.V.-s) between the three plates were taken, where inter-assay % C.V proved to be 2.44% (Table 2), which is highly acceptable.

Discussion

Aspergillosis is the most common invasive mold disease worldwide,⁵ and to date, there is a growing number of various molecular methods to identify biological samples contaminated with traces of *Aspergillus* conidia or DNA.^{23,26,27,30–34} Rapid and noninvasive molecular barcoding methods for detecting and identifying pathogens directly from clinical samples are under the spotlight^{35–39} since most cultured specimens have only a single dominant causative agent;^{44,45} furthermore, the number of clinically relevant *Aspergillus* species may also be limited.⁴⁶ Recent data also support that applications resting on high resolution melting analyses may be ideally suited for barcoding of fungal pathogens.^{44–49}

Species level identification of the *Aspergillus* species may be important especially in case of the cryptic species because some of these strains are associated with special growth

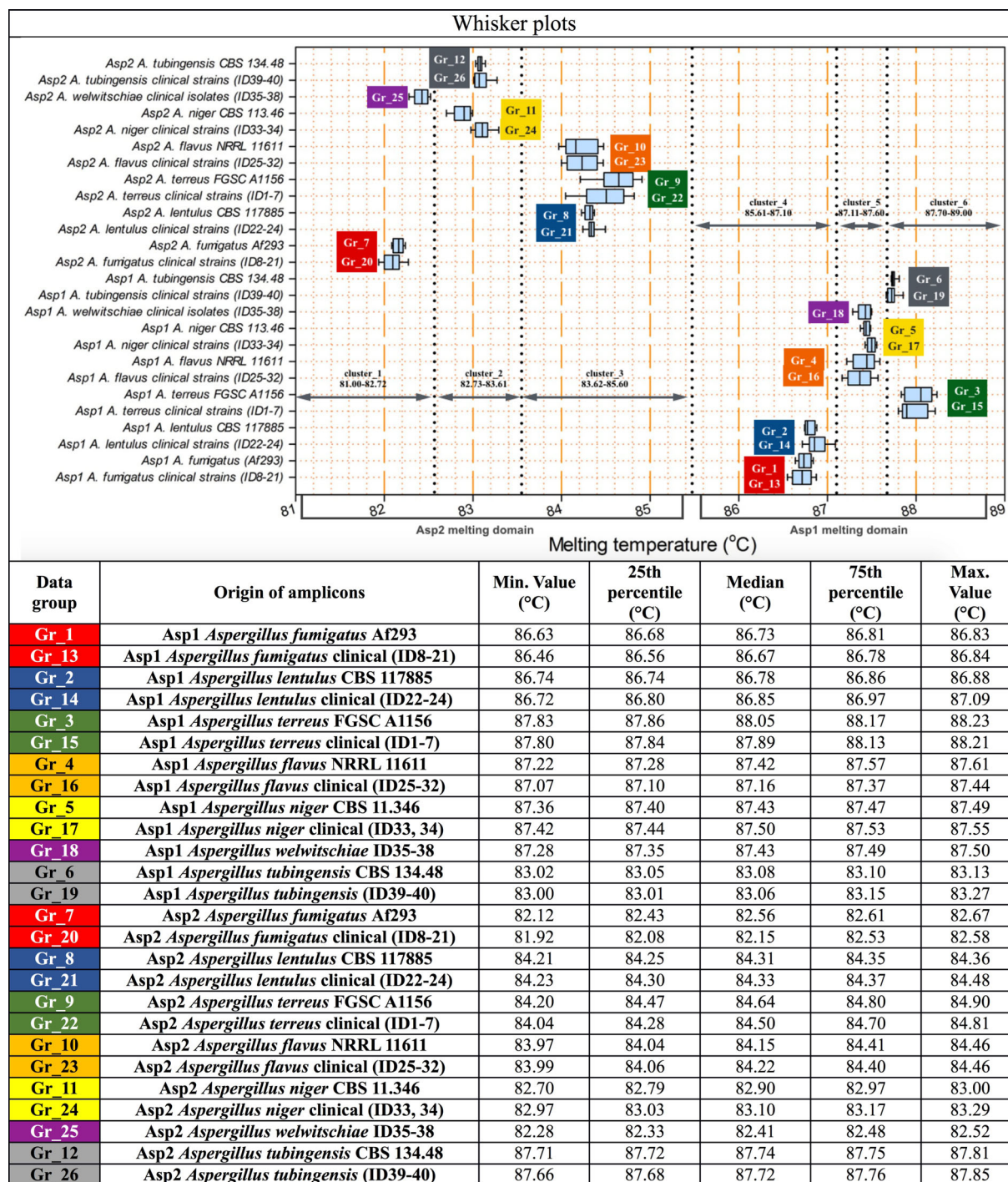


Figure 5. Whisker plots showing the distribution of the melting-temperatures (T_m) of the Asp1 and Asp2 melting domains for the six distinct melting clusters in case of the 22 different datasets. Asp1 and Asp2 amplicon melting temperature data (T_m) were ordered into 26 groups according to the origin of the template DNA target molecules and the Asp1 and Asp2 primer sets used to amplify certain regions of the *Aspergillus benA* genes. Temperatures of melting (T_m) data were subtracted from the analysis of the six *Aspergillus* reference panels (data groups.1-12) and from the three *Aspergillus* clinical.panels (data groups.13-26). Whisker plots were constructed for each data group showing the range of obtained temperatures of melting (T_m); the minimum, the median, and the maximum T_m values with 25th and 75th percentiles. Figure 5 also displays the melting temperature regions of the six pre-set melting clusters (cluster.1 – cluster.6) they were allocated according to the relative localization of the whisker plots. Cluster.1 (81.00 – 82.72°C); cluster.2 (82.73 – 83.61°C); cluster.3 (83.62 – 85.60°C); cluster.4 (85.61 – 87.10°C); cluster.5 (87.11 – 87.64°C); cluster.6 (87.65 – 89.00°C). This Figure is reproduced in color in the online version of *Medical Mycology*.

Table 3. Representation of the six melting peak cluster (cluster_1-cluster_6) T_m ranges of the Asp1 and Asp2 melting domains.

Clinical strains tested	mean Tm°C (±SD)						Binomial codes barcoding the different species		
	Asp2 melting domain Tm°C 81.00 – 85.50			Asp1 melting domain Tm°C 83.61 – 89.00					
	cluster_1 81.00-82.72	cluster_2 82.73-83.61	cluster_3 83.62-85.60	cluster_4 85.61-87.10	cluster_5 87.11-87.64	cluster_6 87.65-89.00			
<i>A. terreus</i> SZMC 2414	-	-	84.13±0.04	-	-	88.10±0.04	3	6	(36) ✓
