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Paralog Re-Emergence: A Novel, Historically Contingent Mechanism in the Evolution of Antimicrobial Resistance

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Abstract

Evolution of resistance to drugs and pesticides poses a serious threat to human health and agricultural production. *CYP51* encodes the target site of azole fungicides, widely used clinically and in agriculture. Azole resistance can evolve due to point mutations or overexpression of *CYP51*, and previous studies have shown that fungicide-resistant alleles have arisen by de novo mutation. Paralogs *CYP51A* and *CYP51B* are found in filamentous ascomycetes, but *CYP51A* has been lost from multiple lineages. Here, we show that in the barley pathogen *Rhynchosporium commune*, re-emergence of *CYP51A* constitutes a novel mechanism for the evolution of resistance to azoles. Pyrosequencing analysis of historical barley leaf samples from a unique long-term experiment from 1892 to 2008 indicates that the majority of the *R. commune* population lacked *CYP51A* until 1985, after which the frequency of *CYP51A* rapidly increased. Functional analysis demonstrates that *CYP51A* retains the same substrate as *CYP51B*, but with different transcriptional regulation. Phylogenetic analyses show that the origin of *CYP51A* far predates azole use, and newly sequenced *Rhynchosporium* genomes show *CYP51A* persisting in the *R. commune* lineage rather than being regained by horizontal gene transfer; therefore, *CYP51A* re-emergence provides an example of adaptation to novel compounds by selection from standing genetic variation.

Key words: **standing variation, gene duplication, resistance, fungicides, triazoles, *Rhynchosporium*.**

Introduction

Resistance to drugs and pesticides is a major challenge in medicine (McKenna 2013) and agriculture (Heckel 2012). Understanding the evolutionary mechanisms involved in the emergence and spread of resistance can aid resistance risk assessment and management (MacLean et al. 2010). One relevant evolutionary question is the extent to which populations adapt to novel environments, such as a new biocide, through de novo mutations or through selection from standing genetic variation, as this will affect the probability and speed of emergence of resistant alleles (Hermisson and Pennings 2005). Previous studies have indicated de novo origins for fungicide resistance mutations (Torriani et al. 2009; Camps et al. 2012). Selection from standing variation can be inferred from the population genetic signature of selection, or by tracing the history of the selected allele (Barrett and Schluter 2008). In this study, we have taken the second approach, following the evolutionary history of *CYP51A* in *Rhynchosporium commune* through comparative genomics and analysis of historical infected plant samples.

Duplicate genes have long been considered an important source of genetic adaptive potential (Ohno 1970; Zhang 2003; Dittmar and Liberles 2010). Extensive research has considered the origin and fate of duplicated genes, but focusing on the likelihood of emergence of copy number variants (Lynch and Conery 2000); loss or change of function through pseudogenization, neofunctionalization, or subfunctionalization (Conant and Wolfe 2008; Innan and Kondrashov 2010); and mechanisms of functional divergence before or after duplication (Hughes 1994; Näsvall et al. 2012); around the time of duplication under given conditions of selection and drift, rather than future changes if selective pressures alter. In this study, the use of historical samples enables changes in duplicate gene frequency over time to be measured directly, revealing responses to changes in selection.

The azoles are widely used as both clinical and agricultural fungicides (Kelly et al. 1993). Clinically, the importance of antifungal therapies has increased as HIV, transplant antirejection drugs, and some cancer treatments leave patients immunocompromised and vulnerable to fungal infections (Denning and Hope 2010). In agriculture, crop diseases result in yield losses of 10–15% of global food production (Oerke and Dehne 2004; Strange and Scott 2005), a continual obstacle to feeding a growing population. In 2005, azoles represented 25.3% of the $8.9 billion global agricultural fungicide

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market (Morton and Staub 2008). The target site of the azoles is fungal CYP51. CYP51 is a cytochrome P450, sterol 14α-demethylase, involved in the biosynthesis of ergosterol in fungi, cholesterol in mammals, and sitosterol in plants (Lepesheva and Waterman 2007).

Recently, some ascomycete fungi have been found to possess multiple *CYP51* paralogs, with two paralogs, *CYP51A* and *CYP51B*, in *Aspergillus fumigatus* (Mellado et al. 2001), *A. nidulans* (Da Silva Ferreira et al. 2005), and *Magnaporthe oryzae* (Yan et al. 2011), and three in *A. oryzae* (Da Silva Ferreira et al. 2005) and *Fusarium* spp. (Deng et al. 2007). The reasons why some species possess multiple *CYP51* paralogs, and others only one, are not yet known. Knocking out the *CYP51A* paralog from wild-type isolates of *A. fumigatus* (Mellado et al. 2005) and *Fusarium graminearum* (Fan et al. 2013) resulted in increased azole sensitivity, suggesting that the presence of additional *CYP51* paralogs can confer reduced intrinsic azole sensitivity.

Acquired reductions in sensitivity to azoles may result from mutations or overexpression of *CYP51*. Various point mutations, with quantitative, interacting effects on azole sensitivity, have been reported in the single *CYP51* paralogs of the clinical pathogen *Candida albicans* (Morio et al. 2010) and the wheat pathogen *Mycosphaerella graminicola* (Cools et al. 2010). Reduced azole sensitivity due to constitutive overexpression of *CYP51* was first reported in citrus green mould, *Penicillium digitatum* (Hamamoto et al. 2000). A combination of promoter changes resulting in overexpression and point mutations in the coding sequence has been reported in the *CYP51A* paralog in clinical isolates of *A. fumigatus* (Mellado et al. 2007). Nontarget-site mechanisms, such as enhanced efflux (de Waard and van Nistelrooy 1980) or altered sterol metabolism (Kelly et al. 1995), may also reduce azole sensitivity. Reduced sensitivity to azoles has now been reported in the field or in field isolates of over 20 plant pathogens (Fungicide Resistance Action Committee 2013, www.frac.info, last accessed April 16, 2014).

