

Prevalence, mechanisms and genetic relatedness of the human pathogenic fungus *Aspergillus fumigatus* exhibiting resistance to medical azoles in the environment of Taiwan

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Summary

Emerging azole resistance in *Aspergillus fumigatus* poses a serious threat to human health. This nationwide surveillance study investigated the prevalence and molecular characteristics of azole-resistant *A. fumigatus* environmental isolates in Taiwan, an island country with increasing use of azole fungicides. Of the 2760 air and soil samples screened from 2014 to 2016, 451 *A. fumigatus* isolates were recovered from 266 samples and 34 isolates from 29 samples displayed resistance to medical azoles (itraconazole, voriconazole or posaconazole). The resistance prevalence was 10.9% and 7.5% in *A. fumigatus*-positive samples and isolates respectively. Most (29, 85.3%) azole-resistant isolates harboured TR₃₄/L98H mutations, which were widely distributed, clustered genetically with clinical isolates, and had growth rates that were similar to those of the wild-type isolates. Microsatellite genotyping revealed both the global spread of the TR₃₄/L98H isolates and the occurrence of TR₃₄/L98H/S297T/F495I isolates belonging to local microsatellite genotypes. *AfuMDR3* and *atrF*, two efflux transporter genes, were constitutively upregulated in two individual resistant isolates without *cyp51A* mutations, highlighting their potential roles in azole resistance. These results emphasize the need for periodic environmental surveillance at the molecular level in regions in which azole fungicides are applied, and agricultural fungicide management strategies that generate less selective pressure should be investigated.

Introduction

Invasive aspergillosis is an important human fungal disease causing substantial mortality that affects an ever-increasing population of immunocompromised patients, and most infections are caused by *Aspergillus fumigatus*, a saprophytic mould that produces airborne conidia and is ubiquitously distributed in the environment (Verweij *et al.*, 2015). The recommended therapies for aspergillosis include voriconazole and other mould-active azoles, which

target sterol 14 α -demethylase encoded by *cyp51A* (Verweij *et al.*, 2015). The prevalence of azole-resistant *A. fumigatus* has significantly increased over the past two decades, threatening the effectiveness of first-line antifungal therapy (Verweij *et al.*, 2016). Azole resistance in *A. fumigatus* may develop during patient therapy with medical azoles or through exposure to azole fungicides in the environment. TR₃₄/L98H and TR₄₆/Y121F/T289A mutations, which combine tandem repeats in the *cyp51A* promoter region and amino acid substitution(s) in its coding sequence, are the two major environmentally derived resistance mutations that are prevalent among both clinical and environmental azole-resistant isolates (Chowdhary *et al.*, 2017). Additionally, *cyp51A*-independent resistance mechanisms, such as the upregulation of drug efflux transporters, have also been increasingly recognized in clinical and environmental settings (Slaven *et al.*, 2002; Fraczek *et al.*, 2013; Meneau *et al.*, 2016). Considering the global emergence of azole-resistant *A. fumigatus* isolates and the dismal clinical outcomes associated with delayed effective treatment, experts discourage voriconazole monotherapy for the treatment of invasive aspergillosis in regions with a high environmental resistance rate (> 10%) (Verweij *et al.*, 2015).

To halt the progression of azole resistance and identify potential targets for the discovery of new antifungals, understanding how resistance develops and evolves in different parts of the world is important. Asian countries contribute 24% of the global agricultural fungicide usage (Stensvold *et al.*, 2012). However, knowledge regarding the prevalence and clonality of azole-resistant *A. fumigatus* in Asia based on nationwide environmental surveillance is limited, except for reports from China and India (Chowdhary *et al.*, 2012; Chen *et al.*, 2016a). Taiwan is an East Asian island country geographically separated from the main Eurasian countries but with frequent international business and travel. Agriculture is one of the main industries in Taiwan, and azole fungicides have been widely used in Taiwan for at least three decades. Our earlier work identified clinical TR₃₄/L98H isolates that were genetically related or unrelated to overseas isolates from azole-naïve patients, suggesting an environmental niche for such isolates (Wu *et al.*, 2015). Thus, undertaking a wide environmental survey of azole resistance in *A. fumigatus* in Taiwan in the era of globalization is warranted and ecologically interesting. In this study, we aimed to delineate the prevalence and resistance mechanisms of azole-resistant *A. fumigatus* environmental isolates in Taiwan and their genetic relatedness to strains from international collections.

Materials and methods

A. fumigatus isolates

Air and soil samples from different locations across Taiwan were investigated from January 2014 to June 2016. Air

samples were obtained from the indoor areas of 28 hospitals, communities and farmlands using an air sampler (1000 L in 10 min) (Air IDEAL®; bioMérieux, Marcy l'Etoile, France), and soil samples were collected from communities and farmlands. Two grams of each soil sample was suspended in 4 ml of 0.85% NaCl and 0.3% Triton X-100, vortexed and allowed to settle for 30 s. The cultivation of *Aspergillus* conidia from the air and soil samples (100 μ l of each supernatant) was performed on Sabouraud dextrose agar (SDA) plates supplemented with 50 mg L⁻¹ chloramphenicol (Sigma-Aldrich, St. Louis, USA), which were incubated at 37°C for one day and then at 50°C for up to 2 days to select for heat-tolerant *A. fumigatus*. A maximum of 3 colonies per plate were purified. *A. fumigatus* was identified by morphological characteristics and sequence analysis of the internal transcribed spacer region and calmodulin genes (Balajee *et al.*, 2007).

Antifungal susceptibility testing

To detect the azole-resistant isolates, azole-containing agar plates were used. The aforementioned colonies were inoculated onto three SDA plates supplemented with respectively itraconazole (4 mg L⁻¹), voriconazole (1 mg L⁻¹) and posaconazole (0.5 mg L⁻¹) (all Sigma-Aldrich, St. Louis, USA) and incubated at 37°C. Colonies that grew after 2–4 days on any of the azole-containing agar plates were selected for susceptibility testing. The minimum inhibitory concentrations (MICs) of the medical azoles (itraconazole, posaconazole and voriconazole), amphotericin B (Sigma-Aldrich, St. Louis, USA) and azole fungicides (difenoconazole and tebuconazole [both Chem Service, Pennsylvania, USA]) were determined using the Clinical Laboratory Standard Institute (CLSI) broth microdilution method (M38-A2 document) (CLSI, 2008). The final drug concentrations used for testing were 0.015 to 8 mg L⁻¹ for amphotericin B, itraconazole, posaconazole and voriconazole and 0.06 to 32 mg L⁻¹ for difenoconazole and tebuconazole. According to the clinical breakpoints recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), an isolate with an itraconazole, voriconazole or posaconazole MIC of >2, >2 or >0.25 mg L⁻¹, respectively, was considered 'azole-resistant' (EUCAST).

