

# Genetic differences between Japan and other countries in *cyp51A* polymorphisms of *Aspergillus fumigatus*

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## Abstract

**Background:** Mutations in *cyp51A* gene are known as main mechanisms of azole resistance in *Aspergillus fumigatus*, whereas azole-susceptible strains also carry *cyp51A* mutations (polymorphisms). The polymorphisms found in Europe mainly consist of two combinations of mutations, that is combinations of five single-nucleotide polymorphisms (SNPs) of *cyp51A*, referred to as *cyp51A*-5SNPs, and combinations of three SNPs of *cyp51A*, referred to as *cyp51A*-3SNPs. Few studies have compared the distributions of *cyp51A* polymorphisms between different regions.

**Objectives:** The aim of this study was to investigate the regional differences of *cyp51A* polymorphisms.

**Methods:** We compared the proportions of *cyp51A* polymorphisms in clinical and environmental strains isolated in various countries, and analysed the strains phylogenetically using short tandem repeats (STRs) and whole-genome sequence (WGS).

**Results:** Among the Japanese strains, 15 out of 98 (15.3%) clinical strains and 8 out of 95 (8.4%) environmental strains had *cyp51A* polymorphisms. A mutation of *cyp51A*<sup>N248K</sup> was the most prevalent polymorphism in both clinical ( $n = 14$ , 14.3%) and environmental strains ( $n = 3$ , 3.2%). Only one environmental strain harboured *cyp51A*-5SNPs, which was reported to be the most prevalent in Europe. For phylogenetic analyses using STRs and WGS, 183 and 134 strains, respectively, were employed. They showed that most of the strains with *cyp51A*<sup>N248K</sup> clustered in the clades different from those of the strains with *cyp51A*-5SNPs and *cyp51A*-3SNPs as well as from those with TR<sub>34</sub>/L98H mutations.

**Conclusions:** This study suggests that there are genetic differences between *cyp51A* polymorphisms of *A. fumigatus* in Japan and Europe.

## KEYWORDS

*Aspergillus fumigatus*, *cyp51A*, microsatellite genotyping, phylogenetic analysis, single-nucleotide polymorphisms, whole-genome sequencing

## 1 | INTRODUCTION

*Aspergillus fumigatus* is the most important of the *Aspergillus* species, and it causes chronic pulmonary aspergillosis (CPA) and invasive pulmonary aspergillosis (IPA).<sup>1</sup> Recently, azole resistance in *A. fumigatus* strains has been increasingly recognised as a cause of treatment failure.<sup>2</sup> Azole resistance mechanisms are mostly correlated with mutations of cytochrome P450 sterol 14 $\alpha$ -demethylase (CYP51A), a target protein of azoles.<sup>3,4</sup> Main mechanisms are tandem repeats (TRs) in the *cyp51A* promoter region. Some environmental strains harbour TR<sub>34</sub>/L98H and TR<sub>46</sub>/Y121F/T289A, which suggests the acquisition of azole resistance in the environment such as in soil and compost with fungicides.<sup>2,5-8</sup> The mutations were also found in environmental strains isolated in Asia.<sup>9-12</sup> Some of the resistant strains with TR<sub>34</sub>/L98H in the environment were reported to expand clonally to different areas in India and Iran, a fact indicating their ability to spread to distant locations.<sup>9,11</sup> Other main mechanisms are point mutations in *cyp51A* regions. The point mutations at G54, G138, P216, M220 and G448 frequently cause azole resistance. Several point mutations in *cyp51A* regions have been reported to be correlated with exposure to azoles in human bodies.<sup>8,13-15</sup> TR<sub>34</sub>/L98H and TR<sub>46</sub>/Y121F/T289A are dominant azole resistance mechanisms in Europe,<sup>2,7</sup> while in Japan, azole resistance is mainly caused by *cyp51A* point mutations such as G54E, G54W, G54R, G138, P216L and G448S.<sup>16,17</sup> Thus, the prevalence of the azole resistance mechanisms seems to differ between the two regions.

On the contrary, not all of the *cyp51A* mutations necessarily cause azole resistance, as some azole-susceptible strains have *cyp51A* polymorphisms, which include substitutions such as F46Y, H147Y, M172V, N248T, N248K, D255E, D343N, E427K and G434C.<sup>4</sup> The polymorphisms in Europe consist mainly of two combinations of mutations. One is a combination of single-nucleotide polymorphisms (SNPs), which lead to five amino acid substitutions (F46Y, M172V, N248T, D255E and E427K) that are referred to as *cyp51A*-5SNPs, and the other consists of SNPs that lead to three amino acid substitutions (F46Y, M172V and D255E) referred to as *cyp51A*-3SNPs.<sup>18,19</sup> Together, they were reported to account for about 10% of clinical azole-susceptible strains and to have elevated MICs against azoles, although they are not azole-resistant.<sup>19,20</sup> On the contrary, *cyp51A*<sup>N248K</sup> was found most frequently among strains in China<sup>21</sup> and among strains recovered from lung transplant recipients in Canada.<sup>22</sup> However, few studies have compared the distributions of *cyp51A* polymorphisms between different regions. Such studies may elucidate the clinical characteristics of *A. fumigatus* in each region, which may be related to the acquisition of azole resistance.

Therefore, the purpose of the present study is to gain insight into *cyp51A* polymorphisms among clinical and environmental strains in Japan and compare them genetically with those in other regions.

## 2 | MATERIALS AND METHODS

### 2.1 | Clinical and environmental strains

All clinical and environmental strains in the present study were provided by the National Bio-Resource Project (NBRP), Japan (<http://www.nbrp.jp>). We randomly selected 100 clinical azole-susceptible strains and 111 environmental strains with unknown *cyp51A* sequences from NBRP. Clinical strains were recovered in Japan between 1998 and 2017. Environmental strains were obtained from soils and air in Japan between 2007 and 2016 (Table S1). All the strains were identified as *A. fumigatus* both by morphological characteristics and by sequencing the  $\beta$ -tubulin gene as previously described.<sup>23</sup> The procedure of this study, including the availability of clinical information, was approved by the Ethics Committee of our institution (approval number MMRC-REC 21-26).

### 2.2 | Microsatellite genotyping

Microsatellite genotyping was performed for all strains as described previously.<sup>24</sup> The regions of nine loci (2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B and 4C) were amplified and sequenced. The numbers of short tandem repeats (STRs) were counted.

### 2.3 | Antifungal susceptibility testing

We determined MICs by the Clinical and Laboratory Institute (CLSI) M38-E3 broth microdilution method,<sup>25</sup> with slight modifications as described previously.<sup>26</sup> In brief, we measured MICs against itraconazole (ITCZ) and voriconazole (VRCZ) in RPMI 1640 medium (pH 7.0) at 35°C using EIKEN plates (Eiken Chemicals). All tests were repeated at least in triplicate. Isolates with an elevated MIC of either ITCZ or VRCZ ( $\geq 4$   $\mu$ g/ml) were defined as azole-resistant, and the others were considered azole-susceptible in this study.

### 2.4 | Screening of *cyp51A* point mutations and TR

The strains were screened for *cyp51A* point mutations by Surveyor Nuclease assay, as described previously.<sup>26</sup> Briefly, the *cyp51A* amplicons of each strain were hybridised with reference *cyp51A* amplicon. They were then treated with Surveyor Nuclease (Integrated DNA Technologies, Inc), an endonuclease that cleaves the sites of DNA mismatch and distortion. Both cleaved and uncleaved fragments were revealed by electrophoresis. For screening TR, we amplified the *cyp51A* promoter region by PCR and analysed the amplicon by electrophoresis. The primers used here are shown in Table S2.