<i>A. terreus</i> SZMC 2424	-	-	84.35±0.04	-	-	87.84±0.04	3	6	(36) ✓
<i>A. terreus</i> SZMC 2460	-	-	84.60±0.13	-	-	87.84±0.04	3	6	(36) ✓
<i>A. terreus</i> SZMC 22546	-	-	84.04±0.05	-	-	88.19±0.04	3	6	(36) ✓
<i>A. terreus</i> SZMC 22547	-	-	84.90±0.07	-	-	87.84±0.06	3	6	(36) ✓
<i>A. terreus</i> SZMC 22548	-	-	84.88±0.06	-	-	87.81±0.05	3	6	(36) ✓
<i>A. terreus</i> SZMC 22549	-	-	84.79±0.05	-	-	88.17±0.04	3	6	(36) ✓
<i>A. fumigatus</i> SZMC 2504	82.18±0.07	-	-	86.81±0.05	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 2490	82.07±0.08	-	-	86.75±0.07	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 2486	82.22±0.08	-	-	86.84±0.07	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 22550	82.17±0.09	-	-	86.77±0.06	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 22551	82.08±0.06	-	-	86.72±0.03	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 22552	82.13±0.04	-	-	86.77±0.05	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 22553	82.12±0.07	-	-	86.75±0.09	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 22554	82.18±0.10	-	-	86.79±0.08	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 3111	81.85±0.09	-	-	86.40±0.09	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 3109	82.12±0.09	-	-	86.71±0.06	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 3100	82.07±0.10	-	-	86.63±0.14	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 3117	82.07±0.15	-	-	86.66±0.12	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 3106	81.99±0.10	-	-	86.60±0.05	-	-	1	4	(14) ✓
<i>A. fumigatus</i> SZMC 3104	81.98±0.04	-	-	86.58±0.03	-	-	1	4	(14) ✓
<i>A. lentulus</i> SZMC 3118	-	-	84.36±0.11	86.82±0.08	-	-	3	4	(34) ✓
<i>A. lentulus</i> SZMC 3123	-	-	84.32±0.05	86.79±0.06	-	-	3	4	(34) ✓
<i>A. lentulus</i> SZMC 20911	-	-	84.10±0.05	87.03±0.06	-	-	3	4	(34) ✓
<i>A. flavus</i> SZMC 22583	-	-	84.12±0.07	-	87.24±0.08	-	3	5	(35) ✓
<i>A. flavus</i> SZMC 22582	-	-	84.12±0.07	-	87.24±0.08	-	3	5	(35) ✓
<i>A. flavus</i> SZMC 22581	-	-	84.16±0.10	-	87.27±0.05	-	3	5	(35) ✓
<i>A. flavus</i> SZMC 22580	-	-	84.10±0.06	-	87.47±0.07	-	3	5	(35) ✓
<i>A. flavus</i> SZMC 22579	-	-	84.06±0.10	-	87.12±0.09	-	3	5	(35) ✓
<i>A. flavus</i> SZMC 22578	-	-	84.03±0.06	-	87.45±0.05	-	3	5	(35) ✓
<i>A. flavus</i> SZMC 22577	-	-	84.16±0.11	-	87.58±0.06	-	3	5	(35) ✓
<i>A. flavus</i> SZMC 22575	-	-	84.05±0.12	-	87.48±0.14	-	3	5	(35) ✓
<i>A. niger</i> SZMC 3119	-	83.01±0.06	-	-	87.52±0.06	-	2	5	(25) ✓
<i>A. niger</i> SZMC 3108	-	83.07±0.10	-	-	87.35±0.12	-	2	5	(25) ✓
<i>A. welwitschiae</i> SZMC 2402	82.26±0.30	-	-	-	87.35±0.12	-	1	5	(15) ✓
<i>A. welwitschiae</i> SZMC 23407	82.37±0.11	-	-	-	87.38±0.11	-	1	5	(15) ✓
<i>A. welwitschiae</i> SZMC 23406	82.48±0.05	-	-	-	87.48±0.04	-	1	5	(15) ✓
<i>A. welwitschiae</i> SZMC 23408	82.48±0.05	-	-	-	87.45±0.05	-	1	5	(15) ✓
<i>A. tubingensis</i> SZMC 2758	-	83.05±0.03	-	-	-	87.77±0.06	2	6	(26) ✓
<i>A. tubingensis</i> SZMC 3127	-	83.11±0.02	-	-	-	87.71±0.06	2	6	(26) ✓

