# Prospective survey of *Aspergillus* species isolated from clinical specimens and their antifungal susceptibility: A five-year single-center study in Japan

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29

**Abstract**[1]

*Aspergillus fumigatus* is the most prevalent species that causes aspergillosis. *A. fumigatus* strains with tandem repeats in the *cyp51A* promoter have emerged in the environment. *Aspergillus* species other than *A. fumigatus* have also been recognized as causative agents of aspergillosis; however, they show lower susceptibility to antifungals compared with *A. fumigatus*. Therefore, it is important to precisely identify *Aspergillus* species and determine their antifungal susceptibility. Herein, we collected 119 mold strains isolated from clinical specimens collected at a hospital between November 2013 and December 2018. The collected strains were identified by sequencing several regions, including internal transcribed spacers, and determined their susceptibility to the antifungals itraconazole, voriconazole, and amphotericin B. Of 119 strains, 107 were *Aspergillus* species, which were identified as *A. fumigatus* (67), *Aspergillus* section *Nigri* (21), *A. flavus* (7), *A. terreus* (6), and *A. nidulans* (6). In *Aspergillus* section *Nigri*, the number of *A. niger* was less than the number of *A. welwitschiae* and *A. tubingensis*. Two azole-resistant *A. fumigatus* samples were included among the isolates. Four of the eight *A. tubingensis* isolates showed less susceptibility to voriconazole; however, all

[1] **Abbreviations**

| | |
|---|---|
| MIC | Minimum inhibitory concentrations |
| TR | Tandem repeat |

47 isolates of *A. niger* and *A. welwitschiae* were susceptible to itraconazole and
48 voriconazole. Because of lack of susceptibility data for non-fumigatus *Aspergillus* and
49 an increasing frequency of antifungal resistance among *A. fumigatus*, our data along
50 with further surveillance may contribute to determining the frequency and susceptibility
51 of *Aspergillus* spp. clinical isolates in Japan.
52
53 **Keywords:** *Aspergillus* species, antifungal susceptibility, clinical specimens,
54 filamentous fungus

55 The *Aspergillus* genus is well-known as the causative agent of aspergillosis, a major
56 mycosis in humans. *A. fumigatus* is the most prevalent species in the genus that acts as a
57 causative agent of aspergillosis [1,2]. Non-*fumigatus Aspergillus* species, such as *A.*
58 *niger*, *A. flavus*, and *A. terreus*, are also important causative agents of aspergillosis.
59 Although azoles have long been used for the treatment of aspergillosis, less susceptible
60 or resistant *Aspergillus* strains have recently emerged. During treatment with azoles,
61 substitution(s) can occur in the *cyp51A* gene in *A. fumigatus*, leading to antifungal
62 resistance [3,4]. In addition to strains that acquire resistance within a host,
63 azole-resistant *A. fumigatus* with a tandem repeat (TR) in the *cyp51A* promoter have
64 been isolated from the environment. These environmental strains are a cause of
65 aspergillosis in azole-naïve patients, which can lead to a delay in selecting the
66 appropriate antifungal for treatment. Furthermore, a proportion of non- *Aspergillus*
67 *fumigatus* strains show reduced susceptibility to antifungals, including azoles [5]. As
68 described above, the precise identification of *Aspergillus* species, their prevalence, and
69 determination of their antifungal susceptibility has become increasingly important. In
70 this study, we prospectively surveyed mold strains isolated from clinical specimens
71 collected in a hospital (number of beds: 651) between November 2013 and December

