1	Azole susceptibility in clinical and environmental isolates of Aspergillus fumigatus from
2	eastern Hokkaido, Japan
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## 23 Abstract

24 Azole antifungals are used not only clinically for fungal infections but also used as 25 agricultural fungicides. Recently, azole-resistant Aspergillus fumigatus containing a tandem 26 repeat in the promoter region of *cyp51A* combined with amino acid substitution(s) appears in 27 the environment in Eurasia, especially in several European countries. Although azole 28 fungicides have been used in Japan, especially in Hokkaido, surveillance and characterization 29 of A. fumigatus in Hokkaido have not been reported. In this study, we collected soil samples 30 from farms that used an azole fungicide in the Tokachi area of eastern Hokkaido, isolated 91 31 A. fumigatus strains, and determined the minimal inhibitory concentrations of medical azoles 32 required for these strains. Moreover, because causative agent A. fumigatus is ubiquitous in the 33 air and acquired from the environment, we collected 22 clinical isolates of A. fumigatus to 34 measure their susceptibility to medical azoles in a hospital in the Tokachi area. Our data show 35 that almost all A. fumigatus isolates retained susceptibility to medical azoles. Clinical isolates 36 OKH34 and OKH6 showed 8 and 2 µg/mL of voriconazole, respectively, as the minimal 37 inhibitory concentration. Both isolates did not contain tandem repeat in cyp51A promoter 38 region. An isolate contained G448S mutation in cyp51A conferring voriconazole resistance, 39 which is the first report from Japan. Our data shows the existence of azole-resistant and low 40 azole-susceptible clinical isolates and highlight the necessity for continuous surveillance in

41	Japan because resistant A. fumigatus strains can arise through clinical or environmental
42	selection or could be introduced from overseas.
43	
44	Keywords: azole, Aspergillus fumigatus, cyp51A
45	

47 Aspergillus fumigatus is a fungus commonly found in the environment, known to be the leading causative agent of aspergillosis. Azole antifungals are used not only to treat 48 aspergillosis and other fungal infections but are also used as agricultural fungicides. Because 49 50 of the extensive use of azole antifungals, azole-resistant A. fumigatus strains have been 51 reported globally (reviewed in [1]). Although some A. fumigatus strains are intrinsically 52 resistant to azoles, acquired resistant strains have also been reported [1, 2]. The mechanisms 53 responsible for the acquisition of azole resistance are roughly classified into the selections in 54 a patient and in the environment [2]. Tashiro and colleagues have reported the correlation 55 between the duration of azole treatment and the susceptibility of A. fumigatus isolates [3], 56 indicating that long-term exposure of azoles in patients increases the likelihood of resistance acquisition. Alternatively, recent studies and evidence suggest that the use of azoles 57 58 fungicides has driven the acquisition of azole resistance (reviewed in [4]). Most 59 environmental isolates with azole resistance harbored the cyp51A mutation consisting of a 60 tandem repeat in the cyp51A promoter region and amino acid substitution(s) named as 61 TR<sub>34</sub>/L98H or TR<sub>46</sub>/Y121F/T289A [1].

As of 2015, 17 azole fungicides have been approved for agricultural use in Japan.
The shipment of tebuconazole was the largest among azole fungicides in Japan from October
2011 to September 2012 (2012 agricultural chemical year [5]). Snelders and colleagues