Resistance mechanisms

All azole-resistant isolates and randomly selected azole-susceptible *A. fumigatus* isolates were examined for mutations in *cyp51A* and its promoter region using previously described methods (Snelders *et al.*, 2010). For resistant isolates without typical *cyp51A* mutations known to be associated with azole resistance, *cyp51A*-independent resistance mechanisms were investigated. *hapE* was sequenced as described previously (Camps *et al.*, 2012b), *srbA* was sequenced using the laboratory-designed PCR primers and conditions described in Supporting Information Table S1 (Willger *et al.*, 2008), and both sequences were compared to those of *A. fumigatus* Af293 to detect any mutations. Biofilms were prepared for the gene expression analysis of *cyp51A*, *cyp51B* and drug efflux transporter genes (*AfuMDR1*, *AfuMDR2*, *AfuMDR3*, *AfuMDR4*, *atrF*, *cdr1B* and *MFS56*). In brief, for each isolate,

three 75T flasks were inoculated with 25 ml of spore suspensions (10^5 spores ml^{-1}) prepared in RPMI-1640 medium and incubated at 37°C until a layer of adherent mycelia (biofilm) formed (usually 16 h). The biofilms were then washed thoroughly three times with sterile PBS by repeated pipetting to remove non-adherent cells. After washing, itraconazole was added to the first flask to a final concentration of 4 mg L^{-1} , voriconazole to the second flask at a concentration of 1 mg L^{-1} , and an equivalent volume of DMSO to the third flask as a control. After 8 h of treatment, the biofilm material was scraped and removed from each flask and placed into a 2 ml screw-cap vial. Excess medium was removed by centrifugation at 13 000 r.p.m. for 2 min. RNA was extracted from 100 mg of the fungal mycelia using a MagCore Total RNA Cultured Cells Kit (RBC Bioscience, New Taipei City, Taiwan). cDNA was subsequently synthesized with GoScript™ reverse transcriptase (Promega, Wisconsin, USA) according to the manufacturer's instructions. The basal and azole-induced mRNA expression levels were then assessed by quantitative reverse transcription (RT)-PCR using the 2X KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Massachusetts, USA) with previously described primers and modified PCR conditions (95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 65°C for 30 s) (Fraczek *et al.*, 2013). For each strain and each gene examined, three biological replicates were performed with three technical replicates. Transcript levels were normalized to a housekeeping gene, β -tubulin, and compared to *A. fumigatus* ATCC MYA-3626, except for expression of the *AfuMDR4* gene, which was compared to ATCC 16903. The results were analysed using the $2^{-\Delta\Delta C_t}$ method. Five susceptible strains and 2 TR₃₄/L98H isolates were also evaluated for *cyp51A*-independent resistance mechanisms as controls.

Microsatellite genotyping

Genotyping was performed based on a cluster analysis of nine *A. fumigatus* short tandem repeat (STR) markers with interlaboratory standardization, as described previously (de Valk *et al.*, 2005; de Valk *et al.*, 2009). For phylogenetic analysis, we included environmentally resistant and randomly selected susceptible isolates in this study along with 13 local clinical isolates (including 7 TR₃₄/L98H isolates) identified in our collection from 2011 to 2016 and 23 isolates from other countries (Chowdhary *et al.*, 2012; Badali *et al.*, 2013; Ahmad *et al.*, 2014; Kidd *et al.*, 2015; Steinmann *et al.*, 2015; Chen *et al.*, 2016a; Alvarez-Moreno *et al.*, 2017; Toyotome *et al.*, 2017). The dendrogram was prepared using the Unweighted Pair Group Method with an Arithmetic mean (UPGMA) algorithm using BioNumerics v7.5 software (Applied Maths, Sint-Martens-Latem, Belgium).

Growth assays

To assess the fitness costs of azole resistance, radial growth and sporulation were observed in selected TR₃₄/L98H isolates of different microsatellite genotypes, resistant isolates without *cyp51A* mutations and susceptible reference and clinical strains. A conidial suspension ($10 \mu\text{l}$, 10^5 conidia ml^{-1}) was inoculated in the centre of a SDA plate and incubated at 35°C.

The diameter of the growing colony and sporulation were examined every 24 h for up to 72 h.

This study was approved by the Institutional Review Board (EC1040502-E) of the National Health Research Institutes, Taiwan.

Results

A total of 2760 environmental samples, comprising mainly soil samples (2480, 89.9%) from farmlands, were collected, among which 564 (20.4%), 452 (16.4%), 1232 (44.6%) and 512 (18.6%) samples were from Northern, Central, Southern and Eastern Taiwan respectively. Overall, 451 *A. fumigatus* isolates were recovered from 266 samples, and 34 isolates from 29 samples displayed azole resistance. Resistant isolates were recovered from all four geographic regions of Taiwan, mainly from farmland soils (31, 91.2%) but also from air in urban communities (3, 8.8%). Overall, the prevalence of azole resistance was 10.9% and 7.5% in *A. fumigatus*-positive samples and isolates, respectively, and compared with that in the other regions, the prevalence was the lowest in Northern Taiwan (Fig. 1; Supporting Information Table S2).

The majority (29, 85.3%) of azole-resistant isolates harboured TR₃₄/L98H mutations with or without S297T/F495I substitutions, which are described as TR₃₄/L98H isolates in this study. All TR₃₄/L98H isolates were resistant to both itraconazole and posaconazole, and 11 (37.9%) isolates (10 with TR₃₄/L98H mutations and one with TR₃₄/L98H/S297T/F495I mutations) were also resistant to voriconazole and exhibited the pan-azole resistant phenotype. Notably, the TR₃₄/L98H/S297T/F495I isolates had lower voriconazole MICs than the TR₃₄/L98H isolates without S297T/F495I substitutions, as demonstrated by the lower geographical mean MIC (1.69 vs. 3.56 mg L^{-1}) and MIC₉₀ (2 vs. 4 mg L^{-1}). A reduced susceptibility to the azole fungicides tebuconazole and difenoconazole was also demonstrated in all TR₃₄/L98H isolates (Table 1).

Five (14.7%) resistant isolates, SD359, SD116A3, TN061-2, KP158A2 and KP183A1, did not harbour the *cyp51A* mutations associated with azole resistance, and all isolates, except for SD116A3, also expressed reduced susceptibility to tebuconazole and difenoconazole (Table 2). SD359 and TN061-2 were pan-azole-resistant, while SD116A3 was resistant to posaconazole and voriconazole, and KP158A2 and KP183A1 were resistant to posaconazole. Of the 45 susceptible isolates, the majority (37, 82.2%) carried wild-type *cyp51A*, and nucleotide polymorphisms were identified in a small number of isolates (8, 17.8%) (Table 1).

