

Original Article

# Ubiquitous Distribution of Azole-Resistant *Aspergillus fumigatus*-Related Species in Outdoor Environments in Japan

Kohei Watanabe<sup>1</sup>, Takashi Yaguchi<sup>2</sup>, and Dai Hirose<sup>1</sup>

<sup>1</sup> School of Pharmacy, Nihon University

<sup>2</sup> Medical Mycology Research Center, Chiba University

## Abstract

*Aspergillus fumigatus*-related species are responsible for causing aspergillosis, which is a fatal infectious disease. Recently, there has been a series of reports of *A. fumigatus*-related species that are resistant to azole drugs used in clinical practice for the treatment of fungal infections. Some of these species have been isolated from outdoor environments. Testing the drug susceptibility of the strains from outdoor environments, therefore, is important. In this study, we isolated and cultured 72 strains of *A. fumigatus*-related species from the outdoor environment in Japan. The isolates identified via morphological observation and molecular phylogenetic analysis were *Aspergillus felis*, *Aspergillus lentulus*, *Aspergillus pseudoviridinutans*, *Aspergillus udagawae*, and *Aspergillus wyomingensis*. The results of the drug susceptibility testing revealed that *A. felis* (6 of 14 strains) and *A. pseudoviridinutans* (13 of 17 strains) were resistant to itraconazole (ITCZ), with 4 mg/L or higher minimum inhibitory concentrations (MICs). The voriconazole (VRCZ)-resistant strains with 4 mg/L or higher MICs were *A. felis* (14 of 14), *A. lentulus* (4 of 4), *A. pseudoviridinutans* (15 of 17), *A. udagawae* (23 of 34), *A. wyomingensis* (1 of 3), and *A. pseudoviridinutans* (1 of 3). Among them, *A. felis* (1 of 14) and *A. pseudoviridinutans* (7 of 17) demonstrated 8 mg/L or higher MICs for ITCZ and VRCZ. These results indicate that *A. fumigatus*-related species resistant to ITCZ and VRCZ are widely distributed in outdoor environments in Japan.

**Key words** : *Aspergillus fumigatus*, drug susceptibility, TUB-2, *Viridinutantes*, voriconazole

## Introduction

The genus *Aspergillus* includes the causative agents of allergic diseases and invasive aspergillosis, which is a fatal infection in humans, cats, and dogs<sup>1-3</sup>. In particular, *Aspergillus fumigatus*, which belongs to *Aspergillus* section *Fumigati*, is the most important causative agent of invasive aspergillosis. Recently, a series of species genetically related to *A. fumigatus* have been isolated in clinical practice to determine the importance of these species<sup>4-7</sup>. The following *A. fumigatus*-related species have been isolated in clinical settings: *Aspergillus aureoles*, *Aspergillus felis*, *Aspergillus hiratsukae*, *Aspergillus lentulus*, *Aspergillus pseudoviridinutans*, *Aspergillus thermomutatus*, *Aspergillus udagawae*, *Aspergillus viridinutans*, and *Aspergillus wyomingensis*<sup>6, 8-16</sup>. These species, except *A. hiratsukae*, *A. thermomutatus*, and *A. lentulus*, are included in the series *Viridinutantes* proposed by

Houbraken et al<sup>17</sup>. In addition to these five species, ser. *Viridinutantes* also includes *Aspergillus acrensis*, *Aspergillus arcoverdensis*, *A. aureoles*, *Aspergillus bezerrae*, *Aspergillus curviformis*, *Aspergillus frankstonensis*, and *Aspergillus siamensis* and currently consists of 12 species<sup>17</sup>. Houbraken et al<sup>17</sup> further proposed eight series, including ser. *Fumigati* to which *A. lentulus* belongs, ser. *Unilaterales* to which *A. hiratsukae* belongs, and ser. *Thermomutati* to which *A. thermomutatus* belongs.