65	reported that epoxiconazole, difenoconazole, propiconazole, bromuconazole, and
66	tebuconazole exhibited similar docking poses to medical triazoles [6]. Of these five
67	fungicides, tebuconazole, propiconazole, and difenoconazole were placed first, second, and
68	fourth, respectively, in terms of their usage in the 2012 agricultural-chemical year in Japan.
69	These three fungicides were mainly shipped to Hokkaido [5]. Recently, Kano and colleagues
70	examined the azole susceptibility of A. fumigatus isolates from a farm in Japan that used
71	tetraconazole twice a year for over 15 years [7]. The study showed that the isolated strains
72	did not exhibit resistance to tetraconazole or itraconazole (ITCZ). In this study, we examined
73	the azole susceptibility in environmental isolates of A. fumigatus from the Tokachi area of
74	eastern Hokkaido. In addition to the examination of environmental isolates, we examined the
75	azole susceptibility of clinical isolates collected at a hospital in the Tokachi area, because the
76	causative agents of aspergillosis are acquired from the environment.
77	Soil samples listed in Supplementary Table 1 were collected in the Tokachi area of
78	eastern Hokkaido, Japan. We identified A. fumigatus by macroscopic observation and
79	subcultured it on PDA. To determine minimal inhibitory concentrations (MIC) of medical
80	antifungals excluding micafungin and minimal effective concentrations (MEC) of micafungin,
81	the broth microdilution method based on CLSI M38-A2 with a slight modification were used.
82	In brief, conidia were diluted in 100 µL of MOPS(NACALAI TESQUE, Inc., Kyoto,

83 Japan)-buffered RPMI 1640 medium (Sigma-Aldrich Co., St. Louis, MO) adjusted to pH 7.0 at  $1 \times 10^4$  cells/mL, and then were inoculated to each well of 96-well microplates, containing 84 medical antifungals at various concentrations (Dry Plate Eiken; Eiken Chemical Co., Ltd., 85 86 Tokyo, Japan). After 48 h of culture at 35°C, the growth inhibition of mold was determined with visual observation. MIC and MEC of antifungals against isolates from soil samples was 87 88 shown in Table 1. All of 91 isolates from these soil samples were susceptible to medical 89 azoles. These results suggest that TR34/L98H- or TR46/Y121F/T289A-type were not prevalent in Tokachi area of eastern Hokkaido. 90

91 Twenty-two of A. fumigatus strains were isolated from clinical specimens in 92 Obihiro-Kosei General Hospital in the Tokachi area from November, 2013 to July, 2015 93 (Supplementary Table 2). To collect and use of these clinical isolates, we received an 94 approval (2013-02) from the ethics committee of Obihiro University of Agriculture and 95 Veterinary Medicine and an approval from the ethics committee of Obihiro-Kosei General 96 Hospital. Conidia of these strains were stocked as glycerol stocks in -80°C until using. 97 Genomic DNA were extracted from these clinical isolates and identified as A. fumigatus by 98 the determination of Internal transcribed spacer and D1/D2 regions. Additionally, partial 99 nucleotide sequences of β-tubulin, *rodA*, and calmodulin genes of OKH6 and OKH34 clinical isolates were determined. Primers used for amplification and sequencing are listed in 100

101	Supplementary Table 3. Twenty-two clinical isolates identified as A. fumigatus were
102	determined the MIC of antifungals (Table 2). MIC of VRCZ against OKH6 and OKH34 were
103	determined at least three times on different days. Although almost all of these isolates were
104	susceptible to antifungals including azoles, the isolate, A. fumigatus OKH6, exhibited low
105	susceptibility to voriconazole (VRCZ) (MIC 2 $\mu$ g/mL). Because MIC of ITCZ against A.
106	fumigatus OKH6 was 1 $\mu$ g/mL, the strain did not exhibit cross-resistance to ITCZ. And
107	among the remaining 21 strains of A. fumigatus, OKH34 exhibited resistance to VRCZ (MIC
108	8 $\mu$ g/mL). The strain OKH34 also did not exhibit cross-resistance to ITCZ (MIC 0.5 $\mu$ g/mL).
109	These results indicate that among 22 clinical isolates collected in this study two isolates
110	OKH6 and OKH34 decreased the susceptibility to VRCZ.
111	To compare cyp51A and the upstream regions of A. fumigatus OKH6, OKH34 and
112	other azole susceptible A. fumigatus strains (Af293, OKH1 and OKH2), we determined these
113	nucleotide sequences. To amplify and determine the nucleotide sequences of <i>cyp51A</i> with the
114	upstream region, primers listed in Supplementary Table 3 were used. As shown in
115	Supplementary Figure 1a, the coding sequence of cyp51A in OKH6 strain was identical to
116	those in OKH1 and OKH2 strains. On the other hand, OKH34 strain has a unique amino acid
116 117	those in OKH1 and OKH2 strains. On the other hand, OKH34 strain has a unique amino acid substitution from glycine to serine in the residue 448 of <i>cyp51A</i> . In the coding sequence of