Of five resistant isolates without *cyp51A* mutations and seven control strains examined for *cyp51A*-independent mechanisms, sequence analysis did not identify amino acid substitutions in *hapE*, but analysis of *srbA* revealed the presence of V37D or E957D amino acid substitutions

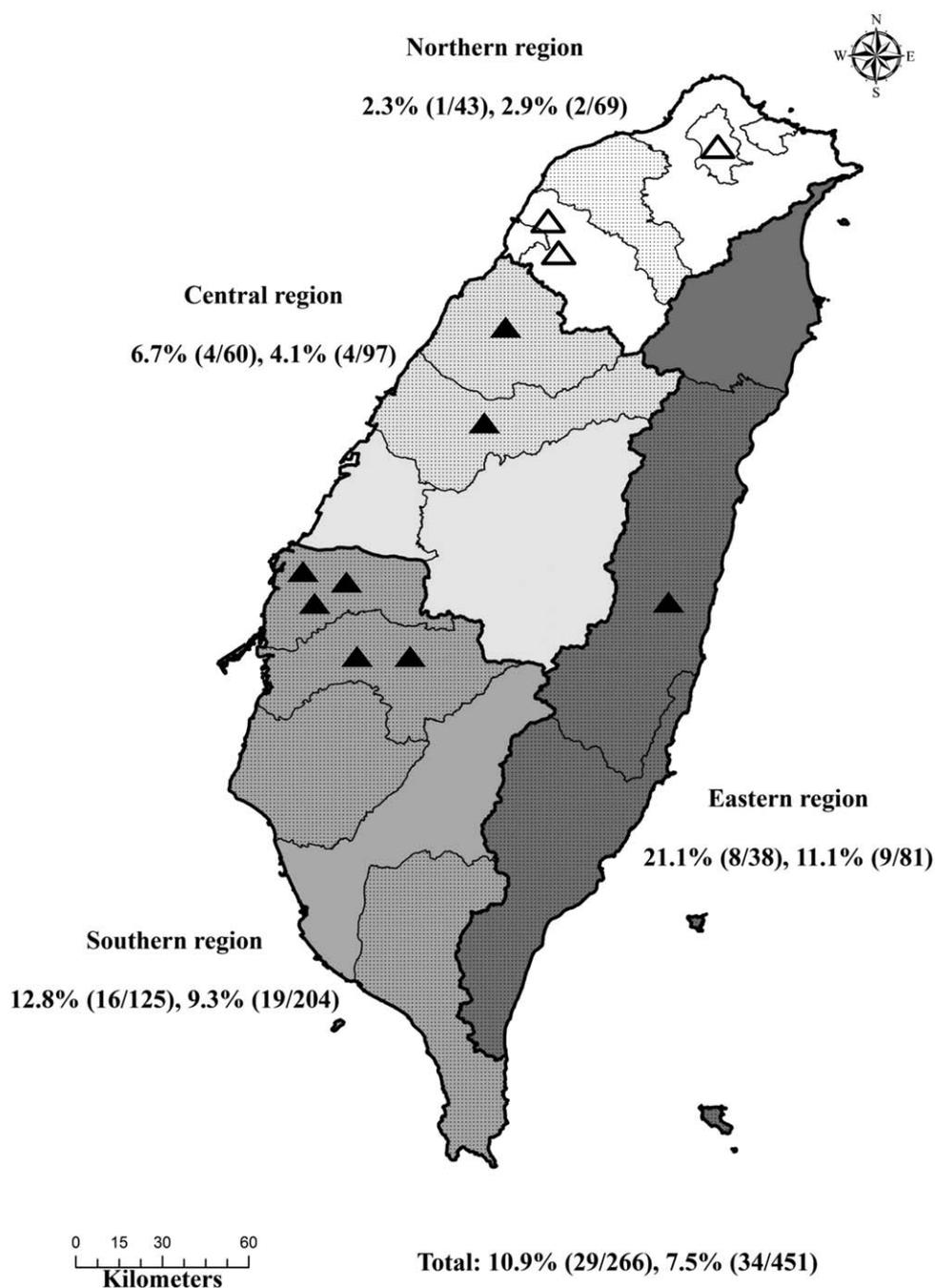


Fig. 1. Prevalence of azole-resistant *A. fumigatus* isolates in the environment of Taiwan. The numbers indicate the prevalence rate (%) of azole-resistance based on *A. fumigatus*-positive samples and *A. fumigatus* isolates respectively. Counties where TR₃₄/L98H isolates have been recovered are illustrated by a dot pattern. The triangles indicate microsatellite genotype A TR₃₄/L98H isolates (▲, environmental isolate and △, clinical isolate).

in both the susceptible and resistant isolates and an additional L68F substitution in TN061-2 (Table 2).

Gene expression analyses revealed remarkable upregulation of *AfuMDR3* expression in SD359 and *atrF* expression in SD116A3 (Fig. 2). SD359 had higher basal (5.5×), itraconazole-induced (25.4×) and voriconazole-induced (4.5×) *AfuMDR3* expression levels; SD116A3 had higher basal (93.7×), itraconazole-induced (20.9×) and voriconazole-induced (20.0×) *atrF* expression levels. In addition, SD359 had 2 to 5.5-fold increases in its basal

expression of *AfuMDR2*, *AfuMDR3* and *atrF*. SD116A3 had increased basal expression of *cyp51A*, *cyp51B*, *AfuMDR1*, *AfuMDR2* and *AfuMDR4* (2.5–5.0×), *cdr1B* (11.2×) and *MFS56* (14.3×). KP183A1 and the susceptible isolate F02411 had similar patterns of increased basal expression of *cyp51B*, *AfuMDR1*, *AfuMDR2*, *AfuMDR4*, *cdr1B*, *atrF* and *MFS56*, which were 2.6–8.3× and 3.6–8.8× higher for KP183A1 and F02411 respectively. The transporters in TN061-2 and KP158A2 were only upregulated to a lesser extent (Supporting Information Figure S1).

Table 1. Minimum inhibitory concentrations (MICs) of antifungal agents and triazole fungicides for *A. fumigatus* environmental strains isolated in Taiwan.

Azole susceptibility, <i>cyp51A</i> characteristics (no.)	MIC or MIC ₅₀ /MIC ₉₀ (Range) (mg L ⁻¹)							
	Amphotericin B	Itraconazole	Posaconazole	Voriconazole	Difenoconazole	Tebuconazole	Difenoconazole	Tebuconazole
Azole-resistant, all (n = 34)	0.5/0.5 (0.25–0.5)	>8/>8 (0.5–>8)	1/2 (0.5–2)	2/4 (1–4)	>32/>32 (4–>32)	32/>32 (8–>32)	>32/>32 (4–>32)	32/>32 (8–>32)
TR ₃₄ /L98H, all (n = 29)	0.5/0.5 (0.25–0.5)	>8/>8 (>8)	2/2 (1–2)	2/4 (1–4)	>32/>32 (16–>32)	32/>32 (16–>32)	>32/>32 (16–>32)	32/>32 (16–>32)
TR ₃₄ /L98H (n = 12)	0.5/0.5 (0.5)	>8/>8 (>8)	1/2 (1–2)	4/4 (2–4)	32/>32 (16–>32)	32/>32 (16–>32)	32/>32 (16–>32)	32/>32 (16–>32)
TR ₃₄ /L98H, S297T, F495I (n = 17)	0.5/0.5 (0.25–0.5)	>8/>8 (>8)	2/2 (1–2)	2/2 (1–4)	>32/>32 (32–>32)	32/>32 (16–>32)	>32/>32 (32–>32)	32/>32 (16–>32)
Wild-type or polymorphisms ^a (n = 5)	0.5/0.5 (0.5)	1/>8 (0.5–>8)	0.5/1 (0.5–1)	4/4 (2–4)	16/32 (4–32)	16/32 (8–32)	16/32 (4–32)	16/32 (8–32)
Azole-susceptible, all (n = 45)	0.5/1 (0.25–1)	0.5/0.5 (0.12–1)	0.12/0.25 (0.06–0.25)	0.5/1 (0.25–1)	2/4 (0.5–8)	4/8 (2–16)	2/4 (0.5–8)	4/8 (2–16)
Wild-type (n = 37)	0.5/1 (0.25–1)	0.5/0.5 (0.12–1)	0.25/0.25 (0.06–0.25)	0.5/1 (0.25–1)	2/4 (1–4)	4/8 (2–8)	2/4 (1–4)	4/8 (2–8)
F46Y, G89G, M172V, N248T, D255E, L358L, E427K, C454C (n = 5) ^b	0.25/1 (0.25–1)	0.5/1 (0.5–1)	0.25/0.25 (0.06–0.25)	1/1 (1)	4/8 (4–8)	8/16 (8–16)	4/8 (4–8)	8/16 (8–16)
D262Y (n = 1)	0.5	0.12	0.06	0.5	0.5	4	0.5	4
F46Y, G89G, M172V, D262Y, L358L, E427K, C454C (n = 1)	0.25	0.25	0.12	1	1	4	1	4
S297T, F495I (n = 1)	0.5	0.25	0.06	1	4	8	4	8

a. For details, see Table 2.