Some strains of *A. fumigatus* have been reported to be resistant to azole antifungals<sup>18</sup>. *A. fumigatus* resistant to itraconazole (ITCZ) was first discovered by Denning et al<sup>19</sup> and is now reported worldwide<sup>20</sup>. For *A. fumigatus*-related species, clinical strains of *A. felis*, *A. lentulus*, *A. pseudoviridinutans*, *A. udagawae*, and *A. viridinutans* have been reported to have MICs of  $\geq 4$  mg/L for both ITCZ and voriconazole (VRCZ)<sup>5, 12, 14, 16</sup>. Lyskova et al<sup>21</sup> and Talbot et al<sup>22</sup> conducted drug susceptibility testing to azole antifungals on fungal

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Address for correspondence: Dai Hirose, PhD

School of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi, Chiba 274-8555, Japan

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E-mail: hirose.dai@nihon-u.ac.jp

species belonging to ser. *Viridinutantes* isolated from outdoor environments and clinical sources. The results indicated that the strains of the following species were resistant to ITCZ or VRCZ, or both, with MICs of  $\geq 4$  mg/L: *A. acrensensis*, *A. arcoveidensis*, *A. aureoles*, *A. felis*, *A. frankstonensis*, *A. pseudoviridinutans*, *A. siamensis*, *A. udagawae*, *A. viridinutans*, and *A. wyomingensis*. However, Lyskova et al<sup>21)</sup> and Talbot et al<sup>22)</sup> did not show the results of drug susceptibility testing separately for environmental and clinical strains. Thus, it remains unclear how many species are resistant to azole antifungals in outdoor environments. In Japan, there have been reports of drug susceptibility tests using clinical strains<sup>16, 23)</sup>, but few studies have been conducted on environmental strains.

From 2012 to 2017, we surveyed the diversity of species of *Aspergillus* in the outdoor environment in Japan and isolated many *A. fumigatus*-related species in the process. In this study, we conducted drug susceptibility tests and molecular phylogenetic analysis using partial nucleotide sequences of the  $\beta$ -tubulin gene (*TUB-2*) on these environmental strains and Japanese clinical strains deposited at the Medical Mycology Research Center (MMRC), Chiba University. Our results indicate that *A. fumigatus*-related species resistant to azole antifungal drugs may be universally distributed in outdoor environments in Japan.

## Materials and methods

### Sampling and fungal isolation

An ecological survey was conducted from 2012 to 2017 to investigate the species diversity of the causative agents of invasive aspergillosis and the distribution pattern of each fungal species in outdoor environments throughout Japan across diverse climates and landscapes. The strains of *A. fumigatus*-related species isolated in that survey from soil and air by the following methods were used in this study. The soil-sampling sites from where the strains of *A. fumigatus*-related species were isolated are listed in Table 1. At each sampling site, 10–15 soil samples at least 5 m apart were obtained. The source of each soil was classified into four types in accordance with the landscape of the sampling site: forest (13 sites), grassland (1 site), bare land (2 sites), and farmland (2 sites) (Table 1). Approximately 100 g of soil was collected from the field after removing any plant litter accumulating on the surface and brought to the laboratory. Approximately 20–40 g of soil was placed in a sterilized culture bottle (5 cm in diameter and 10 cm in height), and then sterilized corn grains were placed on top of the soil and incubated at 35 °C for 7–10 days. After incubation, the conidia possibly identified as *A. fumigatus*-related species, which appeared on the corn grains, were isolated under a stereomicroscope (SZH10; Olympus, Tokyo, Japan) and cultured on corn meal agar (CMA, Nissui Pharmaceutical, Tokyo, Japan) supplemented with chloram-

phenicol (CP) at room temperature. Subsequently, the conidia that formed on the CMA were isolated and cultured on malt extract agar (MA, Nissui Pharmaceutical, Tokyo, Japan). Air sample (200 L) was collected in 2016 from the roof of a building that was approximately 40-m high in Funabashi, Chiba, Japan, and from an adjacent forest (elevation: 25 m) using an air sampler (Mas-100 Eco; Merck, Darmstadt, Germany) with CMA medium supplemented with CP. The strains cultured on MA were identified *via* macroscopic and microscopic morphological observations and the *TUB-2* sequences and are presented in Table 1. Isolates from the same sample were excluded when the strains were identified as the same species. The strains isolated from human and animal clinical specimens in Japan were obtained from MMRC, Chiba University, through the National Bio-Resource Project, Japan (<http://www.nbrp.jp/>) (Table 1).

