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Polymorphism of 14α-demethylase gene (*CYP51*) in the cereal eyespot fungi *Tapesia acuformis* and *Tapesia yallundae*

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Abstract

Cereal eyespot fungi *Tapesia acuformis* and *Tapesia yallundae* are closely related species which show different behaviours upon treatment with sterol 14α-demethylase inhibitors (DMIs). *T. acuformis* is naturally resistant to DMIs belonging to the triazole family and susceptible to the imidazole ones, whilst *T. yallundae* is sensitive to both inhibitors. Cloning of the target enzyme gene, *CYP51*, from the two species revealed an important polymorphism between them. Further sequencing of *CYP51* from sixteen *T. acuformis* and eleven *T. yallundae* strains with different phenotypes with regards to resistance to DMIs confirmed that at least eleven variations are species related. Among them, a conserved phenylalanine residue at position 180, found both in *T. yallundae* and in all known *CYP51* proteins from filamentous fungi and yeast, was replaced in *T. acuformis* by a leucine. Therefore, a leucine at 180 could be possibly involved in natural resistance of *T. acuformis* to triazoles. Other mutations were observed in some resistant strains, sometimes simultaneously, but in contrast to what was reported for other filamentous fungi, where a mutation at the 136 position of the *CYP51* gene product seemed to correlate with resistance to DMIs, we did not find a clear relationship between a given mutation and a particular phenotype. This result suggests that resistance to DMIs could have a polygenic nature in *Tapesia*. We took advantage of species-related variations to develop a PCR-based assay allowing rapid and easy discrimination between field strains of the two species.

Introduction

Tapesia acuformis and *Tapesia yallundae* (teleomorph stages) are the causal agents of cereal eyespot, a damaging disease of autumn-sown wheat and barley crops in several European countries. Although they were formerly considered as two varieties of the same species, usually found together in lesions of infested cereals where they provoke similar symptoms, *T. acuformis* and *T. yallundae* are now considered as two separate species based on recent observations of sexual incompatibility between them (Robbertse et al., 1995; Dyer et al., 1996).

Furthermore, besides a few differences in both morphology and *in vitro* growing rate which, in some cases, were not easy to establish, wild-type strains of *T. acuformis* and *T. yallundae* exhibited distinct behaviours when treated with sterol 14α-demethylase inhibitors (DMIs). DMIs, including triazole or imidazole compounds, are a major class of antifungals used both in medicine and agriculture, which inhibit cytochrome P450 sterol 14α-demethylase, a key enzyme of the sterol biosynthetic pathway (reviewed by Yoshida, 1993). *In vitro* tests performed with wild-type isolates of eyespot fungi have shown that *T. acuformis* isolates were intrinsically less sensitive towards triazole-ring containing DMIs than the *T. yallundae* ones (Leroux et al., 1988; Leroux and Gredt, 1988). Additionally, resistance towards triazole, as well as imidazoles, gradually occurred, that affected the two species, leading to losses of efficacy of DMIs in the field (Leroux and Gredt, 1997).

Our main goal was to clone and sequence the eburicol 14α-demethylase gene (*CYP51*) from field strains

of the two *Tapesia* species in order to assess whether or not mutations at the gene level could account for *T. acuformis* natural resistance towards triazole DMIs or be related to acquired resistance in both species.

Materials and methods

Fungal culture and phenotype characterization

The field strains of *T. acuformis* and *T. yallundae* were collected from winter wheat in France and were maintained without fungicide on agar medium (either corn meal agar or malt agar). Most of them were isolated in 1994 and 1998 but some before 1990 (see Table 1; the two first numbers indicate the date of sampling). For a given phenotype, the tested isolates were often obtained from different plots whereas different phenotypes could be found in the same plot (see Table 1; the first letter identifies the plot). Several strains have been studied previously to determine the molecular basis of benzimidazole resistance (Albertini et al., 1999). The *in vitro* effects of four DMIs (technical grades), including two imidazole molecules, prochloraz and triflumizole, and two triazoles, fluzilazole and triadimenol were tested as previously described (Leroux et al., 1988). For each compound, the concentration that inhibits mycelial growth by 50% (EC_{50} mycelium) was determined from the dose–response curves obtained with at least four experimental concentrations.

Molecular procedures

DNA extraction

DNA was extracted using a CTAB protocol from freeze-dried mycelium of either strains of *T. acuformis* or *T. yallundae* grown in liquid medium at 18 °C as previously described (Albertini et al., 1999).

Obtaining a CYP51 *gene fragment*

After alignment of already available *CYP51* sequences from filamentous fungi, especially *Penicillium italicum* (GenBank accession number AFZ49750), *Uncinula necator* (AFU72657) and *Erysiphe graminis* f. sp. *hordei* (AFO52515), degenerate primers D-CR2 and D-CR6 (Table 2) were designed which correspond to conserved amino-acid sequences LTTPVFG and PYLPFGAG, respectively.

PCR with D-CR2 and D-CR6 were performed in 50 µl reaction volume containing 0.2 µg genomic DNA, 1 µM each primer, 1 mM Mg^{2+}, 0.2 µM each dNTP, 0.1 unit ml^{-1} Amplitaq gold (Perkin Elmer). Amplifications were done as follows: 9 min at 94 °C as a prePCR hotstart step needed to activate the

Table 1. *In vivo* effects on mycelial growth of various DMIs on *T. acuformis* and *T. yallundae* strains