b. Includes KP189A1, which had itraconazole, posaconazole, difenoconazole and tebuconazole MICs of 0.5, 0.12, 1, 8 and 8 mg L⁻¹ respectively.

Table 2. Laboratory characteristics of 5 azole-resistant *A. fumigatus* isolates without *cyp51A* mutations as well as control strains evaluated for *cyp51A*-independent resistance mechanisms.

Strain	Source	<i>cyp51A</i> substitution(s) ^a	Azole susceptibility phenotype	MIC (mg L ⁻¹)							
				Itraconazole	Posaconazole	Voriconazole	Difenoconazole	Tebuconazole	<i>srfA</i> substitution(s) ^a		
Study isolates											
SD359	E	I242V	R	> 8	1	4	32	32	32	V37D	
SD116A3	E	WT	R	0.5	0.5	4	4	8	8	V37D	
TN061-2	E	WT	R	> 8	0.5	4	32	16	16	V37D, L68F	
KP158A2	E	WT	R	1	0.5	2	16	32	32	V37D	
KP189A1	E	Polymorphism ^b	R	1	0.5	2	16	16	16	V37D, E957D	
Control strains											
ATCC MYA-3626	NA	I242V	S	0.25	0.12	0.5	4	8	8	V37D	
ATCC 16903	C	Polymorphism ^c	S	0.25	0.12	0.5	2	8	8	V37D, S82P	
F2509	C	WT	S	0.25	0.12	0.5	2	8	8	V37D	
F02411	C	Polymorphism ^b	S	0.12	0.12	0.5	2	4	4	A865 V	
YL1	C	Polymorphism ^b	S	0.25	0.12	1	4	4	4	E957D	
PE10A1	E	TR34/L98H, S297T, F495I	R	> 8	1	1	> 32	32	32	V37D	
A31	C	TR ₃₄ /L98H	R	> 8	1	4	32	32	32	V37D	

Abbreviations: C, clinical; E, environmental; NA, minimum inhibitory concentration; NA, not available; S, susceptible; R, resistant; and WT, wild-type.

a. The reference sequences for *cyp51A* and *srfA* are GenBank accession numbers AF338659 and AFUA_2g01260 respectively.

b. Strains KP189A1, F02411 and YL1 have F46Y/G89G/M172V/N248T/D255E/L358L/E427K/C454C substitutions in *cyp51A*.

c. ATCC16903 has F46Y/G89G/M172V/L358L/E427K/C454C substitutions in *cyp51A*.

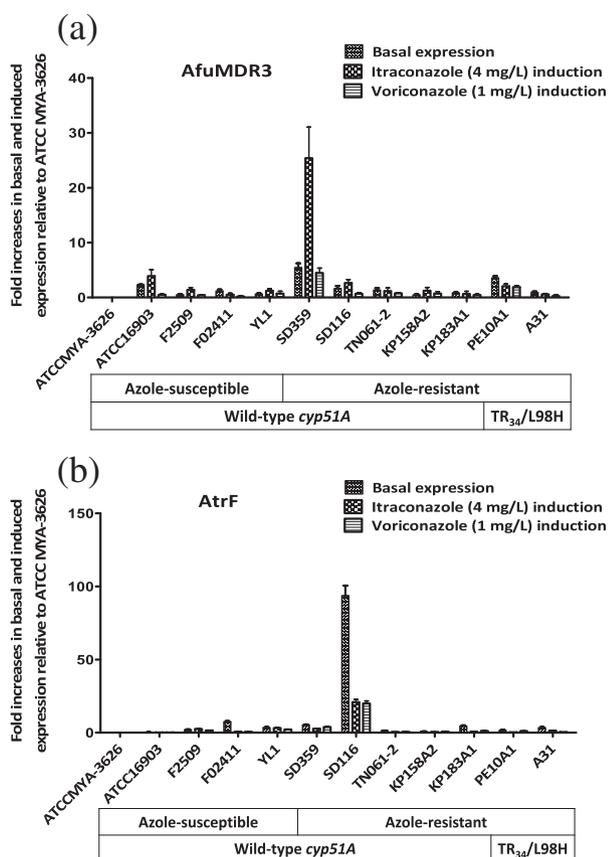


Fig. 2. Expression levels of drug efflux transporter genes (A) *AfuMDR3* and (B) *atrF* in *A. fumigatus* isolates. Expression was evaluated in triplicate biological samples with three technical replicates. The expression levels were normalized to the β -tubulin levels, and compared to *A. fumigatus* ATCC MYA-3626. The error bars represent standard deviation. The wild-type *cyp51A* category includes *cyp51A* polymorphisms.

Microsatellite genotypic analysis revealed heterogeneity and two major clades among the TR₃₄/L98H isolates in Taiwan (Fig. 3). The clade in the upper part of the phylogenetic tree mainly includes TR₃₄/L98H/S297T/F495I isolates from Taiwan and China; the clade in the lower part of the tree mainly contains TR₃₄/L98H isolates without S297T/F495I substitutions from Taiwan and other Eurasian countries, Australia and Colombia. Twenty-nine TR₃₄/L98H isolates could be grouped into 5 major microsatellite genotypes (A-E) (isolates with > 90% similarity based on STR patterns) and others. Using $\geq 90\%$ as a similarity cut-off, the majority (20, 69%) (genotypes A, C, E and others) of the TR₃₄/L98H isolates in this study were genetically distinct from overseas TR₃₄/L98H isolates, while a minority (9, 31%) (SD264A1, TD027-1 and genotypes B and D) clustered with TR₃₄/L98H isolates from other Eurasian countries. Notably, a major cluster was identified (genotype A), which contained 11 isogenic or nearly isogenic clinical ($n = 3$) and environmental ($n = 8$) TR₃₄/L98H/S297T/F495I

isolates recovered from four regions across Taiwan. Genetic clustering of clinical and environmental TR₃₄/L98H isolates was also observed with the genotype B2, C, D and E isolates. Interestingly, allele-sharing of 3 to 6 of the nine microsatellite markers was found between the susceptible isolates and the TR₃₄/L98H isolates, such as KP264A1 with AN0106-6 (6 alleles), KP264A1 with SD246A1 (5 alleles), TD133-1 with SD246A1 (4 alleles) and TY182-1 with SD202A3/SD202A2 (5 alleles). Isolates of mixed microsatellite genotypes or STR patterns may co-exist in the same sample, including HL102-3 and HL102-1 (genotypes B1 and B2), SD202A1 and SD202A2/SD202A3 (genotypes A and C) and TY366B-2 and TY366B-3 (genotype D). Five resistant isolates without *cyp51A* mutations did not cluster. Notably, the low-level azole-resistant isolate KP183A1 shared an identical STR pattern and *cyp51A* polymorphism with susceptible isolate KP189A1, which was recovered from the same geographical area (Tables 1 and 2).