2018. The study was approved by the Ethics Committee of Obihiro-Kosei General Hospital.

A total of 119 mold isolates including 22 *A. fumigatus* clinical isolates reported previously [6], were collected from a hospital in Obihiro (the city population was approxymately 166,000 at the end of May in 2019), Japan (Supplementary Table 1). The sources of isolates were sputum samples (45 strains), otorrhea samples (27), bronchial aspirates (14), wounds samples (10), bronchoalveolar lavage fluid samples (3), pleural effusion samples (3), fecal samples (3), bronchial brushing (2), drain samples (2), pus samples (2), pharyngeal mucus sample (1), urine sample (1), nasal discharge sample (1), lung abscess sample (1), postnasal cavity sample (1), tonsil sample (1), respiratory sample (1), and unknown source sample (1). Genomic DNA from these isolates was prepared using phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation [7]. The internal transcribed spacer regions were amplified and sequenced. Partial gene sequences of β-tubulin, calmodulin, and/or hydrophobin (*rodA*) were determined to identify the species in *Aspergillus* section *Fumigati*. Partial gene sequences of β-tubulin were determined to identify the species in *Aspergillus* sections *Flavi*, *Nigri*, and *Nidulantes*. To confirm whether an isolate belonged to *A. welwitschiae* or *A. niger*,

89 partial calmodulin sequences were analyzed. Primers used for PCR and sequencing are
90 shown in Supplementary Table 2.
91 Minimum inhibitory concentrations (MICs) of amphotericin B (AMPH-B), itraconazole
92 (ITCZ), and voriconazole (VRCZ), against *Aspergillus* isolates (with the exception of
93 one *A. fumigatus* isolate and one *A. nidulans* isolate) were determined using the broth
94 microdilution method based on CLSI M38-A2 with a slight modification using Dry
95 Plate Eiken (Eiken Chemical Co., Ltd., Tokyo, Japan) [7]. The excepted strains did not
96 show spore formation. As recommended by the European Committee on Antimicrobial
97 Susceptibility Testing Antifungal Clinical Breakpoint Table v. 9.0 for *A. fumigatus*, an
98 MIC of more than 2 μg/mL for AMPH-B, ITCZ, and VRCZ was used for the breakpoint
99 to define resistance. Although breakpoints were not suggested for other *Aspergillus* spp.
100 because of insufficient evidence, an MIC of more than 2 μg/mL was also used as a
101 breakpoint.
102 *Aspergillus* was the most frequent genus isolated in this study (107 isolates) (Figure 1).
103 Thirty-four *Aspergillus* strains were isolated from 13 patients at different times
104 (Supplementary Table 1). A further 12 isolates were identified: *Rhizomucor* (2 isolates),
105 *Mucor* (3 isolates), *Scopulariopsis* (*Microascus*) (2 isolates), *Fusarium* (1 isolate),
106 *Lichtheimia* (2 isolates), *Rhizopus* (1 isolate), and *Arthrographis* (1 isolate). Species of

*Aspergillus* were identified as *A. fumigatus* (67 isolates), *Aspergillus* section *Nigri* (21 isolates), *A. flavus* (7 isolates), *A. terreus* (6 isolates), and *A. nidulans* (6 isolates), as shown in Figure 1. Consistent with earlier studies [1,8], *A. fumigatus* was the major *Aspergillus* species isolated. Among the *A. fumigatus* strains, 52 were isolated from respiratory specimens. The others were isolated from samples from wounds (8), otorrhea samples (3), pus samples (2), a faces sample, and a sample from drain. Among isolates, 12 isolates of *Aspergillus* section *Nigri*, 7 strains of *A. flavus*, and 3 isolates of *A. terreus* were obtained from otorrhea samples, indicating that otorrhea samples are another major source of *Aspergillus* spp. Sibling species of *Aspergillus* section *Fumigati*, such as *A. lentulus* and *A. udagawae*, were recently recognized as causative agents of aspergillosis [9,10]; this is problematic because of their morphological similarity to *A. fumigatus* and their differences in antifungal susceptibility compared with *A. fumigatus* [11]. The isolates collected in this study did not contain these sibling species of *Aspergillus* section *Fumigati*. *Aspergillus* section *Nigri* was the second most prevalent, with 21 strains of *Aspergillus* section *Nigri* identified as *A. welwitschiae* (10 isolates), *A. tubingensis* (8 isolates), and *A. niger* (3 isolates). In an earlier study [12], 118 strains of *Aspergillus* section *Nigri* isolated in Japan were used, and were found to comprise 50%

*A. welwitschiae*, 33.1% *A. tubingensis*, and 16.9% *A. niger*. Although only 21 strains were included in this study, the ratio is similar to that of the previous study [12].