Rhynchosporium commune Zaffarano, McDonald, and Linde is an ascomycete fungal pathogen causing barley leaf blotch or scald. Previously known as *R. secalis* (Oud.) Davis (Caldwell 1937), it has recently been shown that *R. secalis s.l.* comprises three distinct host-specialized lineages, with *R. secalis s.s.* now referring only to the rye-infecting species, *R. agropyri* infecting *Agropyron* spp. and *R. commune* infecting barley (Zaffarano et al. 2011). *Rhynchosporium commune* causes necrotic lesions on infected leaves, resulting in grain yield reductions of up to 30% if untreated (Mayfield and Clare 1991). Although some barley varieties provide partial resistance, control is still heavily reliant on fungicides. In 2010, winter barley crops in the United Kingdom received an average of 2.1 fungicide applications, with 98.5% of crop area treated, and 54% of treatments containing an azole (Garthwaite et al. 2011).

The first azole to be used on barley in the United Kingdom was introduced in the mid-1970s. By 1985, surveys of azole sensitivity in *R. commune* revealed a 5- to 10-fold decrease in sensitivity to the earlier azoles relative to baseline testing in 1975–1981 (Kendall et al. 1993). Since the introduction of epoxiconazole in the late 1990s and prothioconazole in the mid-2000s, further sensitivity shifts have affected these compounds, but they retain some effectiveness in the field (Oxley et al. 2008). The mechanisms responsible for these azole sensitivity shifts in *R. commune* were not known.

The classical experiments at Rothamsted Research in Hertfordshire, UK, are field experiments studying the long-term effects of different agricultural practices and other factors affecting crop production and the environment. Broadbalk is the world's oldest continually running agricultural experiment, growing winter wheat since 1843, and the Hoosfield spring barley experiment started 9 years later. Throughout the history of the experiment, plant and soil samples have been retained in the Rothamsted Archive. This enables the use of new analytical techniques on historical samples, such as quantitative polymerase chain reaction (qPCR) to quantify pathogen levels (Bearchell et al. 2005), or pyrosequencing to quantify alleles within populations (Gruber et al. 2002).

Rhynchosporium commune isolates were characterized for sensitivity to four azoles, and a sensitivity shift was confirmed. Two *CYP51* genes were sequenced from *R. commune* and shown to be paralogs *CYP51A* and *CYP51B* (sensu Mellado et al. 2001), along with a pseudogene, *CYP51A-p*. The azole sensitivity shift was not correlated with point mutations or constitutive overexpression of either paralog, but *CYP51A* was present only in less sensitive isolates. *CYP51A* was upregulated in response to tebuconazole and complements yeast *CYP51*; therefore, *CYP51A*, where present, encodes an additional sterol 14-α demethylase with inducible overexpression. Phylogenetic analysis of fungal *CYP51*s indicates that *CYP51A* and *CYP51B* originate from a gene duplication event basal to the filamentous ascomycetes, followed by multiple losses of *CYP51A*. Pyrosequencing analysis of samples from the Hoosfield long-term spring barley experiment revealed that *CYP51A* had declined in the *R. commune* population by the start of the 20th century, but re-emerged in the mid-1980s under selection by azoles.

Results

Ascomycete *CYP51* Paralogs

CYP51 paralogs from fungal genomes on the Broad Institute server, along with *R. commune CYP51* genes and pseudogene, are shown in supplementary figure S1, Supplementary Material online, with gene names according to Nelson (2009). The maximum-likelihood phylogenetic tree is summarized in figure 1 and shown in full in supplementary figure S1, Supplementary Material online. Basidiomycetes and hemiascomycete yeasts each possess a single *CYP51* paralog, apart from an independent duplication in *Coprinopsis cinerea*. *CYP51* gene duplication took place around the origin of the filamentous ascomycetes, forming clades *CYP51A*, *CYP51B*, and *CYP51C*. All species studied have retained a *CYP51B* paralog, but *CYP51A* has been lost from multiple lineages, and *CYP51C* is found only in *Fusarium* spp. Further duplications of *CYP51A* and *CYP51B* have taken place in some *Aspergillus* species. The *A. flavus* "*CYP51C*" described by Liu et al.

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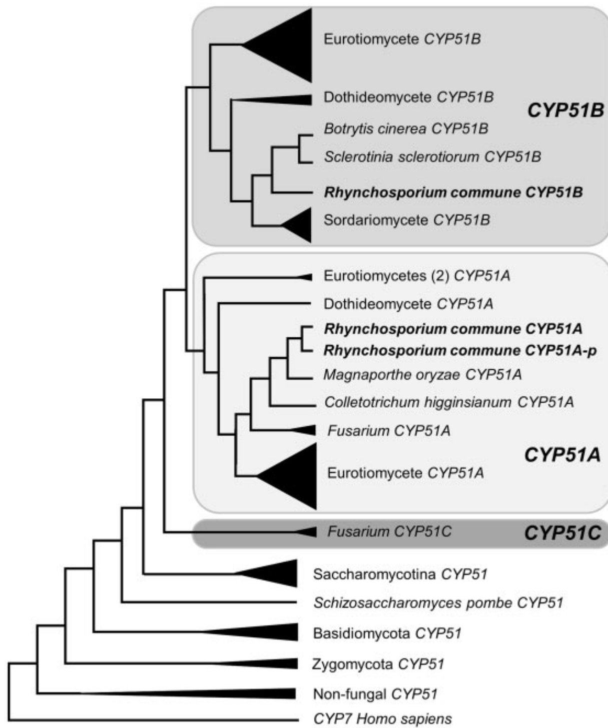

FIG. 1. Summary cladogram of maximum-likelihood phylogeny of coding nucleotide sequences of fungal and outgroup *CYP51* genes, rooted with a *CYP7* outgroup. The full phylogeny is shown in supplementary figure S1, Supplementary Material online.