### DNA extraction and molecular studies

Genomic DNA was extracted from the mycelia cultured on MA overlaid with a cellophane membrane using the cetyltrimethylammonium bromide method<sup>24)</sup>. Polymerase chain reaction (PCR) was performed using Quick Taq HS DyeMix (Toyobo, Osaka, Japan). PCR primer pair Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3')<sup>25)</sup> or T10 (5'-ACGATAGGTTACCTCCAGAC-3')<sup>26)</sup> and BenA2 (5'-AGTTGTCTGGGACGGAAGAG-3')<sup>27)</sup> or Bt2a and Bt2b (5'-ACCCTCAGTGTAGTGACCTTGGC-3')<sup>25)</sup> were used to obtain the sequences of *TUB-2*. DNA fragments were amplified using a PCR thermal cycler (DNA Engine; Bio-Rad, California, USA) with the following thermal cycling schedule: the first cycle of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 54°C for annealing, and 1 min at 72°C, with a final cycle of 10 min at 72°C. PCR products were purified according to the manufacturer's protocol using a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). Purified PCR products were sequenced by Fasmac (Kanagawa, Japan). The sequences obtained in this study were deposited in the DNA Data Bank of Japan (DDBJ) (Table 1).

### Phylogenetic analysis

The sequences were aligned by MAFFT v. 7<sup>28)</sup>, and the dataset was utilized for phylogenetic analysis using maximum-likelihood estimation with RAxML-NG v.1.0.1<sup>29)</sup> implemented in raxmlGUI 2.0.5<sup>30)</sup>. The best-fitting nucleotide substitution model (K80 + I model) was selected using ModelTest-NG v.0.1.6<sup>31)</sup> according to the Akaike information criterion (AICc). The bootstrap procedure of Lemoine et al<sup>32)</sup> was used with 1,000 replicates to estimate clade support.

### Antifungal susceptibility testing

Each strain was tested for susceptibility to ITCZ and VRCZ and cultured for 7–14 days to form conidia, and then conidia suspensions were prepared. The micro-liquid dilution method according to the Clinical and Laboratory Standards Institute's M38-A2 procedure was used with the yeast-like fungi DP