119	The 1kb upstream region of <i>cyp51A</i> of OKH6 did not contain tandem repeats. We examined
120	the expression level of cyp51A and cyp51B in OKH6 and compared with the expression
121	levels in OKH1 and OKH2. After statically culturing for 48 h at 35°C in MOPS-RPMI
122	medium, total RNA were recovered from mycelia with Direct-zol RNA MiniPrep kit with
123	TRI-Reagent (Zymo Research Irvine, CA). Real-time quantitative PCR was performed using
124	THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) and LightCycle 480 II
125	(Roche Diagnostics, Basel, Switzerland). Primers were listed in Supplementary Table 3. The
126	expression levels of cyp51A and cyp51B in OKH6 were comparable to those of OKH1 and
127	OKH2 (Figure 1). These results indicate that the G448S mutation in cyp51A contributed to
128	resistance of OKH34 to VRCZ.

129 cyp51A of A. fumigatus OKH34 possessed a mutation at glycine residue 448 to 130 serine. The point mutation has been described several manuscripts [8-12], but the mutant has not been reported from Japan. Clinical isolates including G448S mutation described in 131 132 several manuscripts were resistant to VRCZ and ITCZ [9-11]. In Japan, to the best of our 133 knowledge, the mutant has not been reported. On the other hand, G448S mutants obtained by in vitro-selection were resistance to voriconazole [8]. Our data also indicated that G448S 134 135 mutant conferred resistance to voriconazole. The patient had received treatment with VRCZ (200 mg daily), and on day 85 after starting VRCZ treatment A. fumigatus OKH34 was 136

137	isolated from a sputum specimen. Before the antifungal treatment, an A. fumigatus strain
138	OKH31 was isolated from the patient and was susceptible to VRCZ, as well as ITCZ. This
139	mutation, therefore, might have been induced during this treatment. Another strain, OKH6,
140	showed low sensitivity to VRCZ and the cyp51A sequence did not contain any mutation,
141	contributing to azole resistance, and the expression levels of cyp51A and cyp51B did not
142	changed in OKH6. In cyp51B coding sequence of OKH6, we found P327S mutation. Since
143	the mutation has not been reported, the importance remains unknown. Recently, the structure
144	of A. fumigatus Cyp51B was resolved [13], indicating that P327-residue located far from the
145	active site of Cyp51B. Therefore, the mutation might not be related the VRCZ low
146	susceptibility. Further investigation is required to elucidate a role of the P327S mutation to
147	azole resistance.
148	In conclusion, we isolated A. fumigatus from soil samples obtained from the Tokachi
149	area, and all of the isolates were susceptible to medical azoles. These results suggest that
150	TR34/L98H- or TR46/Y121F/T289A-type were not prevalent in Tokachi area of eastern
151	Hokkaido. Among clinical isolates, we found two A. fumigatus strains with low VRCZ

of *cyp51A*. Although Wiederhold et al. reported that TR34/L98H- or
TR46/Y121F/T289A-type strains were isolated sporadically in U.S. since 2008, these type

susceptibility. However, these isolates did not possess tandem repeats in the upstream region