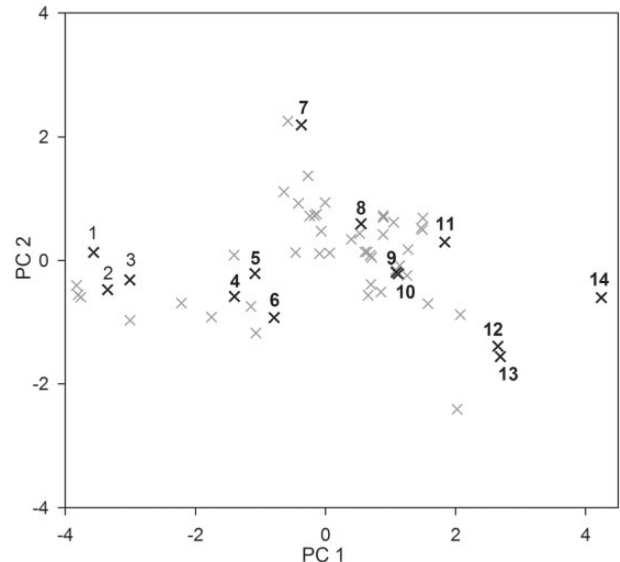

FIG. 2. Biplot of principal component 2 against principal component 1 from PCA of triazole $\log_{10}[EC_{50}]$ data for 61 *Rhynchosporium commune* isolates. Percentage variation and loadings are listed in supplementary table S3, Supplementary Material online. Black crosses indicate isolates selected for sequence analysis. 1: RS 219; 2: K1124; 3: FI12-63; 4: R 9528.4; 5: R 9522.3; 6: QUB 30-10; 7: GKII 18-3-2; 8: GKII 18-2-3; 9: SAC 0003 1.4.8; 10: 788; 11: SAC 09/943/14; 12: QUB 12-3; 13: OSA 28-2-2; 14: RS 783. Bold numbers indicate isolates with a functional *CYP51A*.

(2012) appears from this phylogeny to be a second *CYP51A*. One *R. commune CYP51* falls within the *CYP51B* clade, where its closest relatives are *Botrytis cinerea* and *Sclerotinia sclerotiorum CYP51B*. The other *R. commune CYP51* falls within the *CYP51A* clade, where its closest relatives are sordariomycetes including *M. oryzae* as the sequenced leotiomycetes lack *CYP51A*. The *R. commune CYP51* pseudogene is sister to *R. commune CYP51A*, and is therefore referred to as *CYP51A-p*.

Triazole Sensitivity in *R. commune*

Sensitivity of 57 *R. commune* isolates to the azole fungicides propiconazole, tebuconazole, epoxiconazole, and prothioconazole was measured by in vitro bioassay (supplementary table S2, Supplementary Material online). Triazoles demonstrate partial cross-resistance, so a principal component analysis (PCA) was carried out (fig. 2). 75.4% of variance was encompassed by PC1, representing overall positive cross-resistance against the four triazoles, with all loadings positive. A further 17.2% of variance was encompassed by PC2, with positive loadings for propiconazole and tebuconazole but a negative loading for prothioconazole, showing incomplete cross-resistance between prothioconazole and the other azoles. The most sensitive group of isolates can be separated from the others by PC1 alone, indicating an initial sensitivity shift conferring positive cross-resistance against all four azoles tested. A further shift from intermediate to less-sensitive isolates, and further variation within the less-sensitive group, involves variation in PC2 as well, so this shift shows only partial cross-resistance, with prothioconazole affected differently from the other compounds.

Isolates from each sensitivity group were selected for molecular analysis. Sensitivity profiles for these isolates are shown in figure 3. *CYP51A* and *CYP51B* were sequenced for these isolates (GenBank accession numbers KF753641–KF753674). No single nucleotide polymorphisms (SNPs) correlated with azole sensitivity, but functional *CYP51A* sequences were obtained from the intermediate and less-sensitive isolates, whereas a pseudogene, with a frameshift indel and premature stop codons, was sequenced from the sensitive isolates.

A polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay was developed to distinguish *CYP51A* from *CYP51A-p*. This revealed that sensitive isolates possess *CYP51A-p* but not *CYP51A*, whereas intermediate and less sensitive isolates possess both *CYP51A* and *CYP51A-p* (supplementary fig. S2, Supplementary Material online). This difference in *CYP51A* copy number was confirmed by Southern blot (supplementary fig. S2, Supplementary Material online). *CYP51A-p* was sequenced from additional isolates (GenBank accession numbers KF753666–KF753674) and all contained premature stop codons.

Rhynchosporium commune CYP51A Expression

To test whether *R. commune CYP51A* still functions as a sterol 14α-demethylase, *R. commune CYP51A* and *CYP51B* were expressed in a *Saccharomyces cerevisiae* strain in which the native *CYP51* is under the control of a tetracycline-repressible

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promoter. *Rhynchosporium commune CYP51A* and *CYP51B* were each able to complement *S. cerevisiae CYP51* (fig. 4). Yeast transformants were then tested for triazole sensitivity. There was little difference in fungicide sensitivity, to triazoles or to the non-DMI cycloheximide, between the *CYP51A* and *CYP51B* transformants (fig. 4*b*).

Expression analysis of *R. commune CYP51A* and *CYP51B* was carried out. No differences in constitutive expression of either paralog were correlated with azole sensitivity. However, following the addition of tebuconazole, *CYP51B* was only upregulated around 5- to 10-fold if at all, whereas *CYP51A*, where present, was upregulated around 100-fold. Therefore, *CYP51A* expression is more inducible in response to the effects of azoles than *CYP51B* (fig. 5).

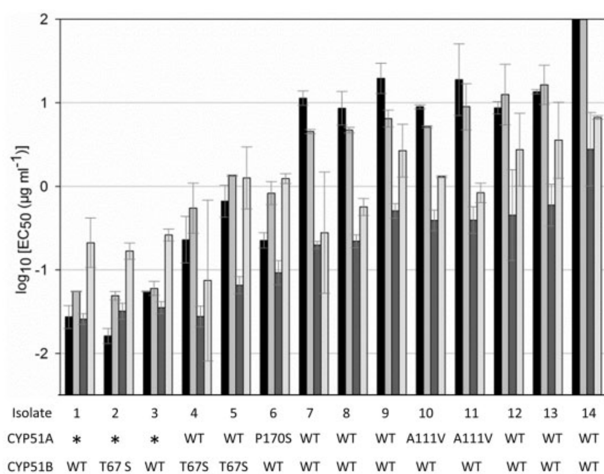

Fig. 3. Azole $\log_{10}[EC_{50}]$ values of *Rhynchosporium commune* isolates selected for target-site sequence analysis, and CYP51 amino acid substitutions. Black: propiconazole, gray: tebuconazole, dark gray: epoxiconazole, light gray: prothioconazole. Error bars indicate standard error between biological replicates. 1: RS 219; 2: K1124; 3: FI12-63; 4: R 9528.4; 5: R 9522.3; 6: QUB 30-10; 7: GKII 18-3-2; 8: GKII 18-2-3; 9: SAC 0003 1.4.8; 10: 788; 11: SAC 09/943/14; 12: QUB 12-3; 13: OSA 28-2-2; 14: RS 783. WT, wild type, identical to reference sequences from isolate 1130 (GenBank accession numbers KF753639 and KF753640); *no functional *CYP51A* sequenced.