Table 1. Strains isolated in this study and used for phylogenetic analysis

Species	Strains No.	Source	Location	Accession No.	Reference
<i>Aspergillus acrensis</i>	IFM 57291	Grassland soil in cattle farm	Brazil	LT795980	Lyskova et al (Unpublished)
<i>A. arcovorderensis</i>	IFM 61334	Caatinga soil	Brazil	AB818845	Matsuzawa et al 2015
<i>A. aureolus</i>	NRRL 2244	Soil	Tafo, Ghana	EF669808	Peterson et al 2008
<i>A. brevipes</i>	NRRL 2439	Soil	Australia	EF669812	Peterson et al 2008
<i>A. felis</i>	CBS 130245	Cat specimen	Australia	MN969363	Houbraken et al 2020
	IFM 54303	Unknown	Japan	AB248299	Yaguchi et al 2007
	IFM 59564	Human specimen	Japan	LT795801	Lyskova et al (Unpublished)
	IFM 60053	Human specimen	Japan	LT795856	Lyskova et al (Unpublished)
	IFM 61579	Human specimen	Japan	LC631859	This study
	IFM 62091	Forest soil	Mt. Norikura, Nagano, Japan	LC631860	This study
	IFM 62092	Forest soil	Mt. Norikura, Nagano, Japan	LC631861	This study
	IFM 62093	Forest soil	Mt. Norikura, Nagano, Japan	LC631862	This study
	IFM 62881	Forest soil	Mt. Noma, Kagoshima, Japan	LC631863	This study
	IFM 62883	Forest soil	Kuninomatsubara, Kagoshima, Japan	LC631864	This study
	IFM 62884	Forest soil	Kuninomatsubara, Kagoshima, Japan	LC631865	This study
	IFM 62885	Forest soil	Kuninomatsubara, Kagoshima, Japan	LC631866	This study
	IFM 62886	Forest soil	Mt. Homan, Fukuoka, Japan	LC631867	This study
	IFM 62893	Bare land soil	Mt. Meakandake, Hokkaido, Japan	LC631868	This study
	IFM 62894	Bare land soil	Mt. Meakandake, Hokkaido, Japan	LC631869	This study
	IFM 62895	Bare land soil	Mt. Meakandake, Hokkaido, Japan	LC631870	This study
	IFM 62993	Bare land soil	Mt. Meakandake, Hokkaido, Japan	LC631871	This study
	IFM 64234	Grassland soil	Miyakejima, Tokyo, Japan	LC631872	This study
	IFM 64235	Grassland soil	Miyakejima, Tokyo, Japan	LC631873	This study
<i>A. franktonensis</i>	CBS DTO 341-F3	Woodland soil	Frankston, Australia	KY808599	Talbot 2017
<i>A. fumigatus</i>	CBS 133.61	Chicken specimen	USA	AY685150	Hong et al 2005
<i>A. fumisynnematus</i>	IFM 42277	Soil	Venezuela	AB248076	Yaguchi et al 2007
<i>A. lentulus</i>	CBS 117885	Human clinical specimens	USA	KJ175522	De Respini et al (Unpublished)
	IFM 47063	Unknown	Japan	AB248074	Yaguchi et al 2007
	IFM 47457	Human specimen	Japan	AB248075	Yaguchi et al 2007
	IFM 58399	Human specimen	Japan	LC631874	This study
	IFM 60648	Human specimen	Japan	LC631875	This study
	IFM 61392	Human specimen	Japan	LC631876	This study
	IFM 61591	Human specimen	Japan	LC631877	This study
	IFM 62073	Forest soil	Mt. Norikura, Nagano, Japan	LC631878	This study
	IFM 62096	Farm soil	Yotsukaido, Chiba, Japan	LC631879	This study
	IFM 62882	Forest soil	Mt. Noma, Kagoshima, Japan	LC631880	This study
	IFM 63961	Air	Funabashi, Chiba, Japan	LC631881	This study
<i>A. pseudoviridinutans</i>	IFM 59502	Human specimen	Japan	LT795936	Lyskova et al (Unpublished)
	IFM 59503	Human specimen	Japan	LT795940	Lyskova et al (Unpublished)
	IFM 60685	Human specimen	Japan	LC631882	This study
	IFM 62074	Forest soil	Mt. Kisokoma, Nagano, Japan	LC631883	This study
	IFM 62075	Forest soil	Mt. Kisokoma, Nagano, Japan	LC631884	This study
	IFM 62076	Forest soil	Mt. Kisokoma, Nagano, Japan	LC631885	This study
	IFM 62078	Forest soil	Mt. Kisokoma, Nagano, Japan	LC631886	This study
	IFM 62079	Forest soil	Mt. Kisokoma, Nagano, Japan	LC631887	This study
	IFM 62080	Forest soil	Mt. Kisokoma, Nagano, Japan	LC631888	This study
	IFM 62083	Forest soil	Mt. Norikura, Nagano, Japan	LC631889	This study
	IFM 62094	Forest soil	Izumo, Shimane, Japan	LC631890	This study
	IFM 64217	Forest soil	Hachijojima, Tokyo, Japan	LC631891	This study
	IFM 64218	Grassland soil	Miyakejima, Tokyo, Japan	LC631892	This study
	IFM 64220	Grassland soil	Miyakejima, Tokyo, Japan	LC631893	This study
	IFM 64225	Grassland soil	Miyakejima, Tokyo, Japan	LC631894	This study
	IFM 64228	Grassland soil	Miyakejima, Tokyo, Japan	LC631895	This study