152

155	resistant strains have not been recovered from the environment in U.S. [14] Very recently, a
156	TR46/Y121F/T289A-type resistant strain was clinically isolated from a patient in Japan [15].
157	In this situation of sporadic isolation, these resistant strains might not be prevalent and might
158	not be isolated from the environment. A. fumigatus TR34/L98H- or
159	TR46/Y121F/T289A-type resistant strain may appear in the environment in Japan through
160	inadequate use of azole pesticides or by importing agricultural products from overseas. For
161	these reasons, we need continuous surveillance of A. fumigatus isolates in Japan, including
162	Hokkaido.
163	
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164 165 166 167 168	Acknowledgments We thank the farm owners for kindly providing soil samples. This study was supported by the Obihiro University of Agriculture and the Veterinary Medicine Fund. The authors would like to thank Enago (www.enago.jp) and B. M. Sarumoh for the English language review.
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<ol> <li>164</li> <li>165</li> <li>166</li> <li>167</li> <li>168</li> <li>169</li> <li>170</li> <li>171</li> </ol>	Acknowledgments         We thank the farm owners for kindly providing soil samples. This study was         supported by the Obihiro University of Agriculture and the Veterinary Medicine Fund. The         authors would like to thank Enago (www.enago.jp) and B. M. Sarumoh for the English         language review.         Conflict of Interest         None

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216	

Figure 1: The relative quantification of *cyp51A* (black bars) and *cyp51B* (white bars) in *A*.

- 218 *fumigatus* OKH1, OKH2, and OKH6 isolates.
- 219 Supplementary Figure 1: Nucleotide sequences of *cyp51A* (a) and *cyp51B* (b) of *A. fumigatus*
- Af293 strain and OKH6, OKH1, OKH2, and OKH34 isolates. Grey-shaded sites indicate that
- the nucleotide of at least one OKH strain is different from the Af293 strain. Boxed sites in (a)
- and (b) indicate the codon at residue 448 of cyp51A and at residue 327 of cyp51B,
- 223 respectively. Coding sequences are underlined in the figure.

Figure 1



	No. of is	olates										
Antifungals	<b></b>	With MIC or MEC (µg/mL) of:										
	Iotai	$\leq 0.015$	0.03	0.06	0.12	0.25	0.5	1	2	4	8	> 8
Itraconazole	91					62	26	3				
Voriconazole	91				2	40	46	3				
Amphothericin B	91					5	60	23	3			
Micafungin	91	91										

Table 1. MIC and MEC against environmental isolates of *A. fumigatus* 

	No. of ise	olates										
Antifungals	Total	With MIC or MEC <sup>a</sup> (µg/mL) of:										
		≤ 0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	> 8
Itraconazole	22					7	12	3				
Voriconazole	22					6	13	1	1		1	
Amphothericin B	22					2	10	9	1			
Micafungin	22	21	1									

Table2. MIC and MEC against clinical isolates of A. fumigatus

<sup>a</sup> MEC are shown for micafungin.

Sample name	Isolated strains	Produce in the farmland	Azole fungicide used to the farmland
Flower bed	12		Unused
SugarBeet-1	13	Sugar Beet	Tetraconazole
SugarBeet-2	21	Sugar Beet	Tetraconazole
SugarBeet-3	5	Sugar Beet	Tetraconazole
SugarBeet-4	1	Sugar Beet	Tetraconazole
Wheat-1	4	Wheat	Unused
Wheat-2	4	Wheat	Unused
Wheat-3	8	Wheat	Unused
Wheat-4	6	Wheat	Tebuconazole
Wheat-5	7	Wheat	Tebuconazole
Wheat-6	10	Wheat	Unused

Supplementary Table 1. Soil samples used in this study.

<sup>†</sup>At the sampling, the farmland was during fallow for one growing season.

Supplementary Table 2. Clinical isolates collected in this study.