## 2.5 | Sequencing of *cyp51A* regions

We inspected the sequences of *cyp51A* regions of the strains suspected of having mutations by screening. The PCR-amplified *cyp51A* regions were sequenced by the use of appropriately designed primers (shown in Table S2). Nucleotide sequences of DNA fragments were determined using Big Dye Terminator v1.1/3.1 (Applied Biosystems) and an ABI automatic sequencer (PerkinElmer). Then, we performed sequencing alignments against the reference sequence from GenBank (GenBank accession no. AF338659) or FungiDB (AFUB\_063960).

## 2.6 | Construction of *cyp51A* mutant strains

To delineate the association between *cyp51A*<sup>N248K</sup> mutation and azole resistance, we replaced the *cyp51A* region of Afs35 strain with the *cyp51A*<sup>WT</sup> or *cyp51A*<sup>N248K</sup> mutation by homologous recombination using Crispr/Cas9 genome editing.<sup>27</sup> In brief, the repair templates (*cyp51A*<sup>WT</sup> and *cyp51A*<sup>N248K</sup>) were constructed by PCR sewing and overlap extension PCR. The primers used to construct the repair template are shown in Table S2. These amplicons were cloned into vector pBluescript II SK(+) digested with HindIII and KpnI, by In-Fusion HD Cloning Kit (TAKARA Bio). Inverse PCR was performed to substitute the nucleobase in the *cyp51A* gene (AAT at 248 codon to AAA) on the plasmid.<sup>28</sup> After the protoplast-polyethylene glycol method, *A. fumigatus* strains were transformed by the Crispr-Cas9 system using the prepared repair template.

## 2.7 | Genotypic analysis

For genotypic comparisons, we analysed nine microsatellite markers of strains, based on the UPGMA minimal spanning tree algorithm of BioNumerics V7.6 software (Applied Math Inc) and constructed a dendrogram. We added some data on strains reported in past articles.<sup>9,11,13,18,29-31</sup>

## 2.8 | Illumina whole-genome sequencing and SNP analysis

Genome DNA was extracted from over-night cultured mycelia by the phenol-chloroform method as described previously.<sup>32</sup> Genomic DNA libraries of 17 *A. fumigatus* strains were constructed using an NEBNext Ultra DNA Library Prep Kit (New England BioLabs) according to the manufacturer's protocol. A 150-bp paired-end sequencing on a HiSeq X Ten system (Illumina) was carried out by GENEWIZ. In addition to the 17 samples, we also used raw data from 23,<sup>33</sup> 27<sup>34</sup> and 67 strains.<sup>35</sup> Raw genomic reads of all 134 samples were quality-controlled and trimmed by fastp (ver. 0.20.1).<sup>36</sup> Filtered reads were aligned against the Af293 reference genome and A1163 reference genomes (retrieved from AspGD, <http://www.aspgd.org/>)<sup>37</sup> using BWA-MEM (ver. 0.7.17-r1188).<sup>38</sup> The detection of SNPs was performed using GATK (ver. 4.1.2.0).<sup>39</sup> According to the best practice workflow for 'Germline short variant discovery' and Zhao et al.,<sup>35</sup> the function 'HaplotypeCaller' was used to call short variants (SNPs and INDELs) with the sorted BAM file for each sample, followed by the function 'GenotypeGVCFs' to combine the vcf files. Next, only SNPs were extracted from the joint-called variant file using the function 'SelectVariants'. To filter out false-positive variant calling, the function 'VariantFiltration' was carried out with the following parameters: 'QD < 25.0 || FS > 5.0 || MQ < 55.0 || MQRankSum < -0.5 || ReadPosRankSum < -2.0 || SOR > 2.5'.

aspd.org/)<sup>37</sup> using BWA-MEM (ver. 0.7.17-r1188).<sup>38</sup> The detection of SNPs was performed using GATK (ver. 4.1.2.0).<sup>39</sup> According to the best practice workflow for 'Germline short variant discovery' and Zhao et al.,<sup>35</sup> the function 'HaplotypeCaller' was used to call short variants (SNPs and INDELs) with the sorted BAM file for each sample, followed by the function 'GenotypeGVCFs' to combine the vcf files. Next, only SNPs were extracted from the joint-called variant file using the function 'SelectVariants'. To filter out false-positive variant calling, the function 'VariantFiltration' was carried out with the following parameters: 'QD < 25.0 || FS > 5.0 || MQ < 55.0 || MQRankSum < -0.5 || ReadPosRankSum < -2.0 || SOR > 2.5'.

## 2.9 | Phylogenetic analysis of WGS SNPs data

The SNP sites with a minor allele frequency  $\geq 5\%$  and no missing data were filtered by VCFtools (ver. 0.1.16)<sup>40</sup> with the options '--maf 0.05 --max-missing 1'. A phylogenetic tree was constructed using multi-threaded RAxML (ver. 8.2.12),<sup>41</sup> the GTRCAT model and 1000 bootstrap replicates, and visualised by iTOL.<sup>42</sup> Phylogenetic analysis was also performed against the A1163 reference.

## 2.10 | Evaluation of virulence against silkworms

We infected silkworm (*Bombyx mori*) larvae with *A. fumigatus* strains as previously described,<sup>43</sup> with slight modifications. Briefly, fifth-instar silkworms were purchased from Ehime Sansyu. They were raised at 30°C for 2–3 days. Inoculum of 0.05 ml diluted to  $3.0 \times 10^6$  CFU/ml was injected into the haemolymph of the silkworms using a 1 ml Terumo Myjector 29G insulin syringe (Terumo). After the infection, they were maintained at 30°C and their survival was evaluated by Kaplan–Meier method using a log-rank test with GraphPad Prism 5 (GraphPad Software Inc). This experiment was performed in duplicate, and the results were shown as mean values.

## 2.11 | Growth under hypoxia

Conidia of each strain were inoculated on glucose minimal medium agar medium for 90 h at 37°C in an Anaeropack System (Mitsubishi Gas Chemical), the concentration of which was maintained at 1%–3%, that is hypoxia. We compared its growth under hypoxia with that under ambient air, that is normoxia.

## 2.12 | Statistical analyses

Quantitative variables were analysed by *t* test and Mann–Whitney test. Categorical variables were compared by chi-squared or Fisher's exact test, and a two-tailed *p*-value of <.05 was considered

significant. Analyses were conducted with GraphPad Prism version 5 and JMP® Pro 16 (SAS Institute Inc).

### 2.13 | Accession numbers

The sequence data of *cyp51A* mutations in this study are available from the DDBJ Sequenced Read Archive (accession numbers LC639871 to LC639893). The whole-genome sequence (WGS) data of *A. fumigatus* strains in this study are available from the DDBJ Sequenced Read Archive under accession numbers DRR290031 to DRR290047.