	T. acuformis			*T. yallundae*		
	Pro^S	Pro^{R1}	Pro^{R2}	Tri^S	Tri^{R1}	Tri^{R2}
Prochloraz[a,b]	0.03–0.06	0.4–0.8	0.5–1.5	0.02–0.08	0.03–0.10	1.5–2.5
	S[d]	**R**	**R**	**S**	**S**	**R**
Triflumizole[a,b]	>25	1.5–2.5	5–20	0.4–1.0	>25	10–20
	R	**S**	**R**	**S**	**R**	**R**
Flusilazole[a,c]	0.3–0.5	0.5–1.0	0.5–1.5	0.04–0.08	0.2–1.0	≥2.5
	R	**R**	**R**	**S**	**R**	**R**
Triadimenol[a,c]	>25	>25	>25	0.8–2.0	>25	>25
	R	**R**	**R**	**S**	**R**	**R**
Phenotypes and names[e] of the various *Tapesia* strains reported in this study						
	85AG8	94N30T	94CA21T	84J1	94AL1M	94M29T
	87H9M	94BE14T	98AG11T	94AH12T	94T17M	98Y5T
	94BR29B	94T7T	98N6TL	94AL24TR	94W19B	
	98AB11ML	98AA19B			98AA23T	
	98AG5M	98AG16B			98AF6B	
	98C16T	98B14T			98O16B	
		98N18T				

[a]Range of EC50 (extreme values) in mg l^{-1}. [b]Imidazole DMI. [c]Triazole DMI. [d]**S** for sensitive and **R** for resistant phenotype (in boldface). [e]The two first numbers indicate the date of sampling whereas the first letter identifies the plot.

Table 2. Primers designed for this study

Primer's name	Orientation: S or R[a]	Nucleotidic sequence (5′ → 3′)/amino-acid sequence (in boldface)	Relative position in *CYP51* (codons numbers)
D[b]-CR2	S	CTGACCACNCCNGTNTTYGG **LTTPVFG**	125–131
D[b]-CR6	R	GCCGGCGCCGAANGGNAR*I*TANGG **PYLPFGAG**	462–455
GSP1up	R	CCAGGAACTCGAGGAAGAGGACGAATG **HSSSSSSSW**	318–310
GSP2up	R	GGAGCGCTTGATGAATGTC **TFIKRS**	177–172
GSP1down	S	CATTCGTCCTCTTCCTCGAGTTCCTGG **HSSSSSSSW**	310–318
GSP2down	S	GTGGGAACCCCACAGATGGG **WEPHRWD**	419–425
BEG	S	GGACGCGACTGCAAGATGGGAATACTC **-----MGIL**	−5–4
END	R	CAGACTGGTGTAATCCGTCTCCACGAC **VVETDYTSL**	506–498
ACU	S	CATCAAGCGCTCCGCAGAATTA **IKRSAEL**	174–180
YAL	S	CATCAAGCGCTCCCCAGAATTY **IKRSPEF**	174–180
UCA	R	TAATTCTGCGGAGCGCTTGATG **IKRSAEL**	180–174
LAY	R	GAATTCTGGGGAGCGCTTGATG **IKRSPEF**	180–174

[a]S for sense, R for reverse. [b]D for degenerate primer; *N* for: A/G/T/C; *R* for A/G; *Y* for: C/T; and *I* for: inosine (able to bind to C or T).

DNA polymerase and 35 cycles of 1 min at 94 °C, 1 min at 58 °C and 2 min at 72 °C in a Gen Amp PCR 2400 thermocycler (Perkin Elmer). Amplified fragments with the roughly expected size of 1 kb were visualized on 1% agarose gels containing ethidium bromide (0.4 mg ml^{-1} gel) in 1X tris-acetate EDTA buffer. Sequencing procedures were performed at the ESGS facility (Cybergène-ESGS, Genopole Evry, France) as described elsewhere (Albertini et al., 1999) and all the primers used in this study (listed in Table 2) were synthesized by Sigma-Genosys biotech (UK).

Genome walking

Genome walker libraries were constructed following manufacturer's instructions (Clontech laboratories Inc., USA) with freshly extracted DNA from two strains, each representing one species, 94BE14T (*T. acuformis*) and 84J1 (*T. yallundae*). Briefly, approximately 2.5 µg genomic DNA were digested to completion with either one of the restriction enzymes *Dra*I, *Stu*I or *Pvu*II. Synthetic DNA adaptors were then ligated to genomic DNA fragments to constitute uncloned genomic libraries. Two PCR amplification steps were performed successively, a primary PCR with an outer adaptor primer AP1 provided in the kit and an outer gene-specific primer, followed by a nested PCR with a nested adaptor AP2 provided in the kit and a nested specific primer. To amplify upstream sequences of *CYP51* we designed GSP1up in the primary PCR and GSP2up in the nested PCR in the antisense direction of the already amplified gene fragment. Similarly, to amplify the downstream sequences of the gene we designed GSP1down and GSP2down in the sense direction of the former obtained amino-acid sequence data. PCR reactions in each case were performed in 50 µl reaction volume containing 0.2 µg digested DNA, 1 µM each primer (one from the kit, the other one specific of the*CYP51* gene), 1 mM Mg^{2+}, 0.2 µM each dNTP and 0.1 unit ml^{-1} *Thermus thermophilus* polymerase mixed with a proof reading activity and *Tth* antibodies to allow hotstart PCR (Clontech), amplifications

were done using a touchdown procedure: seven cycles of 2 s at 94 °C and 3 min at 72 °C were followed by 32 cycles of 2 s at 94 °C and 3 min at 67 °C. Amplified fragments were visualized and sequenced as previously mentioned above and sequences of primers are listed in Table 2.

cDNA cloning

Total RNA was extracted from roughly 100 mg fresh mycelium of strains 94BE14T (*T. acuformis*) and 84J1 (*T. yallundae*) using Qiagen Plant mini Kit. DNA was digested with DNAse I and total purified RNA was then submitted to RT-PCR (Clontech laboratories Inc., USA) following manufacturer's instructions. Then, specific *CYP51* primers, BEG which begins fifteen bases before the start, and END corresponding to the amino-acid sequence VVETDYTSL, were designed (Table 2) to specifically amplify *CYP51* cDNA. PCR with primer pair BEG/END was performed in 50 µl reaction volume containing 0.2 µg total cDNA. PCR conditions and cycling were as previously described for the gene walking procedure (former section). *CYP51* cDNA of the expected 1.6 kb size was visualized in each case as a single band on 1% agarose gel and directly sequenced at the ESGS facility as previously noticed.