Growth assays revealed similar growth rates for the TR₃₄/L98H isolates with different microsatellite genotypes, the resistant isolates without *cyp51A* mutations, except for SD116A3, and the susceptible control strains, except for F02411 (Supporting Information Figure S2). All of the tested strains had apparent sporulation at 48 h.

Discussion

This study represents one of the largest nationwide environmental surveillance studies of azole-resistant *A. fumigatus* in Asia. The prevalence rates of azole resistance, 10.9% and 7.5% based on *A. fumigatus*-positive samples and isolates, respectively, are within the range of environmental resistance rates reported for other Eurasian countries as well as for Colombia (6% to 14%) (Snelders *et al.*, 2009; Mortensen *et al.*, 2010; Chowdhary *et al.*, 2012; Badali *et al.*, 2013; Ahmad *et al.*, 2014; Bader *et al.*, 2015; Alvarez-Moreno *et al.*, 2017; Tangwattanachuleeporn *et al.*, 2017), but they are higher than the environmental resistance rate in China (1.4%) (Chen *et al.*, 2016a). TR₃₄/L98H mutations constitute the major resistance mechanism found in this study, which is similar to the aforementioned reports, except for in Colombia, where the TR₄₆/Y121F/T289A mutations, which were mostly recovered from the azole fungicide-containing soils of flower crops, dominated (Alvarez-Moreno *et al.*, 2017). As expected, the TR₃₄/L98H isolates exhibited resistance to both itraconazole and posaconazole and reduced susceptibility to the azole fungicides difenoconazole and tebuconazole (Snelders *et al.*, 2012; van Ingen *et al.*, 2015). The lower voriconazole resistance rate (37.9%) found here might be explained by the inclusion of a higher portion of TR₃₄/L98H/S297T/F495I isolates that were associated with lower voriconazole MICs than TR₃₄/L98H

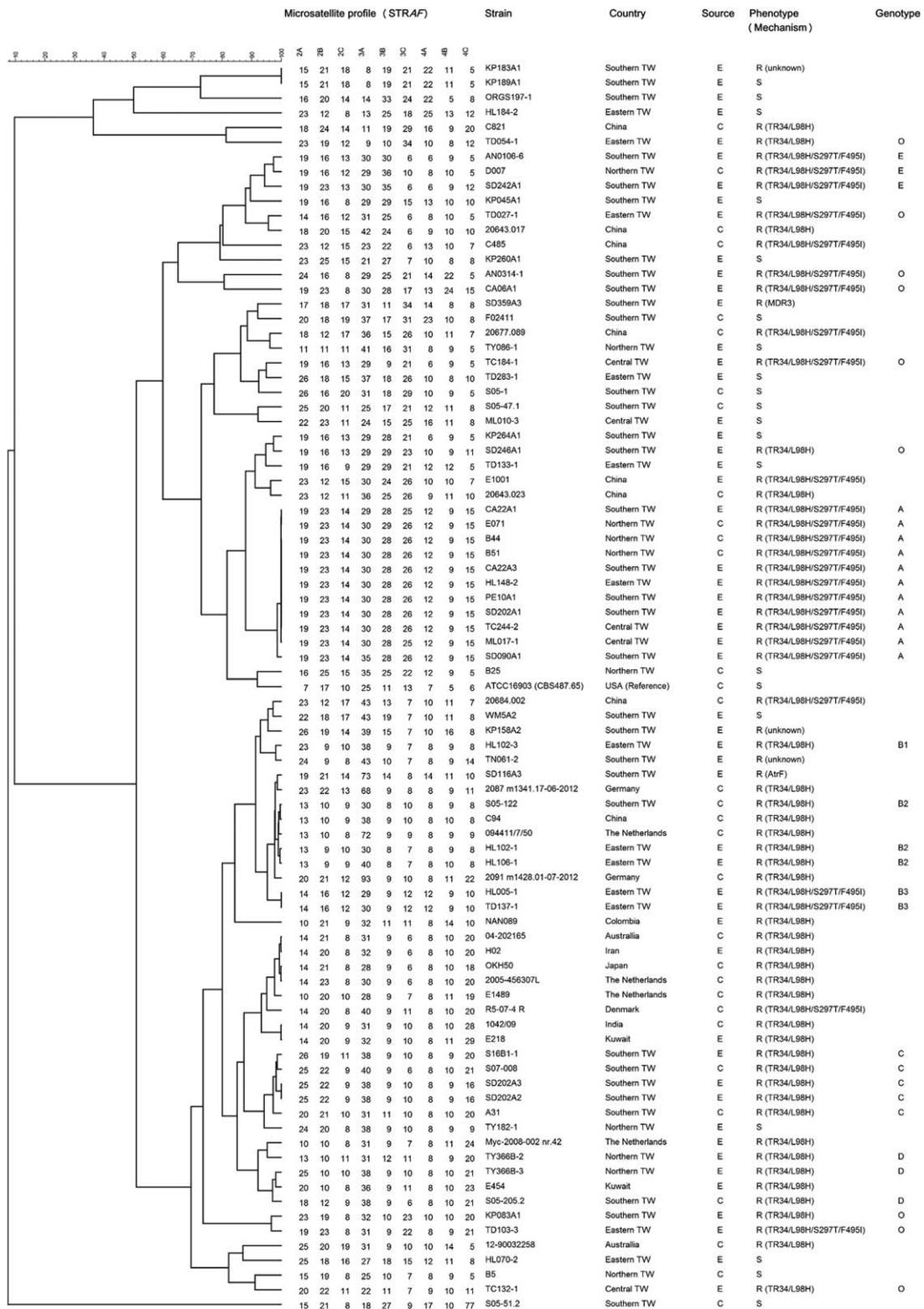


Fig. 3. Genetic relationships between *A. fumigatus* clinical and environmental isolates from Taiwan and published isolates from other countries determined by microsatellite genotyping.

The scale bar indicates the percentage identity. Microsatellite genotypes (A-E) were assigned to isolates from Taiwan with >90% similarity based on short tandem repeat patterns if there were three or more isolates in that cluster; O, other genotypes distinct from A-E. Abbreviations: C, clinical isolate; E, environmental isolate; R, resistant; S, susceptible; and TW: Taiwan.

isolates without S297T/F495I substitutions observed here and in an earlier study (Abdolrasouli *et al.*, 2015).