The MICs of AMPH-B were less than 4 μg/mL against all *Aspergillus* strains (Table 1). Two isolates (3%) of *A. fumigatus* OKH34 (= IFM 64159) and OKH50 (=IFM 64160) were resistant to VRCZ and both VRCZ and ITCZ (Table 1). The *A. fumigatus* OKH34 strain was previously reported to be resistant to VRCZ, but not ITCZ [6]. This strain contained a G448S substitution in Cyp51A, which led to VRCZ resistance. Another *A. fumigatus* OKH31, which was susceptible to VRCZ and ITCZ, were isolated from same patient earlier than OKH34 strain, suggesting that VRCZ treatment of a patient might lead to the development of resistance [6]. Another strain, *A. fumigatus* OKH50, was also previously reported to contain $TR_{34}$/L98H in Cyp51A, which led to resistance to ITCZ and VRCZ [13]. Tsuchido recently reported the isolation of $TR_{34}$/L98H strains in the Shiga/Kyoto region of Japan [8]. The region is approximately 1000 km from Obihiro, Hokkaido. Although the $TR_{46}$/Y121F/T289A strain was not isolated in this study, Hagiwara et al. reported an isolate of *A. fumigatus* from Tokyo, Japan, that carried the TR and mutations [14]. These results indicate that TR-type strains are not locally isolated in Japan and might be hidden among most *A. fumigatus* isolates.

141 The MIC values of VRCZ against four *A. tubingensis* isolates were high (> 2 μg/mL).
142 One of the three strains showed high MICs (> 2 μg/mL) with both VRCZ and ITCZ. We
143 sequenced the *cyp51A* gene from eight *A. tubingensis* strains. Sequences of *cyp51A*
144 genes in these resistant strains had amino acid substitutions identical to the sequences of
145 *A. tubingensis* IFM62856 and IFM62857, which show resistance to ITCZ and VRCZ
146 [12], suggesting that these substitutions in *cyp51A* play a pivotal role in azole resistance
147 in these strains. As discussed by Howard [15] and Hashimoto [12], further studies are
148 necessary to unravel the azole-resistance mechanism.
149 In summary, we performed a prospective, single-center survey of mold isolates, in
150 particular *Aspergillus* spp., in Japan. *A. fumigatus* was the most frequently isolated
151 species, and included two azole-resistant strains. Four *A. tubingensis* strains were
152 resistant to azoles. A resistant strain carrying a $TR_{34}$/L98H mutation was found in the
153 survey. This study had limitations, for example all isolates were collected locally, and
154 the number collected included only around 100 isolates. Continuous nationwide
155 surveillance will be important to determine the current status of the frequency and
156 susceptibility of clinical *Aspergillus* isolates in Japan.
157

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165

166 **Conflict of interest**

167 None

168

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230 **Figure Legends**

231 Figure 1. Frequency of fungal species isolated. Pie charts graphically show the

232 proportion of fungal species isolated at the single center for 5 years. The number of

233 isolates is indicated in parentheses.

234

Table 1. MIC distributions and the geometric means of AMPH-B, ITCZ, and VRCZ against *Aspergillus* isolates.