	IFM 64233	Grassland soil	Miyakejima, Tokyo, Japan	LC631896	This study
	IFM 62889	Farm soil	Uehara, Iriomote Island, Okinawa, Japan	LC631897	This study
	IFM 64230	Grassland soil	Miyakejima, Tokyo, Japan	LC631898	This study
	IFM 64232	Bare land soil	Miyakejima, Tokyo, Japan	LC631899	This study
	NHAVA1	Human specimen	USA	GQ144441	Vinh et al 2009
<i>A. siamensis</i>	CBS DTO 278-B6	Soil	Thailand	KY808582	Talbot 2017
<i>A. spinosus</i>	NRRL 5034	Soil	Nicaragua	EF669844	Peterson et al 2008
<i>A. tatenoi</i>	CBS 407.93	Soil from sugarcane plantation	Brazil	DQ114130	Hong et al (Unpublished)
<i>A. udagawae</i>	CBS 114217	Soil	Brazil	AF132226	Varga et al (Unpublished)
	IFM 5058	Human specimen	Japan	AB248292	Yaguchi et al 2007
	IFM 51744	Human specimen	Japan	AB248293	Yaguchi et al 2007
	IFM 53867	Unknown	Japan	AB248294	Yaguchi et al 2007
	IFM 53868	Human specimen	Japan	AB248295	Yaguchi et al 2007
	IFM 54302	Unknown	Japan	AB248296	Yaguchi et al 2007
	IFM 58400	Unknown	Japan	LC631900	This study
	IFM 59990	Human specimen	Japan	LC631901	This study
	IFM 61606	Human specimen	Japan	LC631902	This study
	IFM 62070	Forest soil	Mt. Kisokoma, Nagano, Japan	LC631903	This study
	IFM 62072	Forest soil	Mt. Kisokoma, Nagano, Japan	LC631904	This study
	IFM 62077	Forest soil	Mt. Kisokoma, Nagano, Japan	LC631905	This study
	IFM 62085	Forest soil	Mt. Norikura, Nagano, Japan	LC631906	This study
	IFM 62086	Forest soil	Mt. Norikura, Nagano, Japan	LC631907	This study
	IFM 62087	Forest soil	Mt. Norikura, Nagano, Japan	LC631908	This study
	IFM 62088	Forest soil	Mt. Norikura, Nagano, Japan	LC631909	This study
	IFM 62089	Forest soil	Mt. Norikura, Nagano, Japan	LC631910	This study
	IFM 62090	Forest soil	Mt. Norikura, Nagano, Japan	LC631911	This study
	IFM 62095	Farm soil	Yotsukaido, Chiba, Japan	LC631912	This study
	IFM 62097	Forest soil	Mt. Takatsuka, Chiba, Japan	LC631913	This study
	IFM 62098	Forest soil	Mt. Takatsuka, Chiba, Japan	LC631914	This study
	IFM 62099	Forest soil	Mt. Takatsuka, Chiba, Japan	LC631915	This study
	IFM 62100	Forest soil	Mt. Takatsuka, Chiba, Japan	LC631916	This study
	IFM 62887	Forest soil	Sumiyoshi, Iriomote Island, Okinawa, Japan	LC631917	This study
	IFM 62888	Farm soil	Uehara, Iriomote Island, Okinawa, Japan	LC631918	This study
	IFM 62890	Forest soil	Midorigaike, Kyoto, Japan	LC631919	This study
	IFM 62891	Forest soil	Sakura, Chiba, Japan	LC631920	This study
	IFM 62892	Forest soil	Tateyama, Toyama, Japan	LC631921	This study
	IFM 62898	Farm soil	Yotsukaido, Chiba, Japan	LC631922	This study
	IFM 62899	Farm soil	Yotsukaido, Chiba, Japan	LC631923	This study
	IFM 62900	Farm soil	Yotsukaido, Chiba, Japan	LC631924	This study
	IFM 62901	Farm soil	Yotsukaido, Chiba, Japan	LC631925	This study
	IFM 62902	Farm soil	Yotsukaido, Chiba, Japan	LC631926	This study
	IFM 62903	Farm soil	Yotsukaido, Chiba, Japan	LC631927	This study
	IFM 62904	Forest soil	Yotsukaido, Chiba, Japan	LC631928	This study
	IFM 62905	Farm soil	Yotsukaido, Chiba, Japan	LC631929	This study
	IFM 62906	Farm soil	Yotsukaido, Chiba, Japan	LC631930	This study
	IFM 62907	Farm soil	Yotsukaido, Chiba, Japan	LC631931	This study
	IFM 62908	Farm soil	Yotsukaido, Chiba, Japan	LC631932	This study
	IFM 62909	Farm soil	Yotsukaido, Chiba, Japan	LC631933	This study
	IFM 63962	Air	Funabashi, Chiba, Japan	LC631934	This study
	IFM 63964	Air	Funabashi, Chiba, Japan	LC631935	This study
	IFM 64237	Forest soil	Hachijojima, Tokyo, Japan	LC631936	This study
<i>A. viridinutans</i>	CBS 127.56	Rabbit specimen	Australia	AF134779	Varga et al (Unpublished)
<i>A. wyomingensis</i>	CBS DTO 332-B1	Coal mine reclamation site soil	Glenrock, USA	KY808589	Talbot et al 2017
	IFM 62081	Forest soil	Mt. Norikura, Nagano, Japan	LC631937	This study
	IFM 62082	Forest soil	Mt. Norikura, Nagano, Japan	LC631938	This study
	IFM 62084	Forest soil	Mt. Norikura, Nagano, Japan	LC631939	This study