The first occurrence of TR₃₄/L98H isolates can be traced to 1998 in the Netherlands (Verweij *et al.*, 2016). Studies have suggested that a common ancestral lineage exists for TR₃₄/L98H isolates across Europe, and a distinct local microsatellite genotype exists for TR₃₄/L98H isolates across India (Camps *et al.*, 2012a; Chowdhary *et al.*, 2012). In contrast to Europe and India, our data revealed both the global spread of TR₃₄/L98H isolates and the occurrence of TR₃₄/L98H/S297T/F495I isolates belonging to local microsatellite genotypes, a phenomenon similar to that observed in China (Chen *et al.*, 2016a). In addition, to the best of our knowledge, this is the first study to demonstrate the clonal expansion of TR₃₄/L98H/S297T/F495I isolates that belong to the locally evolved microsatellite genotype A; these isolates have become widely disseminated and been recovered from patients. Two genotype A isolates, B44 and B51, caused fatal pulmonary aspergillosis in an azole-naïve patient with diabetes and cirrhosis in 2012 (Wu *et al.*, 2015). Notably, most of the TR₃₄/L98H isolates in Taiwan have evolved into several local microsatellite genotypes that are distinct from overseas isolates, including the clones prevalent in India and China (Chowdhary *et al.*, 2012; Chen *et al.*, 2016a). Recent sexual crossing experiments demonstrated that TR₃₄/L98H isolates could outcross with azole-susceptible isolates of different genetic backgrounds, and TR₄₆/Y121F/T289A isolates of opposite mating types could outcross with each other, resulting in a generation of new or microvariant microsatellite genotypes (Camps *et al.*, 2012a; Zhang *et al.*, 2017). The occurrence of sexual crossing is supported by observations of shared alleles at 3 to 6 of the nine microsatellite markers between the susceptible isolates and TR₃₄/L98H isolates in this study. After sexual reproduction, the repeat numbers of microsatellite markers may undergo subtle changes during asexual reproduction (Camps *et al.*, 2012a), which provides a possible explanation for the minor differences observed here for the repeat numbers in microsatellite markers between resistant isolates within each microsatellite genotype or within the same sample. Collectively, the genetic diversity of TR₃₄/L98H isolates in Taiwan may have resulted from continuous sexual and asexual cycles in the field over the years. Moreover, adverse fitness costs were not demonstrated for TR₃₄/L98H isolates of different microsatellite genotypes, which has been previously reported (Verweij *et al.*, 2016), facilitating their survival in the environment and enables their successful dissemination through a large number of easily dispersed airborne spores. The genetic clustering of clinical and environmental TR₃₄/L98H isolates further underlines the risk of acquiring azole-resistant aspergillosis among azole-naïve susceptible hosts.

The absence of TR₄₆/Y121F/T289A isolates in this surveillance may be due to their recent appearance compared to TR₃₄/L98H isolates (Verweij *et al.*, 2016). Another plausible explanation is that nearly all soil samples here were not from compost heaps. A recent Dutch study reported that TR₄₆ isolates were overwhelmingly abundant over TR₃₄ isolates in heat-shock treated compost heaps with residual azole fungicides, which serve as evolutionary incubators that allow the emergence of TR₄₆ isolates with new genotypes through sexual recombination (Zhang *et al.*, 2017). Although not found here, TR₅₃ and G54 mutations, two resistance mechanisms that have arisen from the environment, have been identified in different countries, suggesting their adaptation to the environment (Sharma *et al.*, 2015; Alvarez-Moreno *et al.*, 2017; Tangwattanachuleeporn *et al.*, 2017). Given the history of TR₃₄/L98H isolate dissemination, continuous surveillance is warranted to monitor whether these resistance mechanisms occur.

Five azole-resistant isolates harboured *cyp51A*-independent resistance mechanisms. Although *cyp51B* overexpression and consistent *hapE* or *srbA* mutations were not identified, overexpression of drug efflux transporters, *atrF* in SD116A3 and *AfuMDR3* in SD359, was observed. Fungi can overcome intracellular toxin accumulation with efflux transporters, which include two main categories: ATP-binding cassette (ABC) proteins and major facilitator superfamily (MFS) pumps (Cannon *et al.*, 2009). *AtrF*, an ABC transporter, has been reported to be constitutively upregulated in an itraconazole-resistant *A. fumigatus* clinical isolate, AF72, and two of the four itraconazole-resistant *A. fumigatus* environmental isolates (Slaven *et al.*, 2002; Meneau *et al.*, 2016). The MFS transporter *AfuMDR3* has been reported to be constitutively upregulated in itraconazole-resistant *A. fumigatus* ultraviolet-irradiated mutants with or without co-upregulation of *AfuMDR4* as well as in a clinical isolate (AF2) recovered from a patient chronically treated with itraconazole (Nascimento *et al.*, 2003; Chen *et al.*, 2005). However, the role of *AfuMDR3* in AF2 is unclear because of the coexistence of an M220I mutation in *cyp51A*. The SD359 strain isolated here is valuable because it appears to be the first naturally occurring azole-resistant *A. fumigatus* with significant *AfuMDR3* overexpression that is not accompanied by *cyp51A* mutations or co-upregulation of *AfuMDR4*. These findings provide additional evidence supporting the potential roles of *atrF* and *AfuMDR3* in azole resistance. Future studies validating the phenotypic roles ascribed to *atrF* and *AfuMDR3* and delineating the extent to which *atrF* and *AfuMDR3* overexpression contribute to clinical and environmental resistance are warranted.

Although the role of transporters in KP183A1 resistance is inconclusive, it is interesting to find that low-level posaconazole-resistant KP183A1 is isogenic to the susceptible isolate KP189A1 collected from the same geographical

area, suggesting that KP183A1 might have evolved from a local susceptible strain but has not yet achieved full resistance, which might require the accumulation of additional mutations.

Since the 1970s, azole fungicides have been widely used for crop protection against phytopathogenic moulds in many countries (Stensvold *et al.*, 2012), which have the potential to increase the emergence of azole-resistant *A. fumigatus* threatening human health (Chowdhary *et al.*, 2013). In Taiwan, of the five azole fungicides that have the highest potential to select for the TR₃₄/L98H genotype (Snelders *et al.*, 2012), propiconazole and difenconazole have been used since 1987 and 1993, respectively, and tebuconazole, bromuconazole and epoxiconazole have been used since 1999 (Taiwan Crop Protection Industry Association, 1962–2016). The annual consumption of these 5 azole fungicides in Taiwan was approximately two-thirds that used in the Netherlands in 2008 (Kleinkauf *et al.*, 2013) and has increased since 2012 (20 031 kg, 47 095 kg and 57 396 kg in 2008, 2012 and 2016 respectively) (Supporting Information Table S3). Azole fungicides can persist in soil for a long time and exert selective pressure, driving the development of azole resistance among not only phytopathogenic moulds, such as *Mycosphaerella graminicola* (Cools and Fraaije, 2008), but also human pathogenic fungi, such as *A. fumigatus* (Snelders *et al.*, 2012). In this study, the higher prevalence rates of azole resistance were observed in Central, Southern and Eastern Taiwan, which is consistent with their higher agricultural activities and crop yield than that in Northern Taiwan, a highly urbanized area surrounded by hills and mountains, according to the data from the Council of Agriculture in Taiwan. In addition, the isolates of *Fusarium fujikuroi* in Taiwan, the causative agent of bakanae disease in rice, have already expressed reduced susceptibility to tebuconazole (Chen *et al.*, 2016b). Recent reports revealed the genetic clustering of *Candida tropicalis* isolates exhibiting reduced susceptibility to both fluconazole and azole fungicides recovered from epidemiologically unrelated patients and soil samples (Yang *et al.*, 2012). Together with the diverse TR₃₄/L98H clonality and resistance mechanisms found in this study, it is speculated that for both human and plant pathogenic fungi, resistance traits may emerge and be maintained and then subsequently evolve in fields where azole fungicides have been applied. Hence, studies on reversing the increasing resistance rates by restricting key agricultural azoles might be helpful to identify appropriate public health measures (Kleinkauf *et al.*, 2013). To minimize the risk of fungicide resistance in phytopathogenic moulds, farmers in Taiwan are advised to use tebuconazole in rotation with alternative fungicides that have different modes of action (Chen *et al.*, 2016b), which would theoretically reduce the selective pressure exerted by azole fungicides on bystander human

pathogenic fungi as well. Resistance trends should continue to be monitored to evaluate the effects of fungicide rotation.