| Antifungal Species[a] | No. of isolates MIC (μg/mL) | | | | | | | | Geometric mean MIC (μg/mL) |
|---|---|---|---|---|---|---|---|---|---|
| | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | >8 | |
| Amphotericin B | | | | | | | | | |
| *A. fumigatus* (66) | 0 | 2 | 22 | 34 | 8 | 0 | 0 | 0 | 0.89 |
| *A. welwitschiae* (10) | 0 | 1 | 6 | 3 | 0 | 0 | 0 | 0 | 0.57 |
| *A. tubingensis* (8) | 0 | 2 | 4 | 2 | 0 | 0 | 0 | 0 | 0.50 |
| *A. niger* (3) | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0.71 |
| *A. flavus* (7) | 0 | 0 | 0 | 5 | 2 | 0 | 0 | 0 | 1.3 |
| *A. terreus* (6) | 0 | 0 | 1 | 4 | 1 | 0 | 0 | 0 | 1.2 |
| *A. nidulans* (5) | 0 | 0 | 0 | 3 | 2 | 0 | 0 | 0 | 1.3 |
| Itraconazole | | | | | | | | | |
| *A. fumigatus* (66) | 3 | 31 | 21 | 10 | 0 | 0 | 0 | 1 | 0.39[b] |
| *A. welwitschiae* (10) | 0 | 4 | 3 | 3 | 0 | 0 | 0 | 0 | 0.47 |
| *A. tubingensis* (8) | 0 | 1 | 0 | 4 | 2 | 1 | 0 | 0 | 1.1 |
| *A. niger* (3) | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0.71 |
| *A. flavus* (7) | 0 | 4 | 2 | 1 | 0 | 0 | 0 | 0 | 0.40 |
| *A. terreus* (6) | 3 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0.21 |
| *A. nidulans* (5) | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0.16 |
| Voriconzole | | | | | | | | | |
| *A. fumigatus* (66) | 1 | 21 | 36 | 5 | 1 | 1 | 1 | 0 | 0.44 |
| *A. welwitschiae* (10) | 0 | 2 | 5 | 3 | 0 | 0 | 0 | 0 | 0.53 |
| *A. tubingensis* (8) | 0 | 1 | 0 | 2 | 1 | 3 | 1 | 0 | 1.8 |
| *A. niger* (3) | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0.50 |
| *A. flavus* (7) | 0 | 0 | 4 | 1 | 2 | 0 | 0 | 0 | 0.89 |
| *A. terreus* (6) | 0 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0.30 |
| *A. nidulans* (5) | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0.14 |

[a] The number of isolates is indicated in each parenthesis.

[b] MIC above 8 μg/mL was considered as 16 μg/mL for calculation of the geometric mean.

Supplementary Table 2. Primers used in this study.