“EIKEN” (Eiken Chemical, Tokyo, Japan). The tests were performed according to Tamiya et al.<sup>16)</sup> in triplicate, and *Aspergillus flavus* ATCC MYA-3631 was used as the quality control or reference strain. Four mg/L or higher MICs were determined as resistant.

## Results

We isolated 72 strains of *A. fumigatus*-related species from outdoor environments in Japan. Based on morphological characteristics and molecular phylogenetic analysis, the isolates were identified as *A. felis* (14), *A. lentulus* (4), *A. pseudoviridinutans* (17), *A. udagawae* (34), and *A. wyomingensis* (3) (Table 1). A comparison of the number of isolates of each *A. fumigatus*-related species among the isolation sources demonstrated their widespread distribution; namely, *A. felis*: 28.6% from bare land soil, 14.3% from grassland soil, and 57.1% from forest soil; *A. lentulus*: 50% from forest soil, 25% from farm soil, and 25% from air; *A. pseudoviridinutans*: 5.9% from bare land soil, 35.3% from grassland soil, 52.9% from forest soil, and 5.9% from farm soil; *A. udagawae*: 55.9% from forest soil, 38.2% from farm soil, and 5.9% from air; and *A. wyomingensis*: 100% from forest soil. The clinical strains newly identified in this study were *A. felis* (1), *A. lentulus* (4), *A. pseudoviridinutans* (1), and *A. udagawae* (3).

Molecular phylogenetic analysis based on the *TUB-2* sequences revealed three major clades: *A. felis* and *A. pseudoviridinutans* clade (Bootstrap values (BS) = 94%), *A. lentulus* clade (BS = 85%), and *A. udagawae* and *A. wyomingensis* clade (BS = 95%) (Fig. 1). *A. fumigatus*-related species formed clades with high BS (*A. felis*, BS = 92%; *A. lentulus*, BS = 92%; *A. pseudoviridinutans*, BS = 97%; *A. udagawae*, BS = 100%; and *A. wyomingensis*, BS = 93%) (Fig. 1). For all species, environmental and clinical strains were found to be genetically similar (Fig. 1).