Given the environmental resistance rate (7.5%) found in this study, for patients with invasive aspergillosis in Taiwan, strenuous efforts should be made to obtain a positive culture, followed by accurate species identification and susceptibility testing of causative isolates (Verweij *et al.*, 2015). For empirical therapy, decisions between the current primary agent voriconazole versus a voriconazole-echinocandin combination or liposomal amphotericin B monotherapy should depend on individual clinical conditions. The latter two regimens are particularly considered for critically ill or neutropenic patients, as a delay in initiating effective therapy most likely compromises their survival (Chowdhary *et al.*, 2017).

In conclusion, this study presented both the global spread and local evolution of azole-resistant *A. fumigatus* isolates carrying TR₃₄/L98H mutations on this island and the potential role of efflux transporters in azole resistance. Because resistant isolates were not rare in the environment and were genetically clustered with clinical isolates, periodic environmental surveillance at the molecular level is thus emphasized in regions where azole fungicides are applied to detect epidemiological trends and aid the development of treatment recommendations for human aspergillosis. To retain clinical use of azoles, agricultural fungicide management strategies that generate less selective pressure for antifungal resistance should also be investigated and implemented.

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Transparency declarations

All of the authors declare no conflicts of interest. This study was presented in part at the 27th European Congress of

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References

- Abdolrasouli, A., Rhodes, J., Beale, M.A., Hagen, F., Rogers, T.R., and Chowdhary, A. (2015) Genomic context of azole resistance mutations in *Aspergillus fumigatus* determined using whole-genome sequencing. *MBio* **6**: e00536.
- Ahmad, S., Khan, Z., Hagen, F., and Meis, J.F. (2014) Occurrence of triazole-resistant *Aspergillus fumigatus* with TR₃₄/L98H mutations in outdoor and hospital environment in Kuwait. *Environ Res* **133**: 20–26.
- Alvarez-Moreno, C., Lavergne, R.A., Hagen, F., Morio, F., Meis, J.F., and Le Pape, P. (2017) Azole-resistant *Aspergillus fumigatus* harboring TR₃₄/L98H, TR₄₆/Y121F/T289A and TR₅₃ mutations related to flower fields in Colombia. *Sci Rep* **7**: 45631.
- Badali, H., Vaezi, A., Haghani, I., Yazdanparast, S.A., Hedayati, M.T., Mousavi, B., et al. (2013) Environmental study of azole-resistant *Aspergillus fumigatus* with TR₃₄/L98H mutations in the *cyp51A* gene in Iran. *Mycoses* **56**: 659–663.
- Bader, O., Tünnermann, J., Dudakova, A., Tangwattanachuleeporn, M., Weig, M., and Groß, U. (2015) Environmental isolates of azole-resistant *Aspergillus fumigatus* in Germany. *Antimicrob Agents Chemother* **59**: 4356–4359.
- Balajee, S.A., Houbraken, J., Verweij, P.E., Hong, S.B., Yaghuchi, T., Varga, J., and Samson, R.A. (2007) *Aspergillus* species identification in the clinical setting. *Stud Mycol* **59**: 39–46.
- Camps, S.M., Rijs, A.J., Klaassen, C.H., Meis, J.F., O'gorman, C.M., and Dyer, P.S. (2012a) Molecular epidemiology of *Aspergillus fumigatus* isolates harboring the TR₃₄/L98H azole resistance mechanism. *J Clin Microbiol* **50**: 2674–2680.
- Camps, S.M., Dutilh, B.E., Arendrup, M.C., Rijs, A.J., Snelders, E., Huynen, M.A., et al. (2012b) Discovery of a HapE mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing. *PLoS One* **7**: e50034.
- Cannon, R.D., Lamping, E., Holmes, A.R., Niimi, K., Baret, P.V., Keniya, M.V., et al. (2009) Efflux-mediated antifungal drug resistance. *Clin Microbiol Rev* **22**: 291–321.
- Chen, J., Li, H., Li, R., Bu, D., and Wan, Z. (2005) Mutations in the *cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *J Antimicrob Chemother* **55**: 31–37.
- Chen, Y., Lu, Z., Zhao, J., Zou, Z., Gong, Y., Qu, F., et al. (2016a) Epidemiology and molecular characterizations of azole resistance in clinical and environmental *Aspergillus fumigatus* isolates from China. *Antimicrob Agents Chemother* **60**: 5878–5884.
- Chen, Y.C., Lai, M.H., Wu, C.Y., Lin, T.C., Cheng, A.H., Yang, C.C., et al. (2016b) The genetic structure, virulence, and fungicide sensitivity of *Fusarium fujikuroi* in Taiwan. *Phytopathology* **106**: 624–635.
- Chowdhary, A., Kathuria, S., Xu, J., Sharma, C., Sundar, G., Singh, P.K., et al. (2012) Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR₃₄/L98H mutations in the *cyp51A* gene in India. *PLoS One* **7**: e52871.
- Chowdhary, A., Kathuria, S., Xu, J., Meis, J.F., and Heitman, J. (2013) Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. *Plos Pathog* **9**: e1003633.
- Chowdhary, A., Sharma, C., and Meis, J.F. (2017) Azole-resistant aspergillosis: epidemiology, molecular mechanisms, and treatment. *J Infect Dis* **216**: S436–S444.
- CLSI. (2008) Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi: Approved Standard-Second Edition M38-A2. Wayne, PA: Clinical and Laboratory Standards Institute.
- Cools, H.J., and Fraaije, B.A. (2008) Are azole fungicides losing ground against Septoria wheat disease? Resistance mechanisms in *Mycosphaerella graminicola*. *Pest Manag Sci* **64**: 681–684.
- de Valk, H.A., Meis, J.F., Curfs, I.M., Muehlethaler, K., Mouton, J.W., and Klaassen, C.H. (2005) Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. *J Clin Microbiol* **43**: 4112–4120.
- de Valk, H.A., Meis, J.F., Bretagne, S., Costa, J.M., Lasker, B.A., Balajee, S.A., et al. (2009) Interlaboratory reproducibility of a microsatellite-based typing assay for *Aspergillus fumigatus* through the use of allelic ladders: proof of concept. *Clin Microbiol Infect* **15**: 180–187.
- EUCAST-Subcommittee on Antimicrobial Susceptibility Testing (EUCAST-AFST). (2017) Clinical breakpoints for fungi (v 8.1). [WWW document]. URL http://www.eucast.org/clinical_breakpoints/.
- Fraczek, M.G., Bromley, M., Buied, A., Moore, C.B., Rajendran, R., Rautemaa, R., et al. (2013) The *cdr1B* efflux transporter is associated with non-*cyp51a*-mediated itraconazole resistance in *Aspergillus fumigatus*. *J Antimicrob Chemother* **68**: 1486–1496.
- Kidd, S.E., Goeman, E., Meis, J.F., Slavin, M.A., and Verweij, P.E. (2015) Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses* **58**: 350–355.
- Kleinkauf, N., Verweij, P., Arendrup, M., Donnelly, P., Cuenca-Estrella, M., Fraaije, B., et al. (2013) Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species. In: European Centre for Disease Prevention and Control (ECDC), Stockholm.
- Meneau, I., Coste, A.T., and Sanglard, D. (2016) Identification of *Aspergillus fumigatus* multidrug transporter genes and their potential involvement in antifungal resistance. *Med Mycol* **54**: 616–627.
- Mortensen, K.L., Mellado, E., Lass-Flörl, C., Rodriguez-Tudela, J.L., Johansen, H.K., and Arendrup, M.C. (2010) Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob Agents Chemother* **54**: 4545–4549.
- Nascimento, A.M., Goldman, G.H., Park, S., Marras, S.A.E., Delmas, G., Oza, U., et al. (2003) Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrob Agents Chemother* **47**: 1719–1726.
- Sharma, C., Hagen, F., Moroti, R., Meis, J.F., and Chowdhary, A. (2015) Triazole-resistant *Aspergillus fumigatus* harbouring