Hoosfield Spring Barley Archive

Amplification of a 52-bp fragment of a reference gene (*R. commune β-tubulin*) was successful from samples from 33 different years, from 1892 to 2012. The *CYP51A* nested PCR amplified products from *R. secalis s.l.* and not from *R. orthosporum*. All *R. secalis s.l.* DNA present in these samples was confirmed as *R. commune* with 5% detection limit, except 2012, which was excluded from further testing.

A pyrosequencing assay was developed to distinguish *R. commune CYP51A* from *CYP51A-p* (fig. 6). For most of the 20th century, the majority of the *R. commune* population at the Hoosfield experimental site lacked *CYP51A*, but in 1985, levels of *CYP51A* rapidly increased, and subsequently the majority of the *R. commune* population possesses *CYP51A*. Logit-transformed percentage data were analyzed with a Tukey multiple-comparison test. Levels of functional *CYP51A* in each year from 1985 onwards are significantly higher than in each year from 1892 to 1983. No year up to 1983 has significantly different levels of functional *CYP51A* from control isolates K1124 and FI12-63, which have only *CYP51A-p*, whereas no year from 1985 onwards has significantly different levels of *CYP51A* from isolates 788 and R9528.4, which have both *CYP51A* and *CYP51A-p* (supplementary table S4, Supplementary Material online). A further replicate DNA extraction from the homogenized plant material gave similar results (supplementary table S4, Supplementary Material online).

CYP51A in *Rhynchosporium* Species

The genomes of three *R. commune* isolates, and one isolate each of *R. agropyri*, *R. secalis s.s.*, and *R. orthosporum*, sister species to the *R. secalis s.l.* complex, have been sequenced. The genomes were searched for *CYP51* paralogs (GenBank accession numbers KF753675–KF753687). All contain a single *CYP51B*. Two *R. commune* isolates contain *CYP51A-p* only, and one isolate contains *CYP51A* and *CYP51A-p*.

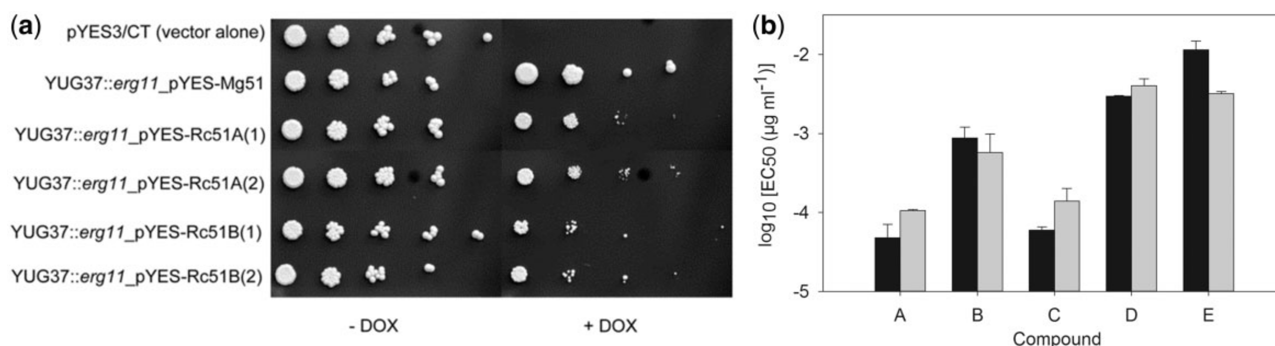

Fig. 4. (*a*) Growth of *Saccharomyces cerevisiae* strain YUG37::*erg11* transformed with pYES3/CT (vector only) or pYES3-CT with *Mycosphaerella graminicola CYP51* (Mg51), *Rhynchosporium commune CYP51A* (Rc51A, two transformants) or *R. commune CYP51B* (Rc51B, two transformants) on SD + GAL + RAF agar, with and without 3 $\mu g\,ml^{-1}$ doxycycline (DOX), with six inoculum concentrations of 1.25×10^6, 2.5×10^5, 5×10^4, 1×10^4, 2×10^3, and 4×10^2 cells μl^{-1}. DOX represses expression of the native *S. cerevisiae CYP51* in strain YUG37::*erg11*. (*b*) Triazole and cycloheximide $\log_{10}[EC_{50}]$ values of yeast transformants *S. cerevisiae* strain YUG37::*erg11* transformed with *R. commune CYP51A* (black) or *R. commune CYP51B* (gray). A: propiconazole, B: tebuconazole, C: epoxiconazole, D: prothioconazole, E: cycloheximide. Error bars indicate standard error of biological replicates.

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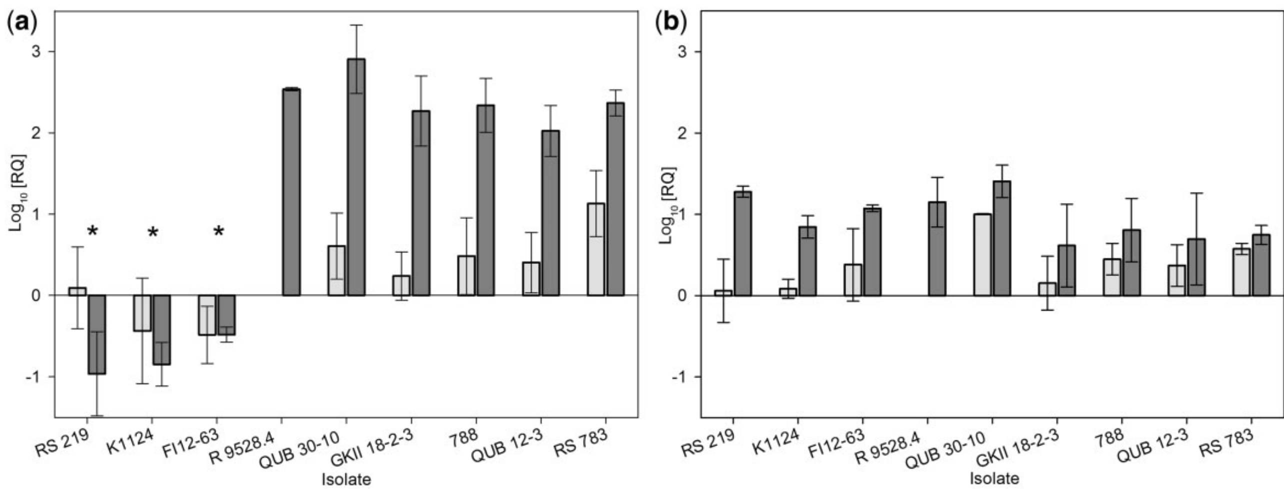