Polymorphism analysis

Primers BEG and END (Table 2) were used to amplify and sequence the whole *CYP51* gene from field strains of *T. acuformis* and *T. yallundae* which show well characterized behaviour towards the triazole DMIs, flusilazole and triadimenol, as well as the imidazoles, prochloraz and triflumizole (Table 1). PCR amplifications were performed in 50 µl reaction volume containing 0.2 µg genomic DNA, 1 µM each primer, 1 mM Mg^{2+}, 0.2 µM each dNTP, 0.1 unit ml^{-1} *Tth* polymerase. Amplifications were done as follows: 3 min at 94 °C as a prePCR hotstart step followed by 35 cycles of 2 s at 94 °C and 2 min at 68 °C. Amplified fragments were visualized on 1% agarose gels containing ethidium bromide in 1X tris-acetate EDTA buffer.

Allele-specific PCR

We designed allele-specific primers, ACU corresponding to *T. acuformis* amino-acid sequence IKRSAEL (position 174–180) and YAL which matches with IKRSPEF (same position) encountered only in *T. yallundae* strains (Table 2). As control primers without any allele-specificity we designed the antisenses UCA and LAY (Table 2).

PCR reactions were performed, with the same cycling parameters as above, using primer END and either ACU or YAL at the final concentration of 1 µM each in 50 µl reaction volume containing 0.2 µg genomic DNA, 1 mM Mg^{2+}, 0.2 µM each dNTP, 0.1 unit ml^{-1} *Tth* polymerase. Control reactions were run with primers BEG and either LAY or UCA under the same conditions.

Results

Phenotype characterization of field strains towards DMIs fungicides

In the early 1980s, DMIs were introduced to control cereal eyespot fungi. From the very beginning of DMI use as a curative treatment, *in vitro* tests conducted with wild-type isolates of eyespot fungi revealed that those belonging to *T. acuformis* were intrinsically less sensitive towards most of the DMIs molecules than the *T. yallundae* ones (Leroux et al., 1988). The natural resistance of *T. acuformis* which concerned all the triazole derivatives and some imidazoles such as triflumizole, but not prochloraz, was confirmed in the tested strains (Table 1).

In addition to natural resistance towards triazole DMIs, acquired resistance to prochloraz gradually occurred in *T. acuformis* populations following a decade of intensive treatments. Further analysis of the *in vitro* effects of triflumizole on *T. acuformis* field strains allowed us to distinguish two different phenotypes, Pro^{R1} and Pro^{R2} among the prochloraz resistant-population (Table 1). The most common one, Pro^{R1} was resistant to triazoles and prochloraz but exhibited hypersensitivity to triflumizole, whereas Pro^{R2} was resistant to both DMIs. Similar analysis performed with *T. yallundae* field strains revealed the major occurrence of two DMI-resistant phenotypes, Tri^{R1} which exhibited resistance towards triazoles and triflumizole but was still sensitive to prochloraz while Tri^{R2} was resistant to both DMIs including prochloraz (Table 1).

The various field strains of *T. acuformis* and *T. yallundae* reported in this study (Table 1) covered the entire phenotype spectrum prevalent in France (Leroux and Gredt, 1997).

Cloning the CYP51 *gene from the two species*

Amplifications with primers D-CR2 and D-CR6 yielded a single major PCR product of the expected 1 kb size with both strains, 94BE14T as a *T. acuformis* (Pro^{R1} phenotype) representative and 84J1 as a *T. yallundae* (Tri^{S}, wild-type) one (data not shown). The D-CR2/D-CR6 fragment potentially encoded, in both cases, a 335 amino-acid polypeptide interrupted by a putative intron of 52 bp in 94BE14T and 56 bp in 84J1. The *T. acuformis* polypeptide shared 98.2% identity and 98.9% similarity with the *T. yallundae* polypeptide, and, when compared to already known *CYP51* protein of other filamentous fungi, displayed 75.3%, 73.4%, 56.4% identity and 87.5%, 82.1%, 71.6% similarity with the corresponding region of *E. graminis* f. sp. *hordei* (Délye et al., 1998), *U. necator* (Délye et al., 1997a) and *P. italicum* (van Nistelrooy et al., 1996).

Genome walking on *T. acuformis* and *T. yallundae* DNA was conducted to obtain complete genomic *CYP51* sequences of the two species by performing PCR amplifications both upstream and downstream to the D-CR2/D-CR6 fragment. Amplifications of *T. acuformis* 94BE14T *Dra*I-digested DNA ligated to synthetic adaptors by using successively primer pairs GSP1up/AP1 and GSP2up/AP2 yielded a main PCR product of roughly 1.2 kb. This fragment encompassed a partial ORF of 647 bp including the first 169 bp of the D-CR2/D-CR6 fragment as well as two putative introns, one of 64 bp and the already mentioned 52 bp intron. Amplifications performed with *T. acuformis* 94BE14T *Pvu*II-digested DNA ligated to the same synthetic adaptors using primer pairs GSP1down/AP1 and GSP2down/AP2 yielded a single PCR product of approximately 600 bp. This fragment, including the last 126 bp of D-CR2/D-CR6, contained a partial ORF of 327 bp terminated by a TGA stop codon. The nucleotide sequence of the *T. acuformis CYP51* gene resulting from joining overlapping sequences of both the upstream 1.2 kb, the 1 kb D-CR2/D-CR6 and the downstream 600 bp fragments was 1694 bp long.