## 3 | RESULTS

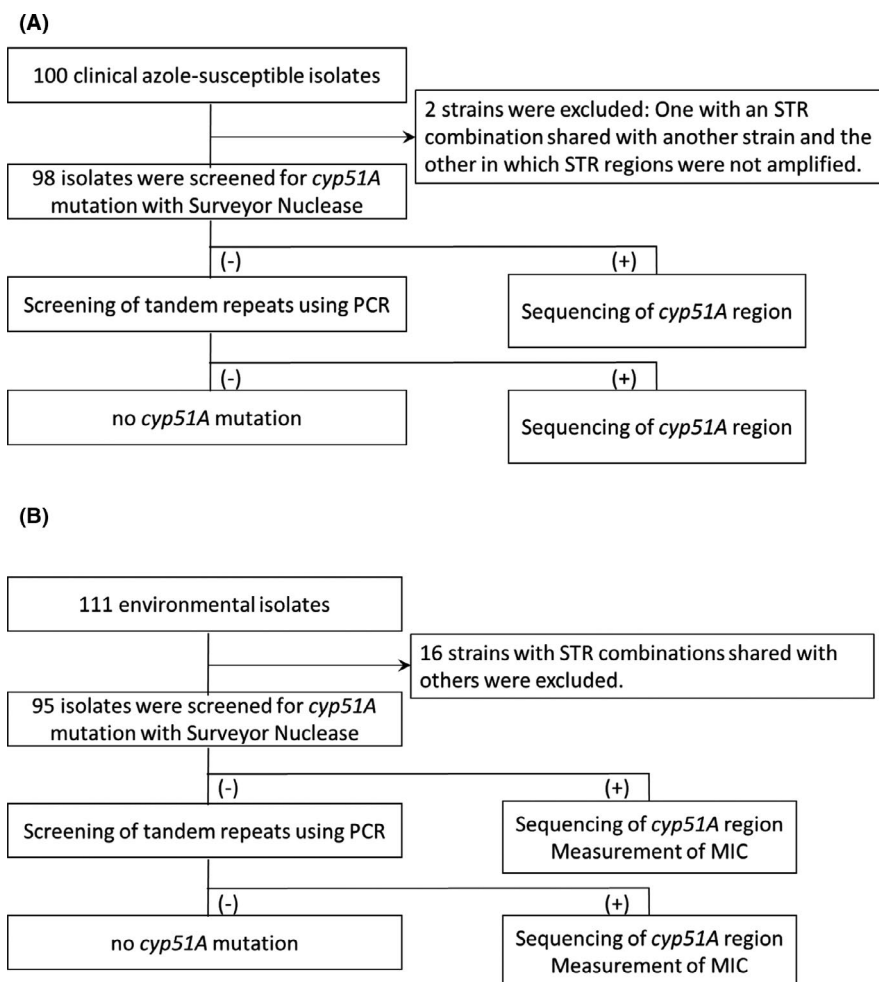
### 3.1 | Samples and clinical information

We investigated the polymorphisms of *cyp51A* of clinical and environmental strains isolated in Japan according to an analysis diagram (Figure 1). We excluded one clinical strain because we could not amplify the STR regions. We also excluded one clinical strain and 16 environmental strains because they had combinations of

STRs that were shared with others. Out of 98 clinical strains, 15 (15.3%) had *cyp51A* polymorphisms (Table 1), and 8 out of 95 environmental strains (8.4%) had *cyp51A* polymorphisms (Table 2). The distributions of the strains screened for *cyp51A* polymorphisms are shown on the maps of Figure S1. *cyp51A*<sup>N248K</sup> was the most prevalent polymorphism in both clinical and environmental strains, with a greater frequency in clinical strains (14 of 98, 14.3%) than in environmental strains (3 of 95, 3.2%). We found *cyp51A*-5SNPs, a prevalent *cyp51A* polymorphism in Europe, in only one environmental strain, and no tendency was found with a clinical background. Among the 14 clinical strains with *cyp51A*<sup>N248K</sup> mutations, 6 were isolated from azole-naïve patients. Interestingly, a frameshift mutation at G465 was found in IFM62405, whose azole susceptibility was improved. We showed a summary of these results in Table S3. Information concerning all the strains is shown in Tables S4 and S5.

### 3.2 | Evaluation of the effect of *cyp51A*<sup>N248K</sup> on MICs to azoles

For the purpose of certifying the correlation of *cyp51A*<sup>N248K</sup> to azole susceptibility, we constructed recombinant strains with *cyp51A*<sup>WT</sup> or *cyp51A*<sup>N248K</sup> using Crispr/Cas9 genome editing.<sup>27</sup>



**FIGURE 1** Analysis diagram of this study. We analysed the *cyp51A* regions of clinical azole-susceptible strains (A) and environmental strains (B). The *cyp51A* genes were screened for point mutations and tandem repeats (TR) by Surveyor Nuclease method and PCR amplification, respectively. When *cyp51A* mutations were found, the alignment of *cyp51A* regions was identified by Sanger sequencing. As for environmental strains with *cyp51A* mutations, MICs were measured

TABLE 1 Characteristics of clinical strains with *cyp51A* mutations and MICs against azoles

| IFM   | <i>cyp51A</i> seq | Tandem repeat | Sample | Clinical form | Underlying diseases                   | Treatment with azole | Year | Hospital | MIC (µg/ml) |       |
|-------|-------------------|---------------|--------|---------------|---------------------------------------|----------------------|------|----------|-------------|-------|
|       |                   |               |        |               |                                       |                      |      |          | ITCZ        | VRCZ  |
| 49895 | N248K             | -             | Lung   | SPA           | Unknown                               | Unknown              | 2000 | Tokyo    | 0.5         | 1     |
| 51748 | N248K             | -             | Sputum | ABPA          | -                                     | -                    | 2002 | Tokyo    | 0.125       | 0.125 |
| 53927 | N248K             | -             | Sputum | CPA           | IP                                    | ITCZ for <1 month    | 2004 | Tokyo    | 0.5         | 0.5   |
| 60369 | N248K             | -             | BALF   | IPA           | Near-drowning                         | -                    | 2011 | Iwate    | 0.5         | 0.5   |
| 60994 | N248K             | -             | Sputum | Colonisation  | Tuberculosis                          | -                    | 2011 | Chiba    | 0.5         | 1     |
| 61407 | N248K             | -             | BALF   | CPA           | COPD                                  | Unknown              | 2012 | Chiba    | 0.25        | 0.25  |
| 62055 | N248K             | -             | Sputum | SPA           | COPD                                  | VRCZ for 45 days     | 2013 | Aichi    | 0.25        | 0.25  |
| 62241 | N248K             | -             | Sputum | IPA           | ALL                                   | VRCZ for <1 month    | 2013 | Aichi    | 0.5         | 0.5   |
| 62521 | N248K             | -             | Lung   | SPA           | BA                                    | ITCZ for 3 years     | 2014 | Tokyo    | 1           | 0.5   |
| 63141 | N248K             | -             | Lung   | SPA           | PTBLD                                 | -                    | 2014 | Osaka    | 1           | 1     |
| 63311 | N248K             | -             | BALF   | CPA           | NTM                                   | -                    | 2013 | Tokyo    | 1           | 0.5   |
| 64302 | N248K             | -             | Sputum | CPA           | NTM                                   | -                    | 2016 | Tokyo    | 0.5         | 1     |
| 64563 | N248K             | -             | Sputum | CPA           | COPD, BA                              | ITCZ for <1 month    | 2016 | Tokyo    | 0.5         | 1     |
| 64666 | N248K             | -             | Sputum | CPA           | PTBLD, COPD                           | ITCZ for 1 month     | 2016 | Tokyo    | 1           | 0.5   |
| 51977 | M39I              | -             | Sputum | Unknown       | BA, Bronchiectasis, Diabetes mellitus | Unknown              | 2002 | Hokkaido | 0.25        | 0.25  |

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; ALL, acute lymphoblastic leukaemia; BA, bronchial asthma; BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; CPA, chronic pulmonary aspergillosis; IP, interstitial pneumonia; IPA, invasive pulmonary aspergillosis; ITCZ, itraconazole; NTM, nontuberculous mycobacteria; PTBLD, post-tuberculosis lung disease; SPA, simple pulmonary aspergilloma; VRCZ, voriconazole.