Note: Representation of the six melting peak cluster (cluster_1-cluster_6) T_m ranges of the Asp1 and Asp2 melting domains with the mean melting temperatures and standard deviations (±SD) assigned to the different clinical strains on completion of the analyses of the three *Aspergillus* clinical panels. Binomial scores were generated in case of the different strains tested according to their Asp1 and Asp2 melting clusters (cluster_1; score_1, cluster_2; score_2, cluster_3; score_3, cluster_4; score_4, cluster_5; score_5, cluster_6; score_6) unequivocally defining the different species according to their binomial scores; (14) for *A. fumigatus*, (34) for *A. lentulus*, (36) for *A. terreus*, (35) for *A. flavus*, (25) for *A. niger*, (15) for *A. welwitschiae*, (26) for *A. tubingensis*.

features and antifungal resistance.^{14,43,50–53} Reliable, species level detection of the typically moderately-growing fungi from cultured specimens may take several days³⁴ delaying adequate diagnosis and setting back the timely initiation of appropriate antifungal treatment. Prompt, correct, species level identification of pathogen fungi furthermore requires nucleic acid based techniques.^{30–34} Although HRM based methods do not have the resolving power as the sequencing or are not as sensitive as the TaqMan probe

based systems, they became more and more attractive to molecular diagnostic laboratories.^{35,39}

Multiple studies have demonstrated the limited utility and enhanced major drawbacks of morphotyping used alone for species identification of clinically relevant *Aspergilli* recognizing that DNA based applications used in tandem with morphological examinations can offer better resolution of species within the genus.¹⁴ The prime aim of this study was to describe a method that may be promising