Similarly, amplifications of *T. yallundae* 84J1 *Pvu*II-digested DNA by using successively the two designed pairs of upstream primers gave a 1.6 kb PCR product that encompassed a partial ORF of 658 bp including the first 173 bp of D-CR2/D-CR6 fragment and two putative introns fairly longer than the *T. acuformis* ones, one of 71 bp and the above mentioned 56 bp intron. Downstream amplifications of *T. yallundae Stu*I-digested DNA following the same procedure with downstream primer pairs yielded a roughly 500 bp fragment containing a partial ORF of 327 bp including the last 126 bp of D-CR2/D-CR6 fragment and terminated by a TGA codon. The whole nucleotide sequence of the *T. yallundae CYP51* gene after joining the three contigs was 1705 bp long.

cDNA cloning

In both species the *CYP51* gene was interrupted by two putative introns. The first intron was at nucleotide position 247–311 and the second at nucleotide position 509–561 in *T. acuformis*, whilst the first intron was at position 247–318, and the second at position 516–572 in *T. yallundae*. A comparison of PCR products obtained after using primer pairs BEG/END on genomic DNA and cDNA from the two *Tapesia* species revealed that the two putative introns were readily excised being at the same position as the two introns in other known *CYP51* from filamentous fungi. The remainder of the cDNA coding sequence was identical in each case to that of the genomic DNA, thus further confirming that the *CYP51* genes we sequenced were not pseudogenes.

T. acuformis (94BE14T) and *T. yallundae* (84J1) complete *CYP51* sequences data can be found in GenBank under the accession numbers AF208657 and AF208658.

CYP51 *sequences analysis*

The inferred 526 amino-acid proteins encoded by the *T. acuformis* 1694 bp and the *T. yallundae* 1705 bp sequences were 98% identical indicating that the two species are indeed closely related. When compared to the known complete *CYP51* sequences they showed a strong homology with *Botrytis cinerea* (76% identity, C. Albertini, GenBank accession number: AF279912), *E. graminis* f. sp. *hordei* (70% identity, Délye et al., 1998) and *U. necator* (69% identity, Délye et al., 1997a). This is much more than the minimal requirement of 40% homology for belonging to the same cytochrome P450 family (Nelson et al., 1993).

Variability of the CYP51 *gene*

Species-specific variations

Comparison of 94BE14T *T. acuformis* nucleotidic sequence of the *CYP51* gene with the 84J1 *T. yallundae*

one showed they share more than 92% homology. When, additionally, the 300 bp sequence upstream of the start that should include the gene promoter was analyzed, sequence homology decreased to less than 88%, mainly due to important deletions. In *T. yallundae*, the deletions were all encountered between 300 and 80 bp before the first coding region. In *T. acuformis* there was a large deletion of 10 bp long closer to the start, i.e., 40 bp upstream of the ATG codon, that was not observed in the *T. yallundae* 84J1 sequence, neither in the two *T. yallundae CYP51* alleles isolated by others (Wood et al., 2001). In the *CYP51* gene itself, comparative analysis revealed 123 differences between the two species. Most of them were silent variations or concerned deletions in *T. acuformis* introns leading to few differences in restriction maps. Fourteen changes were expressed at the amino-acid sequence level (Table 3). These amino-acid differences were principally focused in the N-terminal part of the *CYP51* protein with ten of them comprised within the first 180 amino-acids. They were located at amino-acid positions: 5, where there was a E in *T. acuformis* for a D in *T. yallundae* (5: E/D), 8: S/T, 13: Q/T, 85: D/N, 102: R/P, 103: N/D, 157: E/D, 168: D/N, 178: A/P, 180: L/F, 351: D/E, 387: T/A, 486: H/Y and 496: T/G (Table 3). Most of these residues were also found highly variable in CYP51s from other origins (Table 4). However, the phenylalanine at position 180 is well conserved in known CYP51s of the fungal kingdom, being present both in filamentous fungi and yeast, in contrast to plants and mammals where it is missing. As azole antifungals selectively inhibit yeast and fungal CYP51s over their plants and mammals counterparts (Georgopapadakou, 1998), the replacement of the phenylalanine by a leucine at position 180 in *T. acuformis* strains could be linked to triazole natural resistance through possible interference either with fungicide binding or binding-site accessibility. Other variations could have, however, a synergistic effect in that resistance to triazoles and triflumizole.

Primer pair BEG/END was used to amplify *CYP51* from various field strains to check the possible species-specificity of the fourteen observed amino-acid differences. Subsequent amino-acid sequence analysis of *CYP51* from sixteen *T. acuformis* and eleven *T. yallundae* field strains with well characterized phenotypes as regards to resistance or sensitivity towards four DMIs (see Table 1) confirmed that at least eleven of the fourteen reported changes were species-specifically determined at position 8, 85, 102, 103, 157, 168, 178, 180, 351, 387 and 496 (Table 3). Due to its close proximity to primer BEG, the amino-acid at position 5 could not always be identified in sequencing data. The threonine (T) found at position 13 in the *T. yallundae* 84J1 *CYP51* protein was found to be variable in the *T. yallundae* species and replaced either by a histidine (H) or, like in all the *T. acuformis* strains analyzed in this study, by a glutamine (Q). The histidine observed at position 486 in the 94BE14T strain was not species-specific as it was replaced by a tyrosine (Y) in the other *T. acuformis* strains.

Table 3. Amino-acid variations between 94BE14T (*T. acuformis*) and (84J1) *T. yallundae* CYP51s

Amino-acid position	94BE14T (*T. acuformis*)	84J1 (*T. yallundae*)
5	E (GAA)	D (GAT)
8*	**S** (**T**CT)	**T** (**A**CT)
13	Q (CAG)	T (ACG)
85	**D** (G**A**T)	**N** (**AA**C)
102	**R** (**AA**G)	**P** (**CC**G)
103	**N** (**A**AC)	**D** (**G**AC)
157	**E** (GA**G**)	**D** (GA**C**)
168	**D** (**G**AC)	**N** (**A**AC)
178	**A** (**G**CA)	**P** (**C**CA)
180	**L** (TT**A**)	**F** (TT**C**)
351	**D** (GA**C**)	**E** (GA**A**)
387	**T** (**ACA**)	**A** (**GCG**)
486	H (CAT)	Y (TAT)
496	**T** (**AC**G)	**G** (**GG**G)

*The eleven species-specific variations are in boldface.