| Primer name | Sequence (5' to 3') | References and Notes |
|---|---|---|
| **ITS4** | TCCTCCGCTTATTGATATGC | Used to amplify and sequence ITS region [1] |
| **ITS5** | GGAAGTAAAAGTCGTAACAAGG | Used to amplify and sequence ITS region [1] |
| **NL1** | GCATATCAATAAGCGGAGGAAAAG | Used to amplify and sequence D1/D2 region [2] |
| **NL4** | GGTCCGTGTTTCAAGACGG | Used to amplify and sequence D1/D2 region [2] |
| **Bt2a** | AATAGGTGCCGCTTTCTGG | Used to amplify and sequence partial β-tubulin gene [3] |
| **Bt2b** | AGTTGTCGGGACGGAAGAG | Used to amplify and sequence partial β-tubulin gene [3] |
| **rodA1** | GCTGGCAATGGTGTTGGCAA | Used to amplify and sequence partial hydrophobin gene of *A. fumigatus* [3] |
| **rodA2** | AGGGCAATGCAAGGAAGACC | Used to amplify and sequence partial hydrophobin gene of *A. fumigatus* [3] |
| **cmd5** | CCGAGTACAAGGAGGCCTTC | Used to amplify and sequence partial calmodulin gene [4] |
| **cmd6** | CCGATAGAGGTCATAACGTGG | Used to amplify and sequence partial calmodulin gene [4] |
| **AnBt2a** | GGTAACCAAATCGGTGCTGCTTTC | Used to amplify and sequence partial β-tubulin gene of *Aspergillus* section *Nigri* [5] |
| **AnBt2b** | ACCCTCAGTGTAGTGACCCTTGGC | Used to amplify and sequence partial β-tubulin gene of *Aspergillus* section *Nigri* [5] |
| **AnigCMF** | CCCCACTTTGCGGGGCAGAT | Used to amplify and sequence partial calmodulin gene |
| **AnigCMR** | TCACCGTCCTGGTCCGCCTC | Used to amplify and sequence partial calmodulin gene |
| **cyp51Aup-F** | GAATATATACGTCGATCTGTGTGAC | Used to amplify and sequence *cyp51A* gene of *A. fumigatus* [6] |
| **cyp51Adown-R** | ATCCCAGCAGATACGCTGGTCTCTGC | Used to amplify and sequence *cyp51A* gene of *A. fumigatus* [6] |
| **cyp51Aup-F** | ACAGAATACTGGGCAGCGGGCTGGAG | Used to sequence *cyp51A* gene of *A. fumigatus* [6] |
| **cyp51A-F** | ATGGTGCCGATGCTATGGCTTACGG | Used to sequence *cyp51A* gene of *A. fumigatus* [6] |
| **cyp51A-F2** | TTAGAGTCTCATGTGCCACTTATTGAGAAGG | Used to sequence *cyp51A* gene of *A. fumigatus* [6] |
| **cyp51A-F3** | CTCACAGCCAAAAGTCCTCGAAGAGC | Used to sequence *cyp51A* gene of *A. fumigatus* [6] |
| **cyp51A-F4** | TTTTCAACGTGGATGGAAAGAAAGGAGTCC | Used to sequence *cyp51A* gene of *A. fumigatus* [6] |
| **cyp51Adown-R2** | ACTATCAAAAACAGGTTTTCGCACGAGC | Used to amplify and sequence *cyp51A* gene of *A. fumigatus* [6] |
| **cyp51AFrc** | CCGTAAGCCATAGCATCGGCACCAT | Used to amplify and sequence *cyp51A* gene of *A. fumigatus* [6] |
| **cyp51Aup-F2rc** | CTCCAGCCCGCTGCCCAGTATTCTGT | Used to amplify and sequence *cyp51A* gene of *A. fumigatus* [6] |
| **Nigri_erg11-upF** | TGCTGGTTTCTGTCGGATGGAACCG | Used to amplify and sequence *cyp51A* gene of *A. tubingensis* |
| **Nigri_erg11-downR** | GGATGCTTCCTCCGGGCTTCGTATTCC | Used to amplify and sequence *cyp51A* gene of *A. tubingensis* |

| | | |
|---|---|---|
| **Nigri_erg11-upF2** | GCGGGCGTATCCAATACGATGGC | Used to sequence *cyp51A* gene of *A. tubingensis* |
| **Nigri_erg11-F1** | CTACGCTTTCGCAGCGTTGCTCG | Used to sequence *cyp51A* gene of *A. tubingensis* |
| **Nigri_erg11-F2** | CGACTATCTCCGGGACTCTCCAC | Used to sequence *cyp51A* gene of *A. tubingensis* |
| **Nigri_erg11-F3** | CGAGCCGCTGCAGTATCAGGAC | Used to sequence *cyp51A* gene of *A. tubingensis* |
| **Nigri_erg11-F4** | CCCAACTAGTACCTTGTAATAAGCATC | Used to sequence *cyp51A* gene of *A. tubingensis* |
| **Nigri_erg11-F5** | TAGAACAGCGAGGATAAGGCTAG | Used to sequence *cyp51A* gene of *A. tubingensis* |
| **Nigri_erg11-R1** | CGAGCAACGCTGCGAAAGCGTAG | Used to sequence *cyp51A* gene of *A. tubingensis* |
| **Nigri_erg11-F2r** | GTGGAGAGTCCCGGAGATAGTCG | Used to amplify and sequence *cyp51A* gene of *A. tubingensis* |