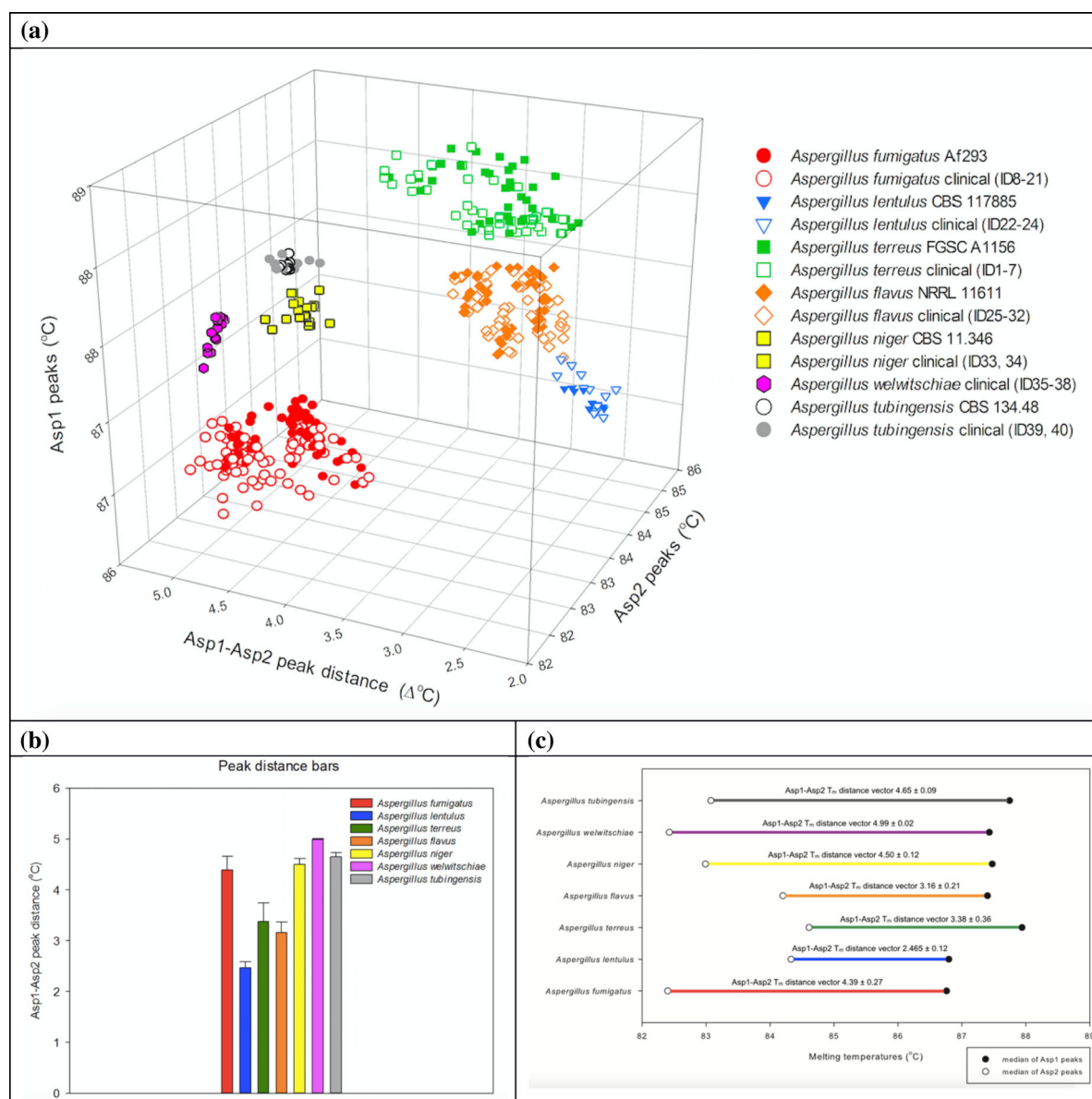


Figure 6. (a). 3D scatter plot representing the distinct habitats of the different type strains and clinical isolates of the 40 *Aspergilli* depicted by seven species specific colors; *A. fumigatus*: red, *A. lentulus*: blue, *A. terreus*: green, *A. flavus*: orange, *A. niger*: yellow, *A. welwitschiae*: pink and *A. tubingensis*: grey. Position of the spots was specified by the section of the three axes; scale_x was determined by T_m data (°C) of the Asp1 peaks, scale_y was determined by the T_m data (°C) of the Asp2 peaks while scale_z was determined by the difference in T_m data (delta °C) of the Asp1 and Asp2 peaks. (b). Bar diagrams showing the difference in Asp1 and Asp2 T_m data with standard deviations. (c). Vectors representing the mean Asp1-Asp2 T_m peak distances with their relative localizations. This Figure is reproduced in color in the online version of *Medical Mycology*.

especially to traditional culturing techniques in virtue of identifying numerous clinical isolates of relevant *Aspergilli* to species with very high accuracy.

Our Asp1–Asp2 HRM method introduced here uses two primer sets (Asp1 and Asp2) targeting two differently conserved regions (Asp1 and Asp2 melting domains) of the *Aspergillus benA* genes. On the basis of the thermodynamic characteristics of the amplicons and the joint appearance of the melting peaks of the Asp1 and Asp2 melting domains and their T_m peak distances, our assay was shown to

have higher resolution power displaying deviations among species than other single locus based HRM systems. This enables us to identify the gDNAs derived from 40 clinical isolates of the relevant opportunistic infectious agents (*A. fumigatus*, *A. lentulus*, *A. terreus*, *A. flavus*, *A. niger*, *A. welwitschiae*, and *A. tubingensis*) based on the thermodynamic characteristics of the amplicons with very high accuracy.