Other variations

The substitutions that we observed in a few strains (Table 5) were as follows: A29T (A being replaced by T at position 29); A29P; A30P; S35T; V37A; Q43H; D78Y; E106K; Q167H; A178S; N244S; S505Q. Because A29T was found associated with T13H and A30P in a *T. yallundae* strain (94AH12T) sensitive to DMIs, these mutations are therefore not likely to be linked to DMIs resistance. Similarly, as A178S was also found in a *T. acuformis* prochloraz-sensitive strain (87H9M) implied that substitution of an alanine by a serine at position 178 does not play any role in acquired resistance to prochloraz. Among these probably neutral variations we did not find the A6T mutation reported in some *T. yallundae* strains (Wood et al., 2001).

Mutations that were not constantly found in correlation with a particular resistant-phenotype, i.e., not always recovered in every strain of a given phenotype, have been observed and could be taken into account.

Table 4. Amino-acid positions affected by polymorphism in fungal CYP51s

Amino-acid position*	**6**	**8**	**13**	**29**	**30**	**35**	**37**	**43**	**76***	**78**	**85**	**102**	**103**	**106**
	1	3	8	24	25	30	32	38	72	74	81	98	99	102
T. acuformis (GenBank accession no. AF208657)	A	**S**	**Q**	**A/P**	**A/P**	S	**V/A**	Q	F	D	**D**	**R**	**N**	E
T. yallundae (AF208658, AF 276660, AF276661**)	**A/T****	**T**	**T**	**A/T**	**A/P**	**S/T**	V	**Q/H**	F	**D/Y**	**N**	**P**	**D**	**E/K**
E. graminis (a) (AF052515)	S	M	Q	L	S	L	F	Q	F	E	N	R	Q	N
U. necator (b) (U72657)	I	S	Q	F	S	A	**G/V**	Q	Y	D	D	L	Q	N
P. italicum (c) (Z49750)	—	—	L	T	G	S	V	Q	F	A	D	V	E	E
C. albicans (d) (X13296)	M	I	I	S	I	P	V	Q	**F/L**	S	D	P	K	E

Amino-acid position*	**109***	**120***	**130***	**132***	**133***	**136***	**147***	**149***	**151***	**153***	**156**	**157***	**167**	**168**	**169***	**178**	**180**	**230***	**244**	**267***
	105	116	126	128	129	132	143	145	147	149	152	153	163	164	165	174	176	229	243	266
T. acuformis	L	E	F	K	D	Y	K	F	K	G	T	**E**	**Q/H**	**D**	E	**A**	**L**	S	N	K
T. yallundae	L	E	F	K	D	Y	K	F	K	G	T	**D**	Q	**N**	E	**P**	**F**	S	**N/S**	K
E. graminis	L	E	F	T	D	**Y/F**	K	F	K	A	T	E	Q	N	E	D	F	T	N	T
U. necator	L	E	F	R	D	**Y/F**	K	F	K	A	**I/T**	E	Q	N	E	V	F	T	N	E
P. italicum	L	E	F	S	D	**Y/F**	K	F	K	G	Q	E	A	D	E	P	F	S	N	S
C. albicans	**F/L**	**D/E**	**F/L**	**K/T**	**G/A**	**Y/H**	**K/R**	**F/L**	**K/R**	**A/V**	T	**D/E**	R	E	**E/Y**	E	F	**T/A**	Y	**E/D**

Amino-acid position*	**268***	**278**	**279**	**287**	**315**	**351**	**387**	**402***	**434***	**444***	**445***	**446***	**448***	**460***	**461***	**463***	**484***	**486***	**496***	**505***
	267	277	278	286	315	354	390	405	437	448	449	450	452	464	465	467	488	490	498	507
T. acuformis	D	M	S	K	S	**D**	**T**	S	T	G	Y	G	I	G	A	R	V	H	**T**	**S/Q**
T. yallundae	D	M	S	K	S	**E**	**A**	S	T	G	Y	G	I	G	A	R	V	**H/Y**	**G**	**S/Q**
E. graminis	Q	M	W	K	S	E	P	A	T	G	Y	G	I	G	A	R	V	S	N	S
U. necator	E	M	W	K	T	E	P	S	N	G	Y	G	I	G	A	R	V	F	Q	S
P. italicum	W	I	S	R	I	K	P	S	S	G	Y	G	V	G	A	R	V	E	E	S
C. albicans	**R/H**	**D/E**	**S/F**	**K/R**	**T/A**	E	P	**S/F**	**V/I**	**G/E**	**F/L**	**G/E**	**V/A**	**G/S**	**G/S**	**R/K**	**V/I**	N	Y	S

Mutations are designed X/Y where Y is the changing amino-acid and are in boldface, *Tapesia* confirmed species-specific variations are also figured in boldface.
*Numbering according to *Tapesia* spp. *CYP51* sequence is given in boldface right above numbering according to *C. albicans*.
**This A6T change in *T. yallundae* has been reported by Wood et al. (2001).
(a) Reported by Délye et al. (1998).
(b) Reported by Délye et al. (1997b).
(c) Reported by de Waard (1996).
(d) Reported *C. albicans* mutations as reviewed by Marichal et al. (1999). Only the mutations that have been demonstrated to be important for fungicide affinity are underlined.