Fig. 5. Expression of (*a*) *CYP51A* and (b) *CYP51B* in *Rhynchosporium commune*, relative to isolate R 9528.4 constitutive expression, with *β-tubulin* as endogenous control. Light gray: constitutive expression, without fungicide; dark gray: induced expression, following the addition of 1 µg ml^{-1} tebuconazole. Error bars indicate standard error of biological replicates. *CYP51A not present in these isolates: A low level of amplicon results from nonspecific amplification of *CYP51A-p*.

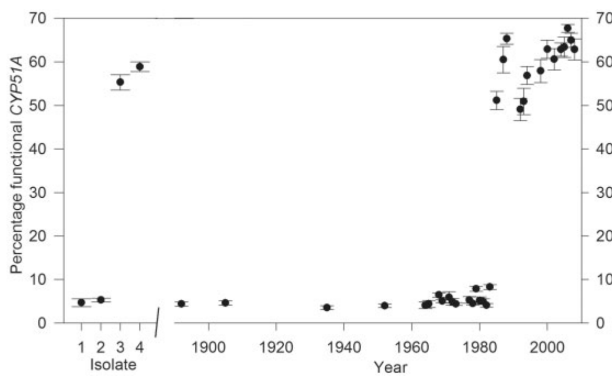

Fig. 6. Percentage of functional *CYP51A* in *Rhynchosporium commune* isolates, and populations sampled from the Hoosfield experiment at Rothamsted Research, UK. Isolate 1: K1124; 2: FI12-63; 3: 788; 4: R 9528.4. Isolates K1124 and FI12-63 contain *CYP51A-p* only; isolates 788 and R 9528.4 contain both *CYP51A-p* and functional *CYP51A*. Error bars indicate standard error of five technical replicates.

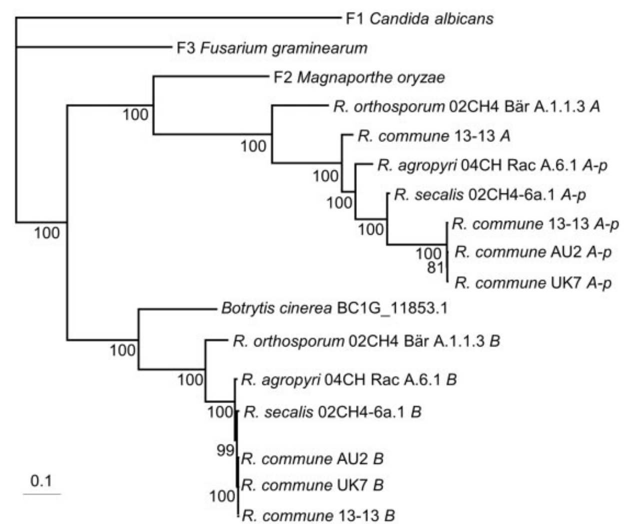

Fig. 7. Maximum-likelihood phylogeny of coding nucleotide sequences of *CYP51* paralogs sequenced from *Rhynchosporium* species, with *Magnaporthe oryzae CYP51A*, *Botrytis cinerea CYP51B*, and *Fusarium graminearum CYP51C*. Rooted with *Candida albicans CYP51* outgroup. Node labels indicate bootstrap support.

Rhynchosporium agropyri and *R. secalis s.s.* contain a *CYP51A* pseudogene, but with different stop codons from *R. commune CYP51A-p*, whereas *R. orthosporum* contained only a functional *CYP51A*. Therefore, a functional *CYP51A* was present in the most recent common ancestor of *R. orthosporum* and *R. commune*, followed by duplication of *CYP51A* and pseudogenization of one copy in the *R. commune* lineage, and loss of the functional copy from some isolates (fig. 7).

Discussion

A 10-fold shift in azole sensitivity in *R. commune* is correlated with the presence of the *CYP51A* paralog. All isolates possess *CYP51B* and a *CYP51A-p* pseudogene, but the functional *CYP51A* is absent from azole sensitive isolates and present in less-sensitive isolates. Further selection for reduced azole sensitivity among isolates with *CYP51A* has resulted in a further 10-fold difference in sensitivity to some azoles, which is not correlated with mutations or overexpression of either *CYP51* paralog, implying that an additional, nontarget-site mechanism is responsible for this second shift.

Analysis of *R. commune* populations in barley samples from Hertfordshire, UK, shows an increase in *CYP51A* frequency in the population from 1985, with *CYP51A* present in the majority of the population thereafter. Similarly, the oldest studied isolates lack *CYP51A*, whereas most modern UK isolates have *CYP51A*. This correlates with a shift in triazole sensitivity reported in UK field populations between baseline monitoring from 1975 to 1981 and further surveys from 1985 onwards (Kendall et al. 1993). This demonstrates the value of the Rothamsted Classical Experiment archives to analyze past evolutionary changes involving mutations which have only now been identified.