The results of the drug susceptibility testing are summarized in Table 2 (See Table S for results of each strain). Of the 72 strains, 19 (26.4%) and 57 (79.2%) were resistant to ITCZ and VRCZ, respectively, with MICs  $\geq 4$  mg/L. No ITCZ-resistant strains of *A. lentulus*, *A. udagawae*, and *A. wyomingensis* were observed in either clinical or environmental sources. In *A. felis* and *A. pseudoviridinutans*, 42.9% (6 of 14) and 76.5% (13 of 17) of environmental strains, respectively, were resistant to ITCZ, although no resistant strains were detected in clinical strains. The VRCZ-resistant strains were identified in *A. felis*, *A. lentulus*, *A. pseudoviridinutans*, *A. udagawae*, and *A. wyomingensis*. In environmental strains, 100% (14 of 14) of *A. felis*, 100% (4 of 4) of *A. lentulus*, 88.2% (15 of 17) of *A. pseudoviridinutans*, 88.2% (15 of 17) of *A. udagawae*, and 33.3% (1 of 3) of *A. wyomingensis* were VRCZ-resistant. In clinical isolates, 50% (2 of 4) of *A. felis*, 50% (3 of 6) of *A. lentulus*, 66.7% (2 of 3)

of *A. pseudoviridinutans*, and 37.5% (3 of 8) of *A. udagawae* were VRCZ-resistant. One environmental strain of *A. felis* and six environmental strains of *A. pseudoviridinutans* were found to be highly resistant to ITCZ with MIC  $\geq 8$  mg/L; these strains were also highly resistant to VRCZ. The molecular phylogenetic tree showed that azole drug-resistant strains were found in various lineages regardless of genetic relatedness (Fig. 1).

## Discussion

In this study, we isolated *A. felis*, *A. lentulus*, *A. pseudoviridinutans*, *A. udagawae*, and *A. wyomingensis*, which are *A. fumigatus*-related species and cause aspergillosis, from outdoor environments in Japan. Among these, *A. fumigatus*-related species that have been reported from clinical isolates are considered to be clinically important owing to their pathogenicity<sup>18)</sup>. The first step in the pathogenesis of aspergillosis is the inhalation of airborne spores and their entry into the human body<sup>33)</sup>. Therefore, it is important to evaluate the species diversity of *A. fumigatus*-related species in outdoor environments and clinical settings since the dominant habitat of *A. fumigatus*-related species is considered to be the outdoor environment. All the *A. fumigatus*-related species identified in this study have been previously reported to be isolated from outdoor environments, such as the following: *A. felis*: soil from coal mine accumulations in the United States, forest soil in Australia, spoil banks and soil crusts in the Czech Republic, water surface in Portugal, and air in Germany and Spain; *A. lentulus*: corn and pepper fields in Korea, soil after herbicide use in Australia, *Coffea* sp. in Denmark, and cocoa beans of unknown origin; *A. pseudoviridinutans*: soils from India and Brazil (environment details unknown) and *Pinus caribaea* in Sri Lanka; *A. udagawae*: soils from prairie and soil from mine waste dump in the United States, plantations in Brazil, spoil bank and soil crust in the Czech Republic, and soils in China, Russia, Thailand, and Turkey (environment details unknown); and *A. wyomingensis*: soils from coal mine accumulations in the United States and soils in China and Russia (environment details unknown)<sup>21, 34, 35)</sup>. Multiple strains of *A. felis*, *A. udagawae*, and *A. wyomingensis* have been reported in Australia, Brazil, and the United States<sup>34, 35)</sup>. Conversely, only one strain each of *A. lentulus* and *A. pseudoviridinutans* has been reported in each of the aforementioned countries. Interestingly, we isolated numerous *A. lentulus* and *A. pseudoviridinutans* species in this study. Reports of isolation of *A. fumigatus*-related species in outdoor environments are incomplete; however, there are many reports of isolation from large animals and also humans<sup>1, 8, 34)</sup>. In Japan, most reports were on *A. fumigatus*-related species obtained from clinical sources<sup>16, 23)</sup>; there were only few reports on these species obtained from outdoor environments. In this study, we isolated





Table 2. Minimum inhibitory concentrations (MICs) of *Aspergillus fumigatus*-related species resistant to ITCZ and VRCZ isolated in Japan