Table 5. Amino-acid mutations encountered in *CYP51* from field strains of *T. acuformis* and *T. yallundae*

Amino-acid position	Amino-acid change[a]	Strains[b] (phenotype)
13	T13H	94W19B (Tri^{R1}); *94AH12T (Tri^{S})
	T13Q	94AL1M (Tri^{R1})
29	A29T	*94AH12T (Tri^{S})
	A29P	98AG11T (Pro^{R2}); 94N30T (Pro^{R1})
30	A30P	*94AH12T (Tri^{S}); 98AG11T (Pro^{R2})
35	S35T	98Y5T (Tri^{R2})
37	V37A	98AA19B (Pro^{R1}); 94N30T (Pro^{R1})
43	Q43H	98Y5T (Tri^{R2})
78	D78Y	98AF6B (Tri^{R1})
106	E106K	94T17M (Tri^{R1})
167	Q167H	98AG11T (Pro^{R2})
178	A178S	*87H9M (Pro^{S})
244	N244S	94W19B (Tri^{R1})
486	Y486H	94BE14T (Pro^{R1})
505	S505Q	98AA23T (Tri^{R1}); 98N18T (Pro^{R1}); 98N6TL(Pro^{R2})

[a]Amino-acid change are designated XNY where Y is the amino-acid substituting X at the *N* position.
[b]Strains with multiple mutations are underlined while DMIs-sensitive strains are indicated by an asterisk.

Mutations in the *CYP51* protein could indeed affect the affinity for an antifungal drug by interfering with the accessibility of its binding-site or alter the binding-site itself either directly or indirectly by a conformational change of the tertiary structure of the protein. Some changes were single amino-acid substitutions in a *CYP51* protein from a DMI-resistant strain as V37A; D78Y; E106K; Y486H; S505Q (Table 5). A V37A mutation was found alone in a *T. acuformis* Pro^{R1} strain, i.e., resistant to triazoles and prochloraz but sensitive to triflumizole (98AA19B). In *U. necator* a mutation at the same position has been observed that was not associated with fungicide resistance, however, the residues involved in the substitution, G37V, were different (Délye et al., 1999). A Y486H substitution was found in a *T. acuformis* strain with a similar Pro^{R1} phenotype (94BE14T). A D78H mutation was present in one *T. yallundae* Tri^{R1} strain sensitive to prochloraz but resistant to triazole DMIs (98AF6B). A E106K substitution was observed in one *T. yallundae* strain with a similar Tri^{R1} phenotype (94T17M). Interestingly these residues, although not conserved ones, were found here affected by mutations that have not been reported before in other filamentous fungi (Table 4). In the case of S505Q, this mutation, which has been found both in two *T. acuformis* strains: a Pro^{R1} (98N18T) and a Pro^{R2} (98N6TL) and also in a *T. yallundae* Tri^{R1} strain (98AA23T), is not likely to be involved in resistance. A Tri^{R1} strain has indeed a very similar phenotype to a Pro^{S} strain, being sensitive to prochloraz and resistant to triazoles and triflumizole (see Table 1), whereas a Pro^{R1} and a Pro^{R2} strain have only in common their resistance to prochloraz, Pro^{R1} being as susceptible as Tri^{S} strains to triflumizole and Pro^{R2} being resistant to it.

Other mutations were found simultaneously in some DMIs-resistant strains (Table 5) as: T13H with N244S in a *T. yallundae* Tri^{R1} strain (94W19B); A29P with A30P and Q167H in a *T. acuformis* Pro^{R2} strain (98AG11T), A29P with V37A in a *T. acuformis* Pro^{R1} strain (94N30T), and S35T with Q43H in a *T. yallundae* Tri^{R2} strain (98Y5T). The T13H substitution is not likely to play any role in acquired resistance to triazole as it was also found in a Tri^{S} strain (94AH12T) as already mentioned earlier in this section. Similarly, because the A30P mutation was present in the same sensitive *T. yallundae* strain (94AH12T) it does not seem to have any involvement in resistance to prochloraz of the Pro^{R2} strain (98AG11T). A A29P change was found in two strains with different phenotypes: a Pro^{R1} in which this mutation was associated with the presence of V37A (94N30T), and a Pro^{R2} (98AG11T) where there is also another mutation, Q167H. As Pro^{R1} and Pro^{R2} phenotypes are distinguished only by triflumizole, i.e., Pro^{R1} being sensitive and Pro^{R2} resistant to that fungicide, A29P could only play a role in the prochloraz acquired resistance displayed by the two phenotypes. V37A could account for the increased sensitivity of the Pro^{R1} strain to triflumizole, on the contrary, the presence of a Q167H in the Pro^{R2} strain could be related to restored triflumizole-resistance. A mutation which affects a conserved residue in known *CYP51* from filamentous fungi and yeasts: Q43H (Table 4) was present in a Tri^{R2} strain (98Y5T), and could be involved in acquired resistance to both DMIs. As this mutation was found associated with a S35T substitution both changes could synergically play a role in DMIs-resistance of that Tri^{R2} strain. A N244S mutation affecting a conserved residue in filamentous fungi but not in yeasts, plants and mammals, was found in a Tri^{R1} strain (94W19B) and could account for acquired resistance to triazoles DMIs.

Strikingly, we have never found the Y136F substitution that has been reported in other filamentous fungi as *P. italicum*, *U. necator*, *E. graminis* f. sp. *hordei* and is believed to be related to DMIs resistance in these organisms (de Waard, 1996; Délye et al., 1997b; Délye et al., 1998). In *Candida albicans*, the replacement of the same tyrosine residue by a histidine, Y132H, has been found in DMIs-resistant mutants (Sanglard et al., 1998). What we observed at this position was a silent variation, Y136 being encoded by a TAT codon in *T. acuformis* strains whereas there was a TAC codon in *T. yallundae* strains. None of the other reported mutations that have been demonstrated in *C. albicans* to be involved in resistance to DMIs, i.e., F105L, T315A, S405F, G464S and R467K (as reviewed by Marichal et al., 1999) were found in this study.

Moreover, it is noteworthy that almost one half of the DMIs-resistant strains analyzed in this study did not show any sequence change at the *CYP51* protein level.