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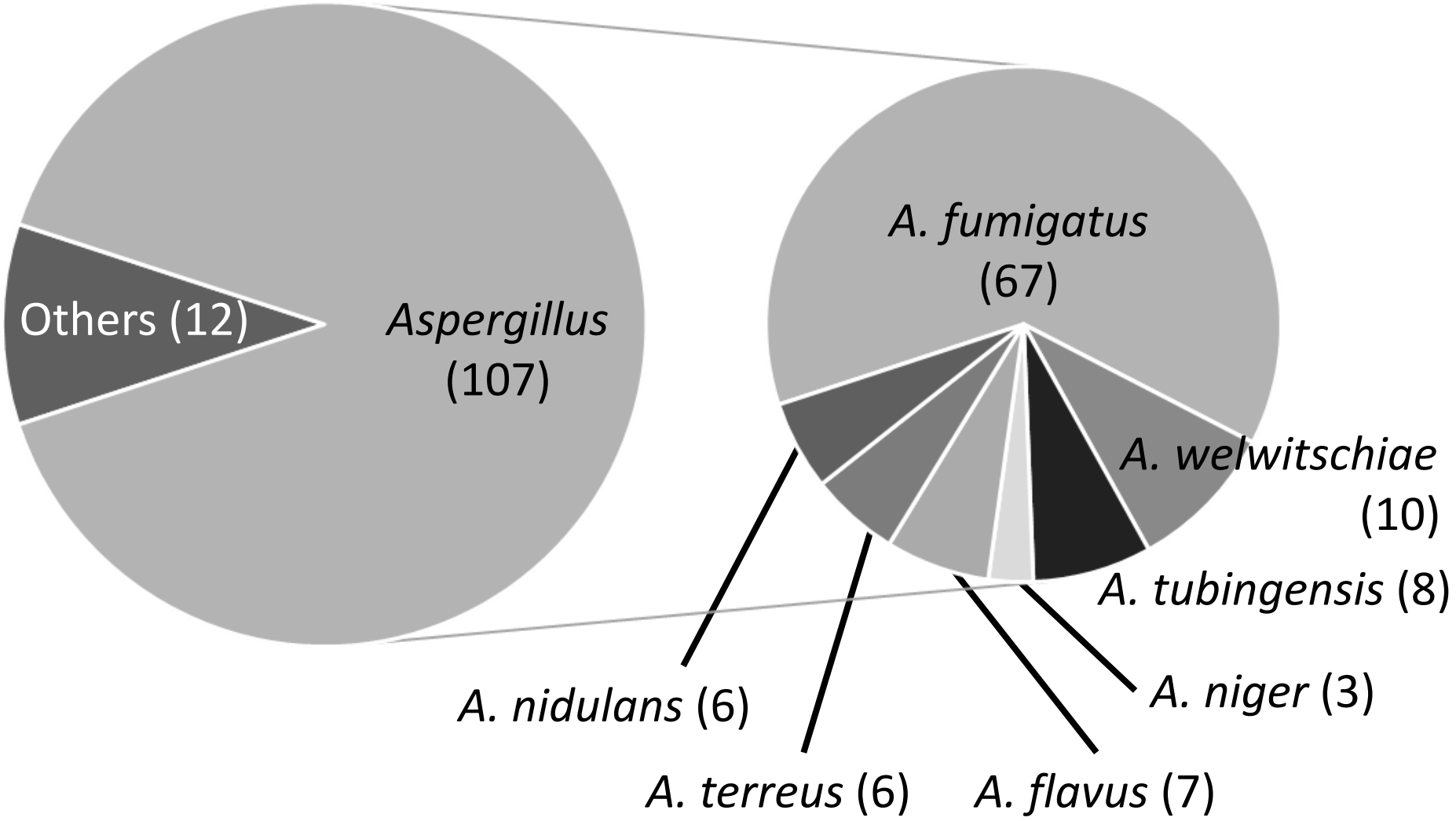
# Figure 1


Others (12)
Aspergillus (107)
A. fumigatus (67)
A. welwitschiae (10)
A. tubingensis (8)
A. niger (3)
A. flavus (7)
A. terreus (6)
A. nidulans (6)