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The mechanism by which *CYP51A* reduces azole sensitivity appears to be inducible target-site overexpression. *Rhynchosporium commune CYP51A* is able to complement *S. cerevisiae CYP51*, so it is a functional sterol 14α-demethylase, and in *R. commune*, *CYP51A* is upregulated following exposure to an azole, so *CYP51A* may be able to maintain *CYP51* function when *CYP51B* is inhibited by azoles. Previously reported cases of reduced azole sensitivity due to target-site overexpression have all involved constitutive overexpression of an existing paralog due to promoter inserts or gene duplication. Acquired reductions in azole sensitivity due to *CYP51* duplication have been reported due to chromosome duplication in *C. glabrata* (Marichal et al. 1997); isochromosome formation, with duplication of chromosome arm 5L containing *CYP51*, two efflux pump encoding genes and one ABC transporter transcription factor, in *C. albicans* (Selmecki et al. 2006); and disomy of chromosome 1, containing *CYP51* and ABC transporter encoding gene *AFR1*, in *Cryptococcus neoformans* (Sionov et al. 2010). However, in all of these cases, this involved duplication of an existing gene, producing a second identical copy rather than a functionally divergent paralog. *CYP51A* demonstrates functional divergence from *CYP51B* through different patterns of transcriptional regulation, including greater upregulation in response to azoles. Neofunctionalization of fungal genes often involves changes in transcriptional regulation rather than changes in substrate (Skamnioti et al. 2008), and often altered expression evolves primarily in one copy whereas the other retains the original pattern (Gu et al. 2005). Intrinsic sequence differences between *CYP51A* and *CYP51B* contribute to reduced intrinsic azole sensitivity in *A. fumigatus* (Martel et al. 2010) and *F. graminearum* (Fan et al. 2013), but *R. commune CYP51A* conferred similar azole sensitivity to *CYP51B* when expressed in yeast, suggesting that upregulation of *CYP51A*, rather than reduced azole binding due to intrinsic sequence differences, is the main mechanism of reduced azole sensitivity in *R. commune* isolates possessing *CYP51A*.

Although *CYP51* paralogs have been extensively studied in a few species, such as *A. fumigatus* where *CYP51A* mutations confer azole resistance, in others species, knowledge of *CYP51* paralog presence is based only on a single isolate selected for genome sequencing. Therefore, genome resequencing of multiple isolates may reveal paralog presence polymorphisms in other species, and possible multiple *CYP51* paralogs should be considered in future when investigating azole sensitivity shifts. Further investigation is also needed concerning the reasons for the emergence of *CYP51A*, and its retention in some fungal lineages, in the absence of selection by azoles. *CYP51A* expression is induced in *CYP51B* deletion mutants (Fan et al. 2013), as well as following *CYP51* inhibition by azoles, suggesting that *CYP51A* is upregulated in response to disruption of ergosterol biosynthesis. Therefore, presence of this inducible *CYP51*, in addition to the constitutively expressed *CYP51B* found in all filamentous ascomycetes, would allow a fungus to respond to fluctuating ergosterol requirements, but it is not yet clear which aspects of fungal biology and lifestyle make this necessary in some species and not others.

Phylogenetic analysis places *R. commune CYP51A* in a monophyletic clade with other filamentous ascomycete *CYP51A*s, which diverged from *CYP51B* around the origin of the filamentous ascomycetes, 320–520 Ma (Lücking et al. 2009). This indicates a single origin of *CYP51A* followed by multiple losses, rather than an independent recent origin in *R. commune*. However, the origin of *CYP51A-p*, and partial loss of *CYP51A*, in the *R. commune* lineage took place far more recently. *Rhynchosporium orthosporum* possesses a functional *CYP51A* and not a pseudogene, whereas the sequenced isolates of *R. agropyri* and *R. secalis s.s.* possess only a pseudogene. The *R. orthosporum CYP51A* forms a sister group to *R. commune CYP51A* and *CYP51A-p*. Therefore, *CYP51A* duplication took place in the *R. commune* lineage after it diverged from *R. orthosporum*, 14.5–35 ka BP (Zaffarano et al. 2008), but before the divergence of *R. agropyri*, *R. secalis s.s.*, and *R. commune* from their most recent common ancestor, 1.3–3.6 ka BP (Zaffarano et al. 2008). This was followed in the *R. commune* lineage by pseudogenization of one copy, and loss of the functional copy in some of the population. Further study of *R. agropyri* and *R. secalis s.s.* would be needed to ascertain whether *CYP51A* pseudogenization occurred independently in these lineages or once in the *R. secalis s.l.* common ancestor, and whether any isolates of these species possess a functional *CYP51A*. The gene phylogeny confirms that *CYP51A* was retained within the *R. commune* lineage, and not regained by horizontal gene transfer, in contrast to the acquisition of antibiotic resistance genes in clinical bacteria by horizontal gene transfer from soil bacteria previously exposed to naturally occurring microbial antibiotics (Forsberg et al. 2012).

Having declined in the *R. commune* population, *CYP51A* later re-emerged under selection by azoles in the late 20th century. Most research into the origin and fate of duplicate genes considers possible outcomes in terms of loss or fixation in a species, and it has been demonstrated that most intraspecific gene copy number variations result from very recent duplication events, as older duplicates have already been lost or fixed, and most established, diverged duplicates are maintained by purifying selection (Schrider and Hahn 2010). Comparative genomics has revealed cases of fixed paralog losses following a change in selection in some taxa, such as repeated gene losses from the olfactory receptor gene family in microsmic mammals (Rouquier et al. 2000), or extensive gene losses in obligate parasites (Spanu et al. 2010). However, the Rothamsted archive samples, covering a time frame including a known change in selection, provide the temporal resolution to detect changes in paralog frequency over time, enabling us to detect the previous near-loss of a paralog followed by its re-emergence due to a further change in selection.

Furthermore, as phylogenetic analyses have shown that the functional *CYP51A* in *R. commune* originated long before azole use, its re-emergence provides an example of an adaptation to a new environment that has been selected from standing genetic variation rather than arising from a de novo mutation (Barrett and Schluter 2008), whereas previous studies of other fungicide resistance alleles have pointed to de novo mutational origins (Torriani et al. 2009; Camps et al.

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2012). Although ancient origins have previously been demonstrated for resistance genes to antibiotics based on natural microbial products (D'Costa et al. 2011), the triazoles are synthetic in origin with no know natural analogs and therefore their use imposes a previously unencountered selective pressure. Standing variation may allow resistance to evolve more quickly, or may result in increased probability of fixation where the fitness benefit is smaller and a de novo mutation may have been lost through genetic drift (Hermisson and Pennings 2005). Furthermore, as this standing variation comprises a trait that has been lost from multiple ascomycete lineages, the availability of *CYP51A* re-emergence as an adaptive pathway is historically contingent upon *CYP51A* still having been present at low levels in the population at the point when selection pressures altered due to anthropogenic fungicide use.