Allele-specific PCR

Considering that a 3′ mismatch does not prime in a PCR reaction under specific annealing temperature (Sommer and Tautz, 1989), we took advantage of constant differences between *T. acuformis* and *T. yallundae CYP51* sequences to design allele-specific primers ACU and YAL (Table 2) in order to develop a PCR-based assay enabling rapid and easy discrimination between field strains of the two species. Preliminary results have shown that using species-specific primer pair PCR amplifications successfully allowed species identification of *Tapesia* field strains (Figure 1). A fragment of around 1 kb was amplified with *T. acuformis* genomic DNA but not with *T. yallundae* DNA using ACU/END primer pair, whilst the same length fragment was amplified with *T. yallundae* DNA but not with *T. acuformis* DNA using YAL/END primers. UCA/BEG and LAY/END were used as non-species specific control primer pairs and led to amplification of a 680 bp fragment in each case.

Discussion

As most CYP51s are membrane bound enzymes, experimental structural information on their active sites (catalytic site, substrate recognition and/or binding-site, drugs binding-sites), that would greatly facilitate developing efficient antifungals, are lacking. The only three dimensional model available so far is based on a soluble isozyme of bacterial origin, *Mycobacterium tuberculosis*, with both different substrate specificity and different folding pathways despite similar spatial folds and some conservation in sequence (Podust et al., 2001, Lepesheva et al., 2001). This *CYP51* soluble ortholog (MTCYP51) has been structurally studied after crystallization in the presence of an azole antifungal, but as emphasized by Podust *et al.* (2001), more structural and biochemical data are required to establish precise features of P450 catalytic cycle as well as drug passage and binding. Actually, none of the mutations that have been identified in *C. albicans* azole-resistant isolates seems to be involved in direct interaction with azole when mapping the protein in a similar conformation as MTCYP51 crystals (Podust et al., 2001).

In spite of these limitations, our plan was to assess whether a relationship between sequence changes and characterized phenotypes of resistance to DMIs could be established in *Tapesia* spp. and whether some conservation of polymorphism positions through the fungal phylum could also be observed.

PCR cloning of the two cereal eyespot agents *T. acuformis* and *T. yallundae CYP51* gene revealed a high degree of polymorphism between these two species that were previously thought as two varieties of the same species. Differences that were confirmed to be species-related were found both in the two introns and in exons. In exons, it turned out to be at least eleven species-specific non silent variations at position 8, 85, 102, 103, 157, 168, 178, 180, 351, 387 and 496. The phenylalanine residue at position 180, as in *T. yallundae*, appeared well conserved in the fungal phylum making this a prioritized substitution for further investigation. Therefore site-directed mutagenesis and complementation experiments are needed to verify to what extent a leucine at position 180 could contribute to triazole resistance.

Concerning acquired resistance to triazoles and/or imidazoles DMIs, our results showed again a high degree of polymorphism without a clear correlation between distinct phenotypes and target-site mutations in the *CYP51* gene. This polymorphism is greater in *Tapesia* spp. than in other reported filamentous fungi. It is likely that all the mutations encountered in DMI-sensitive strains and also, presumably, some of the mutations found only in resistant strains reflect strain variations. Moreover, in roughly half of DMI-resistant field strains we did not detect any expressed mutation