TABLE 2 Characteristics of environmental strains with *cyp51A* mutations and MICs against azoles

| IFM   | <i>cyp51A</i> seq             | Tandem repeat | Place  | Source | MIC ( $\mu\text{g/ml}$ ) |      |
|-------|-------------------------------|---------------|--------|--------|--------------------------|------|
|       |                               |               |        |        | ITCZ                     | VRCZ |
| 60748 | N248K                         | -             | Tokyo  | Air    | 0.5                      | 0.5  |
| 60765 | V396A                         | -             | Chiba  | Air    | 0.25                     | 1    |
| 62345 | D343N                         | -             | Chiba  | Soil   | 1                        | 1    |
| 62400 | N248K                         | -             | Nagano | Soil   | 0.5                      | 0.5  |
| 62405 | c.464_465insgtgg <sup>a</sup> | -             | Nagano | Soil   | 0.25-0.5                 | 0.25 |
| 62432 | N248K                         | -             | Gifu   | Soil   | 0.5                      | 1    |
| 62628 | F46Y                          | -             | Chiba  | Soil   | 2                        | 2    |
|       | M172V                         |               |        |        |                          |      |
|       | N248T                         |               |        |        |                          |      |
|       | D255E                         |               |        |        |                          |      |
|       | E427K                         |               |        |        |                          |      |
| 63915 | c.198+22g>c <sup>b</sup>      | -             | Chiba  | Air    | 0.5                      | 0.5  |

Abbreviations: ITCZ, itraconazole; VRCZ, voriconazole.

<sup>a</sup>c.464\_465insgtgg means insertion of nucleotide 'GTGG' between L464 and G465.

<sup>b</sup>c.198+22g>c means a base substitution in intron.

TABLE 3 MIC profiles of recombinant strains against azoles

| Strain                         | <i>cyp51A</i>                                           | MIC ( $\mu\text{g/ml}$ ) |      |
|--------------------------------|---------------------------------------------------------|--------------------------|------|
|                                |                                                         | ITCZ                     | VRCZ |
| AfS 35<br>(Background strain)  | <i>cyp51A</i> <sup>WT</sup>                             | 0.5                      | 1    |
| <i>cyp51A</i> <sup>WT</sup>    | $\Delta cyp51A^{\text{WT}}::cyp51A^{\text{WT}}::hph$    | 0.5                      | 1-2  |
| <i>cyp51A</i> <sup>N248K</sup> | $\Delta cyp51A^{\text{WT}}::cyp51A^{\text{N248K}}::hph$ | 0.5                      | 2    |

Abbreviations: ITCZ, itraconazole; VRCZ, voriconazole.

Both of the recombinant strains displayed almost the same MICs to azoles (Table 3). Furthermore, the clinical and environmental strains with *cyp51A*<sup>N248K</sup> did not show elevated MICs to azoles compared to strains with *cyp51A*<sup>WT</sup>. Among clinical strains, there was no significant difference in MICs between 83 WT strains and 14 N248K strains by Mann-Whitney test (Figure 2A); among environmental strains, no elevation of MICs to azoles was found in three WT strains (IFM60759, 62370, 63893) or three N248K strains (IFM60748, 62400, 62432) (Figure 2B). These results indicate that *cyp51A*<sup>N248K</sup> does not influence the susceptibility to azoles.

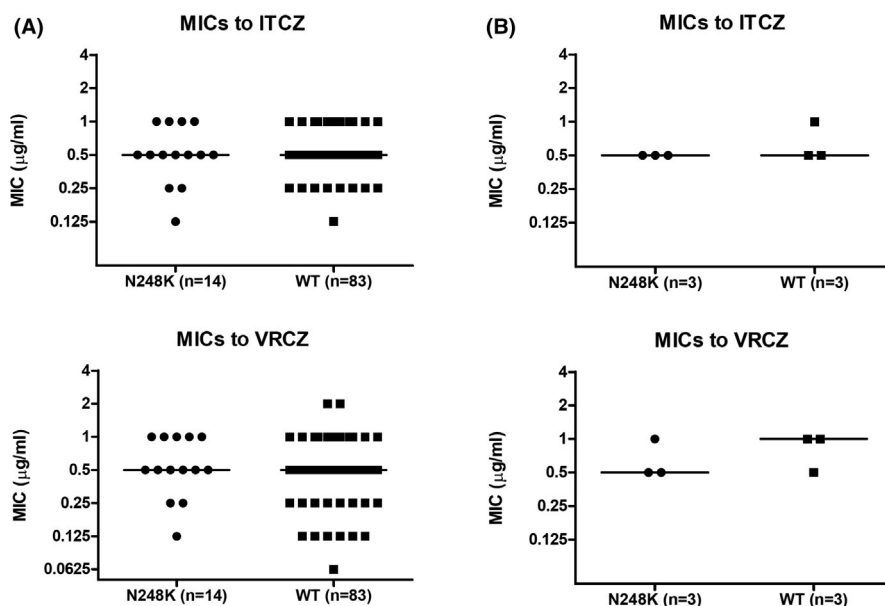


FIGURE 2 MICs against azoles of WT and N248K strains. Among clinical strains, there was no significant difference in MICs between WT strains and N248K strains by Mann-Whitney test. Among environmental strains, no elevation of MICs to azoles was found in three WT strains or three N248K strains. Bars indicate median values