Materials and Methods

Bioinformatic and Phylogenetic Analysis

CYP51 sequences were obtained from fungal genomes on the Broad Institute server (http://www.broadinstitute.org/science/data, last accessed April 16, 2014), along with *Mycobacterium tuberculosis*, *Trypanosoma cruzi*, *Homo sapiens*, *Arabidopsis thaliana CYP51G1*, and *Mus musculus CYP51*, with *Mus musculus CYP7A1* as an outgroup (Aoyama et al. 1996). A text search was carried out for annotated *CYP51* genes, followed by BLAST searches against each genome with *S. cerevisiae CYP51*, and with *CYP51A* and *CYP51C* from the nearest available relative for species where these paralogs were not found. Start sites, introns, and stop sites were checked manually, and introns removed as they were too variable to align unambiguously. Predicted amino acid sequences were aligned using M-Coffee (Notredame et al. 2000), and the corresponding coding nucleotide alignment generated using PAL2NAL (Suyama et al. 2006). Model selection was carried out in jModelTest 0.1.1 (Posada 2008), selecting by AICc. Maximum-likelihood phylogenetic analysis was carried out in PhyML (Guindon and Gascuel 2003), implemented through the TOPALi v2.5 platform (Milne et al. 2009), using a TVM + I + G model, with 100 bootstrap runs.

Fungicide Sensitivity Testing

Isolates used are listed in supplementary table S1, Supplementary Material online. Isolates had been stored as spore suspensions on silica gel at −80 °C. Isolates were grown on Czapek dox agar with 0.5% mycological peptone, with an inoculum density of 1.25×10^5 spores per 90 mm Petri dish, at 18 °C for 10 days. Sensitivity testing was carried out as described by Pijls et al. (1994), using Sabouraud medium, *R. commune* spores at 1.25×10^5 spores ml^{-1}, and the following technical-grade fungicides: epoxiconazole, 50 $\mu g\,ml^{-1}$ with 2.5-fold dilutions; prothioconazole, propiconazole, tebuconazole, 100 $\mu g\,ml^{-1}$ with 3-fold dilutions, repeated with 300 $\mu g\,ml^{-1}$ for less sensitive isolates. Plates were incubated at 18 °C for 7 days, then fungal growth was measured by optical absorbance at 620 nm using an Optima Fluostar plate reader (BMG Labtech, Germany), in well-scanning mode with a 4×4 matrix of scanning points within a 3-mm diameter. The accompanying software was used to fit a dose–response curve (4-parameter fit) and calculate EC_{50} values. Cross-resistance was assessed by PCA using the correlation matrix of $\log_{10}$ transformed EC_{50}s for the four fungicides, in GenStat 14th Edition (VSN International, Hertfordshire, UK).

CYP51 Sequencing

Initial fragments were amplified using degenerate primers CYP51 3F and CYP51A CIGEK rv for *CYP51A*, and DMIRES F1 and 14DM R2 for *CYP51B* (supplementary table S3, Supplementary Material online). The remainder of each gene (GenBank accession numbers KF753639–KF753640) was found by genome-walking using the GenomeWalker Universal Kit (Clontech, CA). Libraries were prepared from isolate 1130 (Rohel et al. 1998) genomic DNA, with restriction enzymes *DraI*, *PvuII*, *EcoRV*, and *StuI*. Three reactions in the 5′-direction and one in the 3′-direction were needed to obtain the complete coding sequence of *CYP51A*, and two reactions in the 5′-direction and one in the 3′-direction for *CYP51B*. Genome-specific primers (GSP1 and GSP2) used in successive reactions are listed in supplementary table S3, Supplementary Material online. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System, ligated into pGEM-T Easy plasmid vector using T4 DNA ligase and cloned in JM109 competent cells (all Promega). Plasmid DNA was extracted and purified using the GeneElute (Sigma) or QIASpin (Qiagen) mini-prep kits, then sequenced by Eurofins MWG (Germany) using sequencing primers M13uni and M13rev (Messing 1983) and the sequences assembled in Vector NTI 10 (Invitrogen, CA).

CYP51B, *CYP51A*, and *CYP51A-p* were sequenced from isolates with a range of triazole sensitivities (fig. 2): K1124, FI12-63, RS-219, QUB 30-10, R 9528.4, 9522.3, 788, SAC 1-4-8 (0003), GKII 18-2-3, GKII 18-3-2, SAC 09/943/14, QUB 12-3, OSA 28-2-2, and RS 783. Isolates were grown in Sabouraud liquid medium for 10 days. Fungal material was harvested by filtration and freeze-dried. DNA was extracted as in Motteram et al. (2009).

CYP51A and *CYP51B* were amplified using PCR, carried out with Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland), with *CYP51B* and *CYP51A* primers Forwards 1 and Reverse 1 (supplementary table S3, Supplementary Material online). Products were purified and sequenced by Eurofins MWG (Germany), with the nested and internal primers listed in supplementary table S3, Supplementary Material online. *CYP51A-p* was amplified using Easy-A High-Fidelity PCR Cloning Enzyme (Stratagene) with primers listed in supplementary table S3, Supplementary Material online. Products were cloned and sequenced as described for genome walking, with additional sequencing reactions using internal primers (supplementary table S3, Supplementary Material online).

PCR–RFLP Assay

Two PCR reactions were carried out per isolate, one to amplify *CYP51A* and one to amplify *CYP51A-p*, using Phusion

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High-Fidelity DNA Polymerase (Finnzymes Oy) with primers listed in supplementary table S3, Supplementary Material online. Restriction digests were carried out with enzymes *PstI* and *HindIII* (Promega), with 3-h incubation time.

CYP51A copy number was confirmed by Southern blot as described by Motteram et al. (2009). Ten micrograms of genomic DNA from isolates 788, K1124, and FI12-63 was digested with high concentration *PstI* and *EcoRV* (Promega). The probe was amplified from pGEM-T Easy plasmids containing *CYP51A* from isolate 788 cDNA, using primers Southern F and Reverse 10D (supplementary table S3, Supplementary Material online) to amplify a 734-bp fragment of exon 3.