Species	Number of strains	Source	MIC value (mg/L)					
			ITCZ			VRCZ		
			2 >	2	≥ 4	2 >	2	≥ 4
<i>Aspergillus felis</i>	14	E*	4	4	6			14
	4	C**	3	1		2		2
<i>A. lentulus</i>	4	E	4					4
	6	C	6			1	2	3
<i>A. pseudoviridinutans</i>	17	E	1	3	13		2	15
	3	C	1	2		1		2
<i>A. udagawae</i>	34	E	32	2			11	23
	8	C	8			2	3	3
<i>A. wyomingensis</i>	3	E	3				2	1

\*: Environmental, \*\*: Clinical

*A. fumigatus*-related species in a wide area of Japan, regardless of the latitude or altitude, as presented in Table 1. So far, although the diversity of this taxon in outdoor environments has not been sufficiently investigated, there have been few reports of its isolation, and it seems to be widely distributed in outdoor environments regardless of the climatic zone.

Through drug susceptibility testing of *A. fumigatus*-related species from outdoor environments using ITCZ and VRCZ, we were able to identify ITCZ-resistant strains of *A. felis* and *A. pseudoviridinutans* and VRCZ-resistant strains of *A. felis*, *A. lentulus*, *A. udagawae*, and *A. wyomingensis*. In *A. felis* and *A. pseudoviridinutans*, we isolated strains that are highly resistant to ITCZ and VRCZ with MICs  $\geq 8$  mg/L. Studies have tested the susceptibility of these species and found resistant strains with the following MICs: *A. felis* (MIC against ITCZ:  $> 16$  mg/L, MIC against VRCZ: 8 mg/L), *A. lentulus* (MIC against ITCZ and VRCZ: 4 mg/L), *A. pseudoviridinutans* (MIC against ITCZ:  $> 16$  mg/L, MIC against VRCZ: 4 mg/L), *A. udagawae* (MIC against ITCZ:  $> 16$  mg/L, MIC against VRCZ: 16 mg/L), and *A. wyomingensis* (MIC against ITCZ:  $> 16$  mg/L, MIC against VRCZ: 4 mg/L)<sup>5, 21, 22, 36</sup>. However, although studies have described the results of the susceptibility testing of clinical and environmental strains, there was no information on the drug susceptibility of environmental strains. In this study, although numerous strains were found to be susceptible to ITCZ, the strains resistant to ITCZ or VRCZ or both were considered to be distributed in the outdoor environment in Japan regardless of the climate and landscape vegetation of the soil-sampling sites. With regard to the clinical strains used in this study, the strains of *A. felis*, *A. lentulus*, and *A. udagawae* were sensitive to ITCZ but resistant to VRCZ with MIC of 8 mg/L. Conversely, the strain of *A. pseudoviridinutans* was sensitive to ITCZ but resistant to VRCZ with MIC of 4 mg/L. This indicates that drug

susceptibility tend to be similar between the clinical and environmental strains.

The molecular phylogenetic analysis based on *TUB-2* sequences showed that the environmental and clinical strains isolated in this study were genetically closely related. There was no clear genetic differentiation between the strains resistant to ITCZ or VRCZ or both and those that were not resistant in the drug susceptibility testing. The mechanism of drug resistance of *A. fumigatus*-related species was recently studied by Talbot et al<sup>22</sup>, and the M172A/V and D255G mutations in the *cyp51A* gene associated with the resistance of *A. fumigatus* to azole were found in the test species (*A. arcoverdensis*, *A. aureolus*, *A. felis*, *A. frankstonensis*, *A. pseudoviridinutans*, *A. siamensis*, *A. udagawae*, *A. viridinutans*, and *A. wyomingensis*). However, the mechanism of drug resistance of *A. fumigatus*-related species has not yet been elucidated. Although the *cyp51A* mutation was not analyzed in this study, it should be considered in future studies to clarify the evolutionary process of drug-resistant strains in outdoor environments.

This study demonstrated that *A. fumigatus*-related species, which adapt to various environments, are widely distributed in outdoor environments and suggested that many of these strains are resistant to ITCZ or VRCZ or both. We are currently conducting ecological studies to elucidate the distribution patterns of *A. fumigatus*-related species in various outdoor environments in Japan.

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### Conflicts of interest

The authors declare that they have no conflict of interest.

### Transparency declarations

All authors: none to declare.

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