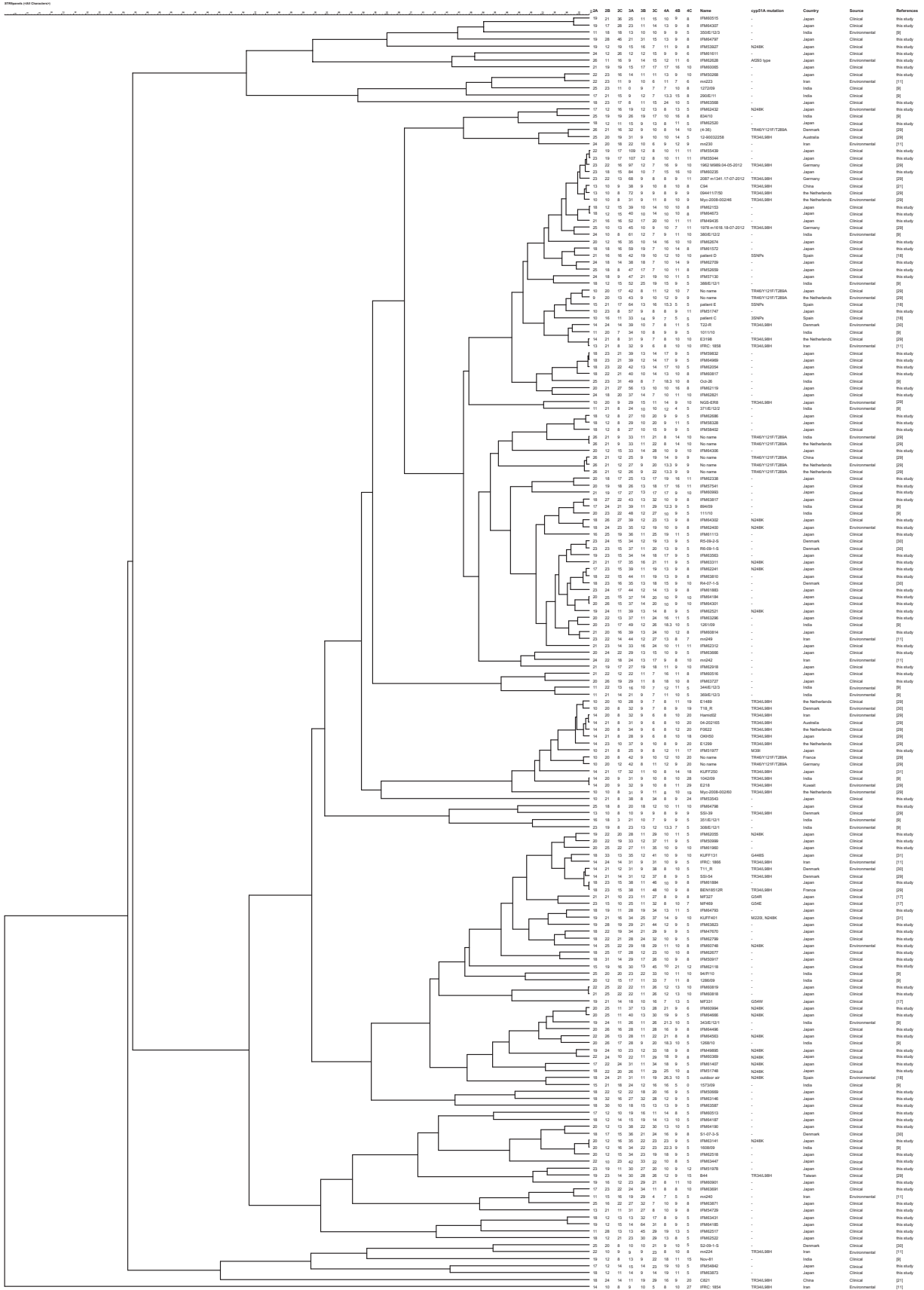
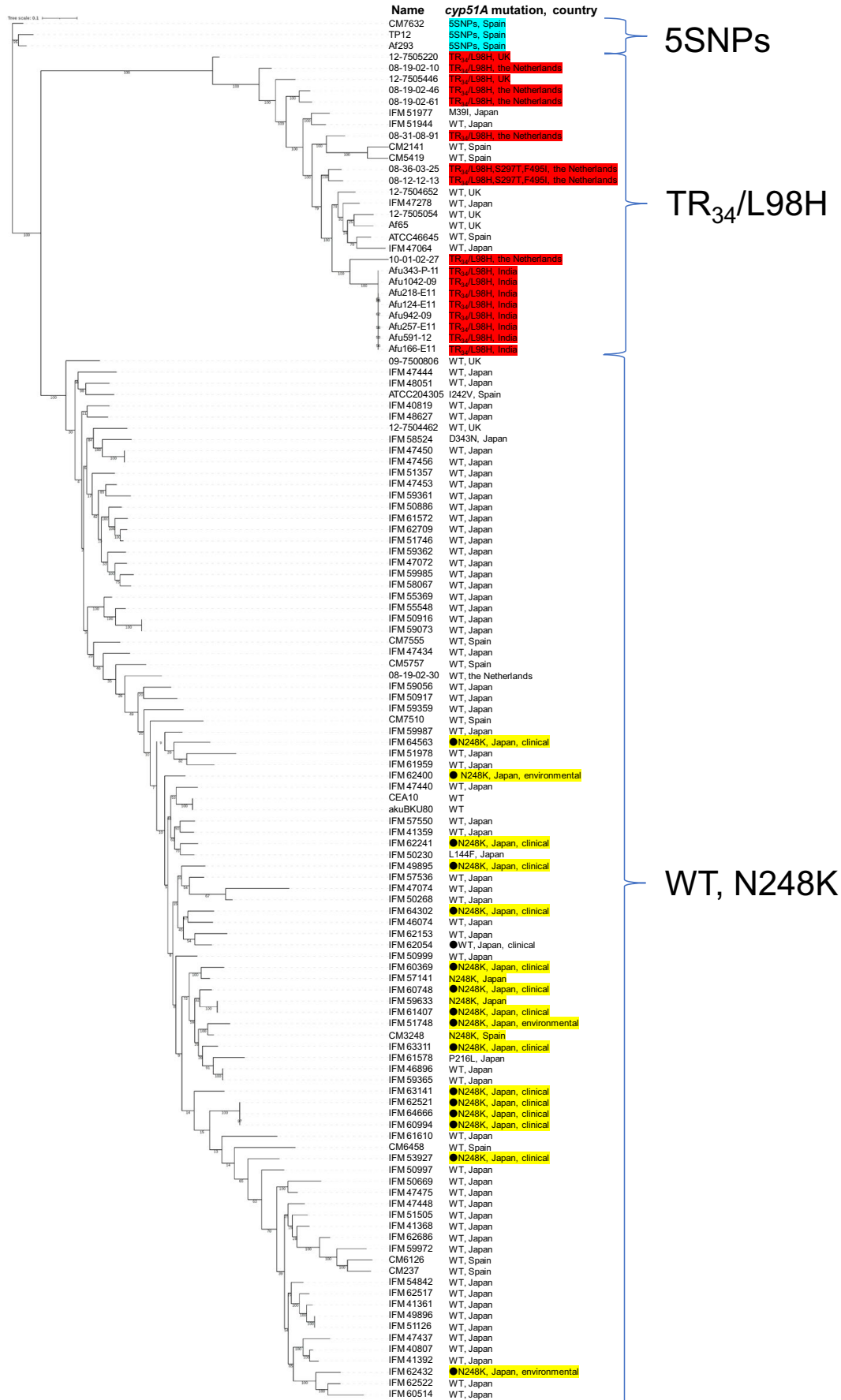


FIGURE 3 Phylogenetic analysis of STR results based on each of the gene variants with the clustering method, using UPGMA and minimal spanning tree algorithm of BioNumerics V7.6 software. Yellow shading indicates *cyp51A*<sup>N248K</sup>. Strains 1042/09 (isolated in India) and mn224 (isolated in Iran) represent other strains that share STR combinations





**FIGURE 4** Whole-genome phylogenetic analysis of the strains with *cyp51A*<sup>N248K</sup> and others isolated in Japan and foreign countries, using Af293 as a reference strain. The strains with *cyp51A*<sup>N248K</sup> (shaded in yellow) clustered in one clade, while the strains with *cyp51A*-5SNPs (shaded in blue) and TR<sub>34</sub>/L98H (shaded in red) clustered in two other clades, respectively. Strains in this study are indicated by circular dots

### 3.3 | Phylogenetic analysis using data of STR combinations

To shed light on the genotypic difference between strains with *cyp51A*<sup>N248K</sup> and others, we phylogenetically analysed a total of 183 strains from various countries using STR combinations. IFM62311 and 55548 were excluded because one locus of STR regions was not amplified appropriately. Among clinical strains, 11 out of 14 strains with *cyp51A*<sup>N248K</sup> clustered in two clades. Out of three environmental N248K strains, one strain clustered there. The two clades to which the N248K strains belonged did not include any European strains with *cyp51A*-5SNPs or *cyp51A*-3SNPs. Further, the two clades did not contain any strains with TR<sub>34</sub>/L98H, whether they were isolated from Europe or Asia (Figure 3).