Isolated strain	Sample	Underlying diseases	Type of aspergillosis
OKH1	Sputum	Abdominal injury	
OKH2	Sputum	Allergic rhinitis, asthma, past history of acute eosinophilic pneumonia	ABPA
OKH6	Sputum	RA, CVD-IP	
OKH8	Sputum	Scleroderma, pneumothorax, CVD-IP	
OKH9	Sputum	Pulmonary tuberculosis, hypertension, AD	SPA
OKH10	Sputum	ITP, heart failure, hypertension	
OKH11	Sputum	Multiple myeloma, hepatitis C	
OKH12 <sup>a</sup>	Sputum	Esophageal cancer, lung metastasis, COPD	SPA, CPPA
OKH13	Bronchial blushing	CPFE, DM	SPA
OKH14 <sup>a</sup>	Sputum	Esophageal cancer, lung metastasis, COPD	SPA, CPPA
$\rm OKH15^{a}$	Sputum	Esophageal cancer, lung metastasis, COPD	SPA, CPPA
OKH16 <sup>b</sup>	Pleural fluid	Lung adenocarcinoma, Hashimoto's disease, asthma	
$OKH17^{b}$	Pleural fluid	Lung adenocarcinoma, Hashimoto's disease, asthma	
OKH21	Sputum	Angioimmunoblastic T-cell lymphoma, aspiration pneumonia	
OKH23	Otorrhea	Allergic rhinitis	Otomycosis
OKH24 <sup>c</sup>	Sputum	NTM infection, DM	SPA
OKH25 <sup>c</sup>	Tracheal aspiration	NTM infection, DM	SPA
OKH29	Tracheal aspiration	Aspiration pneumonia, cerebral hypoxia	
OKH30	Wound	Burn, heat stroke, higher brain dysfunction	
OKH31 <sup>d</sup>	Sputum	CPFE, lung adenocarcinoma (recurrence), ANCA-associated glomerulonephritis,	CPPA
		chronic renal failure, CMV pneumonia	
OKH32	Sputum	Sweet's syndrome, asthma, hypertension, plaque psoriasis	
OKH33	Sputum	AD, prostate cancer, intracerebral hemorrhage	
OKH34 <sup>d</sup>	Sputum	CPFE, lung adenocarcinoma (recurrence), ANCA-associated glomerulonephritis,	CPPA
		chronic renal failure, CMV pneumonia	

Abbreviations: RA: rheumatoid arthritis, CVD-IP: collagen vascular disease associated interstitial pneumonia, AD: Alzheimer-type dementia, ITP: idiopathic thrombocytopenic purpura, COPD: chronic obstructive pulmonary disease, CPFE: combined pulmonary fibrosis and emphysema, DM: diabetes mellitus, NTM: nontuberculous mycobacterial, ANCA: anti-neutrophil cytoplasmic antibody, CMV: cytomegalovirus, ABPA: allergic bronchopulmonary aspergillosis, SPA: simple pulmonary aspergilloma, CPPA: chronic progressive pulmonary aspergillosis

Superscripts (a, b, and c) indicates that these strains were isolated serially from each identical patient.

Supplementary Table 3. Primers used in this study.