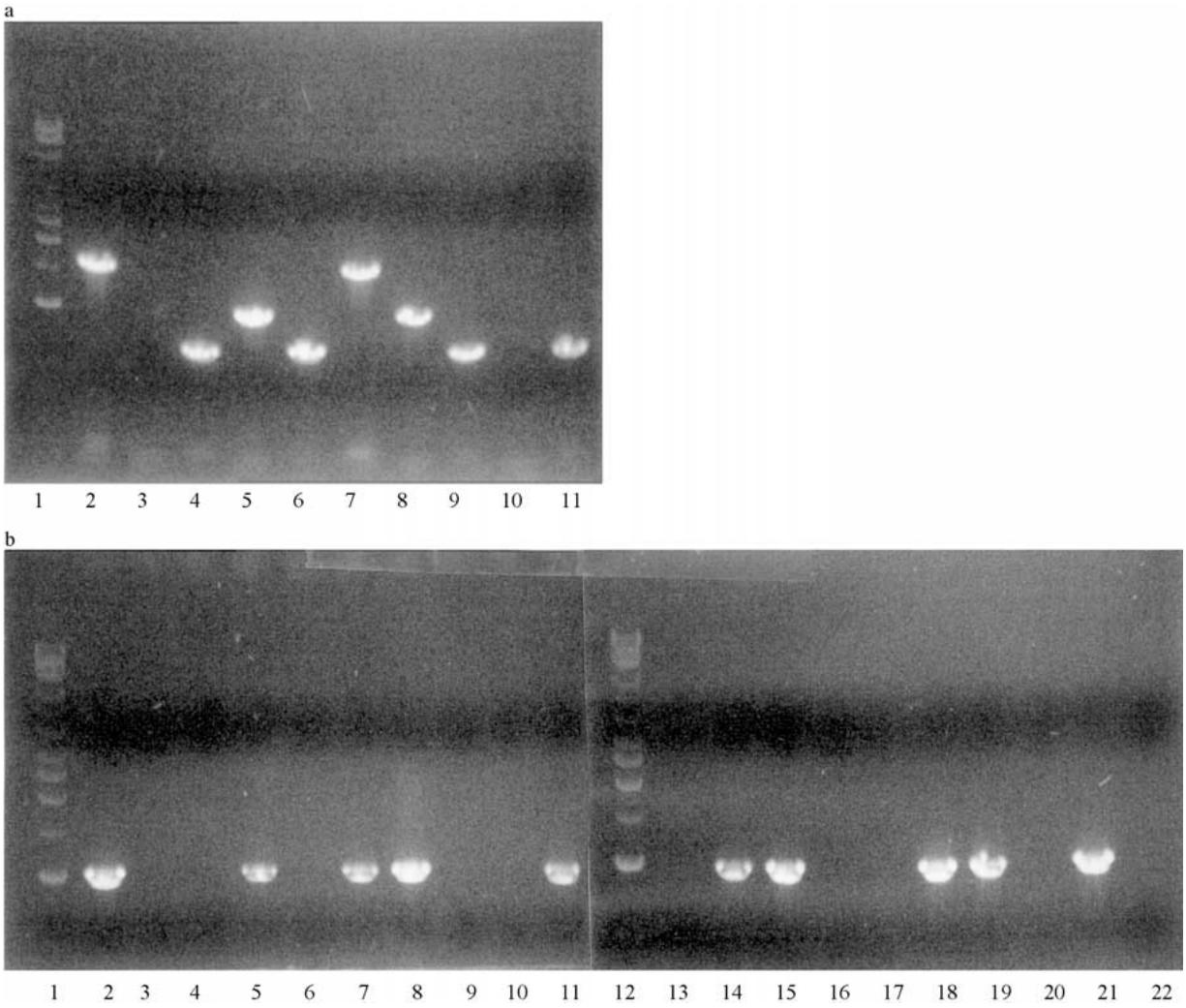

Figure 1. PCR products obtained by amplification of *Tapesia* species genomic DNA using species-specific primers ACU and YAL. (a) PCR amplification products obtained with primer pairs BEG/END (lanes 2 and 7), ACU/END (lanes 3 and 8), UCA/BEG (lanes 4 and 9), YAL/END (lanes 5 and 10) and LAY/BEG (lanes 6 and 11) from 84J1 strain (*T. yallundae*, lanes 2–6) and 94BE14T (*T. acuformis*, lanes 7–11) genomic DNA, lane 1: 1 kb ladder. Primers ACU and YAL are species-specific in 3′ while UCA and LAY are not (see Materials and methods). (b) PCR amplification products obtained with primer pairs ACU/END (lanes 2, 4, 6, 8, 10, 13, 15, 17, 19, 21) and YAL/END (lanes 3, 5, 7, 9, 11, 14, 16, 18, 20, 22) from different strains of *T. acuformis* (94N30T lanes 2–3, 94CA21T lanes 8–9, 94BR29B lanes 15–16, 94T7T lanes 19–20 and 85AG8 lanes 21–22) and *T. yallundae* (94AL24TR lanes 4–5, 94W19B lanes 6–7, 94M29T lanes 10–11, 94AL1M lanes 13–14 and 94AH12T lanes 17–18) genomic DNA, lanes 1 and 12: 1 kb ladder.

besides the species-related ones. Recently, another group working on *T. yallundae* resistance to prochloraz isolated two different alleles of *CYP51* which differed from each other by a A6T substitution as well as few differences in the sequence upstream of the start. However, they failed to find any correlation between sequence changes and alteration in fungicide sensitivity (Wood et al., 2001). These findings are quite different from what was reported in *P. italicum*, *U. necator* and *E. graminis* f. sp. *hordei* where the replacement of a tyrosine by a phenylalanine at position 136 was found to correlate with resistance to DMIs (de Waard, 1996; Délye et al., 1997b; Délye et al., 1998). This correlation, however, has not been confirmed to be

directly responsible for resistance in these organisms. Furthermore, it should be emphasized that *U. necator* resistance was directed against the triazole triadimenol and that different phenotypes, i.e., highly and moderately resistant, were equally concerned by such a mutation, as reported also for *P. italicum*, implying that the Y136F was not the sole mechanism of resistance in these two filamentous fungi. In *C. albicans*, the replacement of the tyrosine at the same position by a histidine, Y132H, has been confirmed to be involved in DMI resistance by site-directed mutagenesis followed by complementation. It has been shown that Y132H affected CYP51 enzyme affinity both for triazole and imidazole-ring DMIs. However, in *C. albicans* DMIs-resistant strains several other mutations (Loëffler et al., 1997; Sanglard et al., 1998; Marichal et al., 1999), each involved in resistance, were usually found simultaneously and/or in association with other mechanisms such as overexpression of *CYP51* mRNA and/or multidrug transporters, therefore demonstrating the polygenic nature of resistance to DMIs at least in that yeast. Overexpression of *CYP51* wild-type gene has recently been also found related to DMIs resistance in *Penicillium digitatum* (Hamamoto et al., 2000) and in *Venturia inaequalis* (Schnabel and jones, 2001). Analysis of *CYP51* gene expression should therefore be performed, as it would be of interest to check whether the numerous differences we observed between the two *Tapesia* species in sequences upstream of the start might be linked to differences in expression regulation or not.

We cannot exclude that the few mutations that were observed only in some *Tapesia* DMI-resistant strains and located at positions which have not yet been reported, i.e., A29P; S35T; Q43H; D78Y; E106K; Q167H; N244S; Y486H, could not be involved in resistance. However, most of these changes are located in the N-terminal region of the protein that is likely to be buried into the membrane (Marichal et al., 1999) and could reflect only strain variations. As the C terminal part of the protein seems to be of greater importance for DMIs resistance (Marichal et al., 1999; Podust et al., 2001), the Y486H mutation could play a role in altering antifungal binding either directly or not. It is possible, also, that multiple mutations, as we found in a few strains, could synergically affect interactions between antifungal drugs and *CYP51*. More experiments are therefore needed to investigate the possible involvement in resistance of these mutations both separately and simultaneously.

On the other hand, we took advantage of specific polymorphisms to design species-specific primers that could allow rapid, easy and unambiguous distinction between field strains of the two species. Previous tests relied upon ribosomal DNA internal transcribed spacers variations and were based either on RFLP techniques or on a combination of PCR and restriction enzymes treatment (Poupard et al., 1993; Gac et al., 1996). Our assay might be a useful complement as it is based on a *CYP51* signature that allows to discriminate *Tapesia* strains having triazole natural resistance from strains that do not have this phenotype.

In another work dealing with the *Botrytis cinerea CYP51* gene, we have also found several variations enabling us, in addition with morphological observations, to distinguish two different genetic entities in *B. cinerea* that might form two different species (Albertini et al., in press). Therefore, we believe that *CYP51* polymorphism could constitute a general tool to identify closely related fungal species.

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