### 3.4 | Phylogenetic analysis using SNV data

We also analysed the WGS data of the 17 Japanese strains with the *cyp51A*<sup>N248K</sup> mutation or *cyp51A*<sup>WT</sup> phylogenetically, comparing them with the previously reported data (117 strains).<sup>33-35</sup> 159,163 polymorphic loci were predicted after hard filtering for the 134 strains (Figure S2). 69,412 polymorphic loci were predicted for 122 strains (Figure 4), excluding the 12 strains with *cyp51A*-3SNPs, in which there was a large number of SNPs against Af293 (134,515–147,066 SNPs): CM2495, CM2730, CM2733, CM3249, CM3249b, CM3262, CM3720, CM4602, CM4946, CM7560, CM7570 and TP32. All the strains were divided into four clusters, as already reported.<sup>34</sup> Three

major clades mainly consisted of the strains with *cyp51A*<sup>WT</sup> (Cluster I), *cyp51A* with TR<sub>34</sub>/L98H mutation (Cluster II) and *cyp51A*-5SNPs (Cluster III) (Figure 4, with Af293 as reference). The 12 strains with *cyp51A*-3SNPs clustered in a different clade from the others (Cluster IV) (Figure S2, with Af293 as reference). Most of the Japanese WT strains belonged to Cluster I. One clade of Cluster I included most of the strains with *cyp51A*<sup>N248K</sup>. These four clades were maintained when A1163 was used as reference (Figures S3 and S4).

### 3.5 | Evaluation of virulence of the strains with *cyp51A*<sup>N248K</sup> mutations

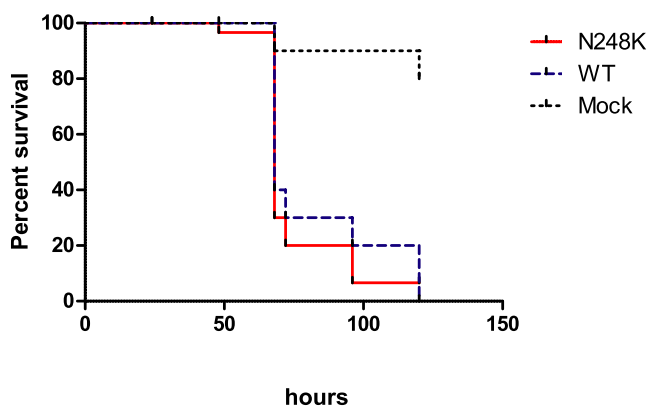
We evaluated the virulence of the strains using the silkworm model. Silkworms were inoculated with WT strains or N248K strains. IFM64190, 64673 and 64797 were used as WT strains, and IFM64302, 64563 and 64666 were used as N248K strains. Each strain was injected into five silkworms. We also made Mock (five silkworms) as an uninfected group. There was no difference in virulence between the WT and N248K strains (Figure 5).

### 3.6 | Growth under hypoxia

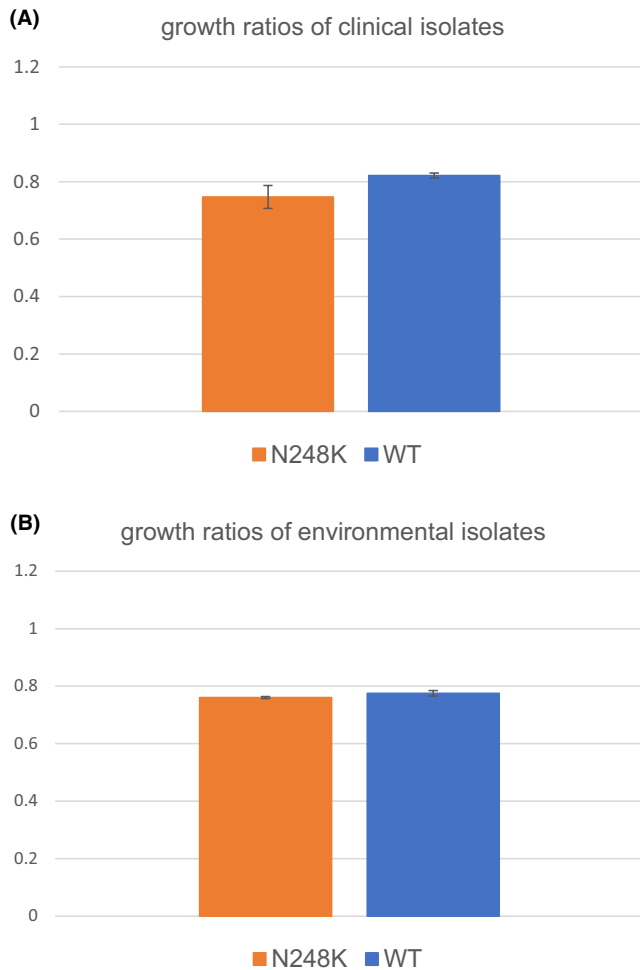
Although growth of *A. fumigatus* strains under hypoxia was reported to be related to their virulence,<sup>44</sup> we did not observe any difference in alteration of growth under hypoxia between the two groups (Figure 6). Clinical strains included five N248K strains (IFM62241, 63141, 64302, 64563, 64666) and six WT strains (IFM62517, 64190, 64301, 64496, 64673, 64797). Environmental strains included three N248K strains (IFM60748, 62400 and 62432) and three WT strains (IFM60759, 62370 and 63893).

## 4 | DISCUSSION

In the present study, we demonstrated that *cyp51A*<sup>N248K</sup> was the most prevalent *cyp51A* polymorphism among *A. fumigatus* strains in Japan, while only one strain harboured *cyp51A*-5SNPs. Since *cyp51A*-5SNPs and *cyp51A*-3SNPs were reported to be prevalent in Europe, this result indicates that strains in Japan have a different constitution of *cyp51A* polymorphisms from those in Europe. For phylogenetic analyses with STR and WGS, 183 and 134 strains, respectively, from various countries were employed. They suggested that most of the strains with *cyp51A*<sup>WT</sup> and *cyp51A*<sup>N248K</sup> in Japan clustered in the clades different from those of the strains with *cyp51A*-5SNPs and *cyp51A*-3SNPs as well as from those with TR<sub>34</sub>/L98H mutations.



**FIGURE 5** Killing rates of silkworms infected with clinical strains with *cyp51A*<sup>N248K</sup> mutations. The survival rates of silkworms inoculated with WT strains or N248K strains were evaluated. We also made Mock (five silkworms) as an uninfected group. The experiments were performed twice, and the results were shown as mean values. No significant difference in survival was detected between the WT ( $n = 15$ ) and N248K ( $n = 15$ ) groups



**FIGURE 6** Growth alteration of N248K and WT strains under hypoxia. We measured the ratios of colony diameters of N248K and WT strains under hypoxia and normoxia. No significant differences were observed between the two groups of strains among clinical strains (A) or environmental strains (B) by *t* test. Error bars indicate standard errors

The prevalence of *cyp51A*<sup>N248K</sup> polymorphisms was reported to be 13.1% among clinical strains in China and 14.4% among those from lung transplant recipients in Canada,<sup>21,22</sup> percentages similar to our report (14.3%). The constitutions of *cyp51A* polymorphisms may be similar in these areas.