- G54 mutation: Is it de novo or environmentally acquired? *J Glob Antimicrob Resist* **3**: 69–74.
- Slaven, J.W., Anderson, M.J., Sanglard, D., Dixon, G.K., Bille, J., Roberts, I.S., and Denning, D.W. (2002) Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, *atrF*, in the presence of itraconazole in an itraconazole resistant clinical isolate. *Fungal Genet Biol* **36**: 199–206.
- Snelders, E., Huis In 't Veld, R.A., Rijs, A.J., Kema, G.H., Melchers, W.J., and Verweij, P.E. (2009) Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl Environ Microbiol* **75**: 4053–4057.
- Snelders, E., Karawajczyk, A., Schaftenaar, G., Verweij, P.E., and Melchers, W.J. (2010) Azole resistance profile of amino acid changes in *Aspergillus fumigatus* CYP51A based on protein homology modeling. *Antimicrob Agents Chemother* **54**: 2425–2430.
- Snelders, E., Camps, S.M., Karawajczyk, A., Schaftenaar, G., Kema, G.H., van der Lee, H.A., et al. (2012) Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS One* **7**: e31801.
- Steinmann, J., Hamprecht, A., Vehreschild, M.J., Cornely, O.A., Buchheidt, D., Spiess, B., et al. (2015) Emergence of azole-resistant invasive aspergillosis in HSCT recipients in Germany. *J Antimicrob Chemother* **70**: 1522–1526.
- Stensvold, C.R., Jorgensen, L.N., and Arendrup, M.C. (2012) Azole-resistant invasive aspergillosis: Relationship to agriculture. *Curr Fungal Infect Rep* **6**: 178–191.
- Taiwan Crop Protection Industry Association. (1962–2016) *Domestic Manufacturers Production & Sale of Pesticides*. Taipei, Taiwan: Taiwan Crop Protection Industry Association. [In Chinese]
- Tangwattanachuleeporn, M., Minarin, N., Saichan, S., Serm Sri, P., Mitkornburee, R., Groß, U., et al. (2017) Prevalence of azole-resistant *Aspergillus fumigatus* in the environment of Thailand. *Med Mycol* **55**: 429–435.
- Toyotome, T., Hagiwara, D., Kida, H., Ogi, T., Watanabe, A., Wada, T., et al. (2017) First clinical isolation report of azole-resistant *Aspergillus fumigatus* with TR₃₄/L98H-type mutation in Japan. *J Infect Chemother* **23**: 579–581.
- van Ingen, J., van der Lee, H.A., Rijs, T.A., Zoll, J., Leenstra, T., Melchers, W.J., and Verweij, P.E. (2015) Azole, polyene and echinocandin MIC distributions for wild-type, TR₃₄/L98H and TR₄₆/Y121F/T289A *Aspergillus fumigatus* isolates in the Netherlands. *J Antimicrob Chemother* **70**: 178–181.
- Verweij, P.E., Ananda-Rajah, M., Andes, D., Arendrup, M.C., Brüggemann, R.J., Chowdhary, A., et al. (2015) International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. *Drug Resist Updat* **21–22**: 30–40.
- Verweij, P.E., Chowdhary, A., Melchers, W.J.G., Meis, J.F., and Weinstein, R.A. (2016) Azole resistance in *Aspergillus fumigatus*: Can we retain the clinical use of mold-active antifungal azoles? *Clin Infect Dis* **62**: 362–368.
- Willger, S.D., Puttikamonkul, S., Kim, K.H., Burritt, J.B., Grahl, N., Metzler, L.J., et al. (2008) A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. *Plos Pathog* **4**: e1000200.
- Wu, C.J., Wang, H.C., Lee, J.C., Lo, H.J., Dai, C.T., Chou, P.H., et al. (2015) Azole-resistant *Aspergillus fumigatus* isolates carrying TR₃₄/L98H mutations in Taiwan. *Mycoses* **58**: 544–549.
- Yang, Y.L., Lin, C.C., Chang, T.P., Lauderdale, T.L., Chen, H.T., Lee, C.F., et al. (2012) Comparison of human and soil *Candida tropicalis* isolates with reduced susceptibility to fluconazole. *PLoS One* **7**: e34609.
- Zhang, J., Snelders, E., Zwaan, B.J., Schoustra, S.E., Meis, J.F., van Dijk, K., et al. (2017) A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. *MBio* **8**: e00791-17.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Expression levels of drug efflux transporter genes in *A. fumigatus* isolates (a–g). Expression was evaluated in triplicate biological samples with three technical replicates. The expression levels were normalized to the β -tubulin levels, and compared to the ATCC MYA-3626 *A. fumigatus* strain, except for those of *AfuMD4*, which were relative to ATCC 16903. The error bars represent standard deviation. The wild-type *cyp51A* category includes *cyp51A* polymorphisms.

Fig. S2. Comparison of the radial growth of *A. fumigatus* isolates on PDA at 35°C. Radial growth was measured as the radius of the expanding colony in millimeters relative to the time (hours) of incubation. The isolates are ordered in the figure by their colony radius sizes at 72 h, with larger radii at the top. Abbreviations: C, clinical isolate; E, environmental isolate; R, resistant; and S, susceptible.

Table S1. PCR primers for amplifying the complete *srbA* sequence. The primers were designed according to the complete sequence of *srbA* in *A. fumigatus* Af293 (AFUA_2 g01260), which contains 2967 base pairs. PCR conditions were: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 2.5 min, with a final extension step at 72°C for 10 min.

Table S2. Prevalence of azole-resistant environmental *A. fumigatus* (ARAF) in different sample types from Taiwan. AF, *A. fumigatus*.

Table S3. Annual consumption of five azole fungicides in Taiwan during 2003–2016 according to the data from 'Domestic Manufacturers Production & Sale of Pesticides' published by the Taiwan Crop Protection Industry Association.