Primer name	Sequence (5' to 3')	References and Notes
ITS1	TCCGTAGGTGAACCTGCGG	Used to amplify and sequence ITS region [1]
ITS4	TCCTCCGCTTATTGATATGC	Used to amplify and sequence ITS region [1]
ITS5	GGAAGTAAAAGTCGTAACAAGG	Used to amplify and sequence ITS region [1]
NL1	GCATATCAATAAGCGGAGGAAAAG	Used to amplify and sequence D1/D2 region [2]
NL4	GGTCCGTGTTTCAAGACGG	Used to amplify and sequence D1/D2 region [2]
BenA1	AATAGGTGCCGCTTTCTGG	Used to amplify and sequence partial $\beta$ -tubulin gene of <i>A. fumigatus</i> [3]
BenA2	AGTTGTCGGGACGGAAGAG	Used to amplify and sequence partial $\beta$ -tubulin gene of <i>A. fumigatus</i> [3]
rodA1	GCTGGCAATGGTGTTGGCAA	Used to amplify and sequence partial hydrophobin gene of <i>A. fumigatus</i> [3]
rodA2	AGGGCAATGCAAGGAAGACC	Used to amplify and sequence partial hydrophobin gene of <i>A. fumigatus</i> [3]
cmd5	CCGAGTACAAGGAGGCCTTC	Used to amplify and sequence partial calmodulin gene of <i>A. fumigatus</i> [4]
cmd6	CCGATAGAGGTCATAACGTGG	Used to amplify and sequence partial calmodulin gene of <i>A. fumigatus</i> [4]
cyp51Aup-F	GAATATATACGTCGATCTGTGTGAC	Used to amplify and sequence <i>cyp51A</i> gene of <i>A. fumigatus</i> .
cyp51Adown-R	ATCCCAGCAGATACGCTGGTCTCTGC	Used to amplify and sequence <i>cyp51A</i> gene of <i>A. fumigatus</i> .
cyp51Aup-F	ACAGAATACTGGGCAGCGGGCTGGAG	Used to sequence <i>cyp51A</i> gene of <i>A. fumigatus</i> .
cyp51A-F	ATGGTGCCGATGCTATGGCTTACGG	Used to sequence <i>cyp51A</i> gene of <i>A. fumigatus</i> .
cyp51A-F2	TTAGAGTCTCATGTGCCACTTATTGAGAAGG	Used to sequence <i>cyp51A</i> gene of <i>A. fumigatus</i> .
cyp51A-F3	CTCACAGCCAAAAGTCCTCGAAGAGC	Used to sequence <i>cyp51A</i> gene of <i>A. fumigatus</i> .
cyp51A-F4	TTTTCAACGTGGATGGAAAGAAAGGAGTCC	Used to sequence <i>cyp51A</i> gene of <i>A. fumigatus</i> .
cyp51Adown-R2	ACTATCAAAAACAGGTTTTCGCACGAGC	Used to sequence <i>cyp51A</i> gene of <i>A. fumigatus</i> .
cyp51AFrc	CCGTAAGCCATAGCATCGGCACCAT	Used to amplify and sequence <i>cyp51A</i> gene of <i>A. fumigatus</i> .
cyp51Aup-F2rc	CTCCAGCCCGCTGCCCAGTATTCTGT	Used to amplify and sequence <i>cyp51A</i> gene of <i>A. fumigatus</i> .
Afu_cyp51a-rtF	ACAGAACCGCCAATGGTCTT	Used to quantify the expression of <i>cyp51A</i> in <i>A. fumigatus</i> .
Afu_cyp51a-rtR	CGCCATACTTTTCTCTGCACG	Used to quantify the expression of <i>cyp51A</i> in <i>A. fumigatus</i> .
cyp51Bup-F	TATCCAGCAAAGTGTGGGCCCCGCCAG	Used to amplify and sequence <i>cyp51B</i> gene of <i>A. fumigatus</i> .
cyp51Bdown-R	TCGTGATATGAAAAAGCACGCCAGC	Used to amplify and sequence <i>cyp51B</i> gene of <i>A. fumigatus</i> .
cyp51Bup-F2	TTTGTGTCTTCCAGTTTGCTTGGATCC	Used to sequence <i>cyp51B</i> gene of <i>A. fumigatus</i> .