It has been suggested that azole treatment in patients is not correlated with the emergence of *cyp51A*<sup>N248K</sup>, since *cyp51A*<sup>N248K</sup> mutations were found in both environmental strains and clinical strains isolated from azole-naïve patients. Phylogenetic analysis based on WGS indicated that most of the N248K strains clustered in the same clade, while the strains with TR<sub>34</sub>/L98H clustered in a clade to which most of the azole-susceptible strains did not belong. A. Chowdhary and F. Ahangarkani reported the clonal spread of strains with TR<sub>34</sub>/L98H in India and Iran.<sup>9,11</sup> In previous papers, analysis based on genotyping and WGS revealed more genetic diversity of azole-susceptible strains compared to strains carrying TR<sub>34</sub>/L98H mutations.<sup>11,33,45,46</sup> Further investigations of *cyp51A* polymorphisms of azole-susceptible

strains and *cyp51A* mutations of azole-resistant strains in various countries will be helpful for delineating the characterisation of the pathogen and the spread of azole resistance in each region.

Our study suggested that *cyp51A*<sup>N248K</sup> does not affect azole susceptibility. These results are compatible with other reports.<sup>4,47</sup> The amino acid of N248 was reported to be located in the surface of CYP51A protein.<sup>4,47</sup> It is thought that the substitution of this amino acid has little influence on interacting with azole agents or the protein structure.<sup>4,47</sup> On the contrary, strains harbouring *cyp51A*-5SNPs or *cyp51A*-3SNPs show slightly elevated MICs against azoles.<sup>19</sup> This suggests that the strains with *cyp51A*-3SNPs and *cyp51A*-5SNPs can affect the clinical treatment of azoles, and this emphasises the importance of surveying the distribution of these polymorphisms. It is noteworthy that, in the present study, we compared *cyp51A* regions with the sequence of A1163 as a reference strain. Since Af293, the other reference strain, has a polymorphism of *cyp51A*-5SNPs,<sup>19,34</sup> clarification of the chosen reference strain is essential.

Interestingly, we demonstrated that the polymorphisms of *cyp51A*<sup>N248K</sup> were more prevalent in clinical strains than in environmental ones. Although the result may be correlated with many factors such as the exposure to agricultural fungicides in the environment, regional distributions of the *cyp51A* polymorphism inside Japan, and the clinical entity of aspergillosis of the patients, one possible explanation for this is that the strains with *cyp51A*<sup>N248K</sup> might have pathogenic factors. However, we could not detect any strong pathogenicity in the experiments with silkworms, nor in any features of growth under hypoxia, which were reported to be factors of pathogenicity in *A. fumigatus*.<sup>44</sup> More detailed insights into these polymorphisms may lead to elucidation of the factors related to pathogenesis. Silkworms have innate cellular immunity such as phagocytic and humoral immune responses.<sup>48</sup> They have been used as an experimental animal model for evaluating the features of pathogenic microorganisms, such as pathogenetic genes of *Candida albicans* and *Cryptococcus neoformans*.<sup>48,49</sup>

This study has several limitations. First, it contains bias in the selection of strains. Some clinical strains were cultured from patients who had treatment difficulties, and environmental strains were cultured from a limited number of locations. More precise results will be gained by random surveys of strains from many patients and places. Second, more information about clinical strains may enable us to explore correlations of the polymorphisms and clinically pathogenic features.

In conclusion, we clarified genetic differences between the strains in Japan and Europe in terms of *cyp51A* polymorphisms. Further investigations may lead to obtaining better insights into the features of *A. fumigatus* strains as clinical pathogens and the pathophysiology of azole resistance in *A. fumigatus*.

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## CONFLICT OF INTEREST

The authors have no conflicts to disclose.

## AUTHOR CONTRIBUTIONS

**Hidetaka Majima:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Resources (equal); Visualization (equal); Writing-original draft (lead). **Tepei Arai:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Resources (equal); Writing-original draft (supporting). **Yoko Kusuya:** Investigation (equal). **Hiroki Takahashi:** Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Visualization (equal); Writing-original draft (supporting). **Akira Watanabe:** Funding acquisition (equal); Project administration (equal); Writing-review & editing (equal). **Yasunari Miyazaki:** Supervision (equal); Writing-review & editing (equal). **Katsuhiko Kamei:** Project administration (equal); Supervision (equal); Writing-review & editing (equal).