The specificity of the Asp1–Asp2 HRM assay was tested thoroughly *in silico*. The Asp1 and Asp2 amplicons of the six reference strains (*A. fumigatus* Af293, *A. lentulus* CBS

117885, *A. terreus* FGSC A1156, *A. flavus* NRRL 11611, *A. niger* CBS 113.46, *A. tubingensis* CBS 134.48) were sequenced; then the sequences were aligned surveying potential sequence deviations within species. Based on our data we presumed that our Asp1 and Asp2 amplicons will display enough sequence divergence between closely related species but at the same time may be conserved enough targeting the different clinical isolates within species.

In the work presented here the gDNA of six *Aspergillus* reference and further 40 clinical isolates were used as a proof of concept. Furthermore, our Asp1–Asp2 duplex HRM assay was tested and optimized experimentally on human gDNA and on relevant *Candida*, *Fusarium*, *Scedosporium* strains (Table 1). Neither the human gDNA, nor the clinical isolates resulted misidentification with our assay. We also proved that even the presence of excess human gDNA did not affect assay results.

Molecular barcoding of the different strains was conducted by real-time PCR amplification; then species discrimination was performed by analyzing of the characteristic thermodynamic profiles using generic double-stranded DNA binding fluorescent dyes. When testing the gDNA panels of the six type strains (*A. fumigatus*, *A. lentulus*, *A. terreus*, *A. flavus*, *A. niger*, *A. tubingensis*) along with the *A. welwitschiae* we managed to identify altogether six distinct melting clusters. Asp1 melting domain contains cluster_4; 85.61–87.10°C, cluster_5; 87.11–87.64°C, cluster_6; 87.65–89.00°C, while Asp2 melting domain contains cluster_1; 81.00–82.72°C, cluster_2; 82.73–83.61°C, cluster_3; 83.62–85.60°C.

When testing DNA samples of the numerous *Aspergillus* clinical strains (ID1–40) they displayed conclusive, species specific double melting peaks in the presence of 5–15 ng *Aspergillus* gDNA. Conversely, the peaks of the Asp1 and Asp2 domains of the examined *Aspergilli* were then assigned to the respective melting clusters. Binomial scores given to the strains unequivocally identified all the 40 examined *Aspergilli* (Table 3); thus, the species barcoding accuracy of the method proved to be 100%. The analytical sensitivity of this assay was also measured and proved to be 10² or lower GE on all reference panels but the *A. fumigatus* panel. This information may be necessary when testing different liquid tissue samples that yield low copy numbers of fungal DNA, which is often the case when fungal gDNA is extracted from bronchoalveolar lavage (BAL), whole blood, serum, or plasma samples.

We also estimated the identification limit of the Asp1–Asp2 HRM assay by analyzing the overlaying melting peaks of the different melting clusters. From the observed cluster patterns, we concluded that our HRM assay is specific, provides highly reproducible thermodynamic characteris-

tics (Fig. 4), and could detect the subtle sequence differences of the Asp1 and Asp2 melting domains preferentially when extracting gDNA from young fungal cultures followed bead beating of the hyphae and conidia. In-house assay performance measurements were also conducted confirming the high accuracy and reproducibility of the Asp1–Asp2 assay. We also confirmed that Asp1 and Asp2 HRM assay results may be consistent over time using the same PCR–HRM platform.

Our prime purpose was to introduce a simple, robust, and highly reproducible molecular barcoding tool that relies on HRM analysis. This article describes a rapid, practical and precise DNA typing method with a high resolution power for the molecular identification of relevant *Aspergillus* clinical isolates to the species level. We believe that this method should be also applied in other research or diagnostic laboratories when more strains were available to test further relevant *Aspergillus* clinical isolates and to prove its applicability or to reveal possible drawbacks. Asp1–Asp2 may be capable to distinguish all clinically relevant strains of the above tested *Aspergilli* even at limiting initial template concentrations so the diagnostic power of our method should be further investigated on liquid tissue specimens. Nevertheless, just like other HRM based applications our molecular barcoding method introduced here possesses inherent simplicity so may be amenable for automatization. We hope that our method will help to identify and discriminate causative agents of aspergillosis more promptly and accurately giving more insight into the pathogenesis and treatment of infection.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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