cyp51B-F	ATGGGTCTCATCGCGTTCATTCTCG	Used to sequence <i>cyp51B</i> gene of <i>A. fumigatus</i> .
cyp51B-F2	AGGTAAGCGACTTTGGCAGAAACAC	Used to sequence <i>cyp51B</i> gene of <i>A. fumigatus</i> .
cyp51B-F3	ATGATGATTGCCTTGTTGATGGC	Used to sequence <i>cyp51B</i> gene of <i>A. fumigatus</i> .
cyp51B-F4	GCAATTTGCATATCTTCAGCTTGGC	Used to sequence <i>cyp51B</i> gene of <i>A. fumigatus</i> .
cyp51Bdown-R2	CTACGGCCGGTTTCTTTTCTACTATAGGG	Used to amplify and sequence <i>cyp51B</i> gene of <i>A. fumigatus</i> .
cyp51B-MR	GCCATCAACAAGGCAATCATCAT	Used to sequence <i>cyp51B</i> gene of <i>A. fumigatus</i> .
Afu_cyp51b-rtF	GACTGCCGCGCAAAGTATG	Used to quantify the expression of <i>cyp51B</i> in <i>A. fumigatus</i> .
Afu_cyp51b-rtR	GCAGCTTGCCGTTTAGGATG	Used to quantify the expression of <i>cyp51B</i> in <i>A. fumigatus</i> .

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## Supplementary Figure 1a

A C A A C T T G A T A T C T C G G G C A T C A T C G C T	G C A A C A G T A T G A A T T T C T C T G T G A G A G A A A A T T C C T A G C A G T T T G C G A G C C A T G C T G G G A G G A A T C T C
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## Supplementary Figure 1b

<i>сур51В</i> ОКН6	G T G A C C C G G T G T T T G A G G A A G A C A A C A A G T C A T T A T A A G T G A C C G G T G G T C T T T T T C G T A T C T A A A T C A C A G C A T T G T T A A G T G G G A T G T	100
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cyp51B OKH6	<u>CATAT GT GAT CCCTACAT CT CATAAT GTT CT CT CT CT CT CCAGGC GT CACT GCCAGGT CT GAGGAGCACT T CCCAAAT CCACT CGAAT GGAACCCT CACCG</u>	2300 2299
OKH1 OKH2	G. <u>G</u> .	2300 2300
cyp51B OKH6	<u>T T G G G A T G A G A A C A T T G C A G C T A G C G C T G A G G A C G A C G A G A A A G T T G A T A T G G C T A C G G G T T A G T A G C C A A T A G C C C G T A C T C C C G T T T T A G C C A A A A C T T G C A A G T T G C T A C C C G T A C T C C C G T T T A C C A A G C C A A A A C T T G C A A G T T G C A A G T T A G C C A A A A C T T G C A A G T T G C A A G T T A G C C A A A A C T T G C A A G T T G C A A G T T A G C C A A A A C T T G C A A G T T G C T A C C C G T A C C C G T A C C C G T A C C C G T A C C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C G T A C C C C C C C C C C C C C C C C C C</u>	2400 2399
OKH1 OKH2		2400
cyp51B OKH6 OKH1	<u>GGGGGT GGACGGCATAGGT GCATT GGCGAGCAATTT GCATATCTT CAGCTT GGCACAATCACT GCT GT ACTT GT GCGGTT ATT CAGATT CCGT AATTT GC</u>	2500 2499 2500
OKH2		2500
OKH6 OKH1		2598
OKH2 cyp51B	<u>CAAACCGCTTGGCCGATCTTTTGTGGAGTTTGAAAAGCGCGAGTCCGCTACCAAAGCCTGA</u> TCGTGATCACTCGATGCCTCTTCTTGATAGACTTGC	2599 2699
OKH6 OKH1 OKH2	······································	2698 2700 2699
cyp51B	AT G G A A G G T A A - T T T A G T C A T T C T C G G G T A T T C T G C C G T C T T G T T G C C G T T T C C A G A C C T T G T A C A G C A T T T A A T T T C T A G A T G T T C A A C	2798
OKH1 OKH2	A	2797 2799 2799
<i>cyp51B</i> OKH6	ТТТ GA A GTT CT A GTT CT A T A CT GT A T T G A T T C A T A C C T T A G C A T A T T A T G A G G A T A G A T A A T GT T T T	2898 2897
OKH1 OKH2		2899