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## REFERENCES

- Denning DW, Chakrabarti A. Pulmonary and sinus fungal diseases in non-immunocompromised patients. *Lancet Infect Dis*. 2017;17(11):e357-e366.
- Chowdhary A, Sharma C, Meis JF. Azole-resistant aspergillosis: epidemiology, molecular mechanisms, and treatment. *J Infect Dis*. 2017;216(suppl\_3):S436-S444.
- Hagiwara D, Watanabe A, Kamei K, Goldman GH. Epidemiological and genomic landscape of azole resistance mechanisms in *Aspergillus* fungi. *Front Microbiol*. 2016;7:1382.
- Dudakova A, Spiess B, Tangwattanachuleeporn M, et al. Molecular tools for the detection and deduction of azole antifungal drug resistance phenotypes in *Aspergillus* species. *Clin Microbiol Rev*. 2017;30(4):1065-1091.
- Zhang J, Snelders E, Zwaan BJ, et al. A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. *MBio*. 2017;8(3):e00791-17. doi:10.1128/mBio.00791-17
- Snelders E, van der Lee HA, Kuijpers J, et al. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med*. 2008;5(11):e219.
- Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. The global problem of antifungal resistance: prevalence, mechanisms, and management. *Lancet Infect Dis*. 2017;17(12):e383-e392.
- Resendiz Sharpe A, Lagrou K, Meis JF, et al. Triazole resistance surveillance in *Aspergillus fumigatus*. *Medical Mycol*. 2018;56(suppl\_1):S83-S92.
- Chowdhary A, Kathuria S, Xu J, et al. Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR<sub>34</sub>/L98H mutations in the *cyp51A* gene in India. *PLoS One*. 2012;7(12):e52871.
- Chowdhary A, Sharma C, Kathuria S, Hagen F, Meis JF. Azole-resistant *Aspergillus fumigatus* with the environmental TR<sub>46</sub>/Y121F/T289A mutation in India. *J Antimicrob Chemother*. 2014;69(2):555-557.
- Ahangarkani F, Badali H, Abbasi K, et al. Clonal expansion of environmental triazole resistant *Aspergillus fumigatus* in Iran. *J Fungi*. 2020;6(4).
- Onishi K, Muhammad Sarumoh B, Hagiwara D, Watanabe A, Kamei K, Toyotome T. Azole-resistant *Aspergillus fumigatus* containing a 34-bp tandem repeat in *cyp51A* promoter is isolated from the environment in Japan. *Med Mycol J*. 2017;58(2):e67-e70.
- Tashiro M, Izumikawa K, Hirano K, et al. Correlation between triazole treatment history and susceptibility in clinically isolated *Aspergillus fumigatus*. *Antimicrob Agents Chemother*. 2012;56(9):4870-4875.
- Bellec B, Raberin H, Morel J, Flori P, Hafid J, Manhungs RT. Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma. *Med Mycol*. 2010;48(1):197-200.
- Howard SJ, Cerar D, Anderson MJ, et al. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg Infect Dis*. 2009;15(7):1068-1076.
- Hagiwara D. Current status of azole-resistant *Aspergillus fumigatus* isolates in East Asia. *Med Mycol J*. 2018;59(4):E71-e76.
- Tashiro M, Izumikawa K, Minematsu A, et al. Antifungal susceptibilities of *Aspergillus fumigatus* clinical isolates obtained in Nagasaki, Japan. *Antimicrob Agents Chemother*. 2012;56(1):584-587.
- Escribano P, Recio S, Pelaez T, Bouza E, Guinea J. *Aspergillus fumigatus* strains with mutations in the *cyp51A* gene do not always show phenotypic resistance to itraconazole, voriconazole, or posaconazole. *Antimicrob Agents Chemother*. 2011;55(5):2460-2462.
- Garcia-Rubio R, Alcazar-Fuoli L, Monteiro MC, et al. Insight into the significance of *Aspergillus fumigatus cyp51A* polymorphisms. *Antimicrob Agents Chemother*. 2018;62(6):e00241-18. doi:10.1128/AAC.00241-18
- Alanio A, Cabaret O, Sitterle E, et al. Azole preexposure affects the *Aspergillus fumigatus* population in patients. *Antimicrob Agents Chemother*. 2012;56(9):4948-4950.
- Chen Y, Lu Z, Zhao J, et al. Epidemiology and molecular characterizations of azole resistance in clinical and environmental *Aspergillus fumigatus* isolates from China. *Antimicrob Agents Chemother*. 2016;60(10):5878-5884.
- Henry B, Guenette A, Cheema F, et al. CYP51A polymorphisms of *Aspergillus fumigatus* in lung transplant recipients: prevalence, correlation with phenotype, and impact on outcomes. *Med Mycol*. 2021;59(7):728-733.
- Yaguchi T, Horie Y, Tanaka R, Matsuzawa T, Ito J, Nishimura K. Molecular phylogenetics of multiple genes on *Aspergillus* section Fumigati isolated from clinical specimens in Japan. *Nihon Ishinkin Gakkai Zasshi*. 2007;48(1):37-46.
- de Valk HA, Meis JF, Curfs IM, Muehlethaler K, Mouton JW, Klaassen CH. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. *J Clin Microbiol*. 2005;43(8):4112-4120.
- Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi*, 3rd Edition M38. CLSI; 2017.
- Arai T, Majima H, Watanabe A, Kamei K. A simple method to detect point mutations in *Aspergillus fumigatus cyp51A* gene using a surveyor nuclease assay. *Antimicrob Agents Chemother*. 2020;64(4):e02271-19. doi:10.1128/AAC.02271-19
- Umeyama T, Hayashi Y, Shimosaka H, et al. CRISPR/Cas9 genome editing to demonstrate the contribution of Cyp51A Gly138Ser to azole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother*. 2018;62(9):e00894-18. doi:10.1128/AAC.00894-18

28. Silva D, Santos G, Barroca M, Collins T. Inverse PCR for point mutation introduction. *Methods Mol Biol.* 2017;1620:87-100.
29. Nakano Y, Tashiro M, Urano R, et al. Characteristics of azole-resistant *Aspergillus fumigatus* attached to agricultural products imported to Japan. *J Infect Chemother.* 2020;26(10):1021-1025.
30. Mortensen KL, Jensen RH, Johansen HK, et al. *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *J Clin Microbiol.* 2011;49(6):2243-2251.
31. Tsuchido Y, Tanaka M, Nakano S, Yamamoto M, Matsumura Y, Nagao M. Prospective multicenter surveillance of clinically isolated *Aspergillus* species revealed azole-resistant *Aspergillus fumigatus* isolates with TR34/L98H mutation in the Kyoto and Shiga regions of Japan. *Med Mycol.* 2019;57(8):997-1003.
32. Takahashi-Nakaguchi A, Muraosa Y, Hagiwara D, et al. Genome sequence comparison of *Aspergillus fumigatus* strains isolated from patients with pulmonary aspergilloma and chronic necrotizing pulmonary aspergillosis. *Med Mycol.* 2015;53(4):353-360.
33. Abdolrasouli A, Rhodes J, Beale MA, et al. Genomic context of azole resistance mutations in *Aspergillus fumigatus* determined using whole-genome sequencing. *MBio.* 2015;6(3):e00536. doi:10.1128/mBio.00536-15
34. Garcia-Rubio R, Monzon S, Alcazar-Fuoli L, Cuesta I, Mellado E. Genome-wide comparative analysis of *Aspergillus fumigatus* strains: the reference genome as a matter of concern. *Genes.* 2018;9(7):363.
35. Zhao S, Ge W, Watanabe A, Fortwendel JR, Gibbons JG. Genome-wide association for itraconazole sensitivity in non-resistant clinical isolates of *Aspergillus fumigatus*. *Front Fungal Biol.* 2021;1(6). doi:10.3389/ffunb.2020.617338
36. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics.* 2018;34(17):i884-i890.
37. Cerqueira GC, Arnaud MB, Inglis DO, et al. The *Aspergillus* Genome Database: multispecies curation and incorporation of RNA-Seq data to improve structural gene annotations. *Nucleic Acids Res.* 2014;42(Data base issue):D705-710.
38. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics.* 2009;25(14):1754-1760.
39. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 2010;20(9):1297-1303.
40. Danecek P, Auton A, Abecasis G, et al. The variant call format and VCFtools. *Bioinformatics.* 2011;27(15):2156-2158.
41. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics.* 2014;30(9):1312-1313.
42. Letunic I, Bork P. Interactive tree of life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res.* 2019;47(W1):W256-w259.
43. Nakamura I, Kanasaki R, Yoshikawa K, et al. Discovery of a new antifungal agent ASP2397 using a silkworm model of *Aspergillus fumigatus* infection. *J Antibiot (Tokyo).* 2017;70(1):41-44.
44. Kowalski CH, Beattie SR, Fuller KK, et al. Heterogeneity among isolates reveals that fitness in low oxygen correlates with *Aspergillus fumigatus* virulence. *MBio.* 2016;7(5):e01515-16. doi:10.1128/mBio.01515-16
45. Chang H, Ashu E, Sharma C, Kathuria S, Chowdhary A, Xu J. Diversity and origins of Indian multi-triazole resistant strains of *Aspergillus fumigatus*. *Mycoses.* 2016;59(7):450-466.
46. Chowdhary A, Kathuria S, Xu J, Meis JF. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. *PLoS Pathog.* 2013;9(10):e1003633.
47. Liu M, Zheng N, Li D, et al. *cyp51A*-based mechanism of azole resistance in *Aspergillus fumigatus*: illustration by a new 3D STRUCTURAL Model of *Aspergillus fumigatus* CYP51A protein. *Med Mycol.* 2016;54(4):400-408.
48. Matsumoto Y, Sekimizu K. Silkworm as an experimental animal for research on fungal infections. *Microbiol Immunol.* 2019;63(2):41-50.
49. Ishii M, Matsumoto Y, Sekimizu K. Usefulness of silkworm as a model animal for understanding the molecular mechanisms of fungal pathogenicity. *Drug Discov Ther.* 2015;9(4):234-237.

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