Yeast Complementation

Rhynchosporium commune CYP51A and *CYP51B* were amplified from a 1/10 dilution of isolate 788 cDNA using Easy-A High-Fidelity PCR Cloning Enzyme (Stratagene), with primers *CYP51A* pYES F2 and pYES R and *CYP51B* pYES F and pYES R (supplementary table S3, Supplementary Material online). PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega), and a sample of the *CYP51A* product digested with *PstI* and *HindIII* to confirm it was *CYP51A* and not *CYP51A-p*. *KpnI* and a *SacI* restriction sites were incorporated by the forward and reverse primers, respectively. An internal *KpnI* site was removed from *CYP51B* using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, CA, USA) with primer pYES mut (supplementary table S3, Supplementary Material online). Cloning in yeast expression vector pYES2/CT (Invitrogen), transformation into *S. cerevisiae* YUG37:*erg11* strain, and complementation analysis were carried out as described by Cools et al. (2010), with pYES-Mg51wt as a positive control. Fungicide sensitivity testing was carried out as in Cools et al. (2010), but with maximum fungicide concentrations of 0.005 $\mu g\,ml^{-1}$ for epoxiconazole and propiconazole, 0.05 $\mu g\,ml^{-1}$ for prothioconazole and tebuconazole and 5 $\mu g\,ml^{-1}$ for cycloheximide, all with 2-fold serial dilutions.

CYP51 Expression

Rhynchosporium commune isolates QUB 30-10, 788, K1124, QUB 12-3, FI12-63, R 9528.4, GKII 18-2-3, and RS 783 783 were grown liquid culture, in 100-ml Sabouraud liquid medium at 2.5×10^4 spores ml^{-1} at 20 °C, 150 rpm, with two biological replicates of treatment and control culture for each isolate. After 7 days, tebuconazole solution in acetone to a final concentration of 1 $\mu g\,ml^{-1}$ was added. An equal volume of acetone without tebuconazole was added to the control cultures. After 10 days, cultures were harvested by vacuum-filtration, then freeze-dried. RNA extraction was carried out with TRIzol Reagent (Invitrogen), according to manufacturer's instructions, followed by overnight precipitation with 4 M lithium chloride and treatment with the TURBO DNA-free Kit (Applied Biosystems, CA). cDNA was synthesized with the Superscript III first-strand synthesis system (Invitrogen). End-point PCR reactions were carried out using Red Hot Taq (ABgene, Epsom, UK) with intron-spanning primer pair *CYP51B* Expression F and *CYP51B* Expression R (supplementary table S3, Supplementary Material online) to check for genomic DNA contamination.

Quantitative PCR was carried out using SYBR Green JumpStart Taq ReadyMix (Sigma), with *CYP51B*, *CYP51A*, and *β-tubulin* primers Expression F and Expression R (supplementary table S3, Supplementary Material online). Relative quantification was calculated by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001), with *β-tubulin* as endogenous control and isolate R9528.4 without fungicide as calibrator sample.

Hoosfield Long-Term Experiment and Pyrosequencing Assay

Spring barley has been grown on the Hoosfield plot at Rothamsted Research, in Harpenden, UK, every year since 1852, apart from 1912, 1933, 1943, and 1967 when it was left fallow to control weeds. Dried leaf, grain, and soil samples have been retained each year. Straw samples used were from Plot 4A, receiving 48 kg N ha^{-1} in the form of ammonium sulfate. From 1968, this plot was divided into four subplots receiving different nitrogen doses in the form of calcium ammonium nitrate, and samples used were from the subplot receiving 48 kg N ha^{-1}.

One gram of material was taken from the archive sample, ground using a sterilized pestle and mortar, then extraction buffer was added and DNA extractions carried out as described by Motteram et al. (2009), with two replicate extractions from each homogenized sample. *Rhynchosporium secalis s.l.* DNA presence and quality were tested by end-point PCR amplification of a 520-bp fragment of reference gene *β-tubulin* using primers Rs F3 and Rs R3 (supplementary table S3, Supplementary Material online), and up to 3 years per decade were selected for analysis. *Rhynchosporium secalis s.l.* species present were identified based on a C/T SNP at nucleotide 391 of *β-tubulin* (Zaffarano et al. 2011), with a nested PCR using primers RhyUF1 and RhyUR1 then PyroRhF1Bio and PyroRHR1, followed by a pyrosequencing assay using the PyroMark system (Qiagen) and the PSQ96 instrument (Biotage, Uppsala, Sweden), programmed to analyze the sequence TC/TGCAGATCCAAGGTAGAACTTACA CT, using the nucleotide dispensation order ATCTGCAGA, with primer PyroRhS1 (supplementary table S3, Supplementary Material online).

To distinguish *CYP51A* from *CYP51A-p*, a pyrosequencing assay was developed to detect a 4-bp indel at nucleotide 418. A nested PCR was carried out with primers Pyro F3 and Pyro R2 then primers Del F1 and Del R1 (supplementary table S3, Supplementary Material online) and the products pyrosequenced using the PyroMark system (Qiagen) and the PSQ96 instrument (Biotage), to analyze the sequence CA[TTTC]CTTCCAGCCCAAGATAAACAGTCAT, using the nucleotide dispensation order GCATCGTCA, with primer Del S1 (supplementary table S3, Supplementary Material online).

Rhynchosporium Spp. Genome Analysis

The genomes of *R. commune* isolates UK7, AU2 (Lehnackers and Knogge 1990), and 13-13 (A. Avrova, The James Hutton

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Institute); *R. secalis s.s.* isolate 02CH4-6a.1 (Zaffarano et al. 2008); *R. agropyri* isolate 04CH Rac A.6.1; and *R. orthosporum* isolate 02CH4 Bär A.1.1.3 (B. McDonald, ETH Zurich) have been sequenced and are currently prepared for publication. A BLAST search was carried out against *R. commune CYP51A*, *CYP51A-p*, and *CYP51B*. Introns were predicted and coding sequences translated. Each *CYP51* was cloned and resequenced by Sanger sequencing, as described for *CYP51A-p* sequencing with primers listed in supplementary table S3, Supplementary Material online, to confirm the genome assembly. Predicted amino acid sequences, along with *B. cinerea CYP51B* and *M. oryzae CYP51A* (the closest relatives of *R. commune* in supplementary fig. S1, Supplementary Material online), *F. graminearum CYP51C*, and *C. albicans CYP51* as an outgroup were aligned with M-Coffee, and coding nucleotides were aligned with PAL2NAL. Substitution models were predicted with jModeltest, and phylogenetic reconstruction carried out in PhyML with the GTR + G model and 100 bootstrap runs.

Supplementary Material

Supplementary tables S1–S4 and figures S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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