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Analysis of regulation of pentose utilisation in *Aspergillus niger* reveals evolutionary adaptations in *Eurotiales*

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Abstract: Aspergilli are commonly found in soil and on decaying plant material. D-xylose and L-arabinose are highly abundant components of plant biomass. They are released from polysaccharides by fungi using a set of extracellular enzymes and subsequently converted intracellularly through the pentose catabolic pathway (PCP).

In this study, the L-arabinose responsive transcriptional activator (AraR) is identified in *Aspergillus niger* and was shown to control the L-arabinose catabolic pathway as well as expression of genes encoding extracellular L-arabinose releasing enzymes. AraR interacts with the D-xylose-responsive transcriptional activator XlnR in the regulation of the pentose catabolic pathway, but not with respect to release of L-arabinose and D-xylose.

AraR was only identified in the *Eurotiales*, more specifically in the family *Trichocomaceae* and appears to have originated from a gene duplication event (from XlnR) after this order or family split from the other filamentous ascomycetes. XlnR is present in all filamentous ascomycetes with the exception of members of the *Onygenales*. Since the *Onygenales* and *Eurotiales* are both part of the subclass *Eurotiomycetidae*, this indicates that strong adaptation of the regulation of pentose utilisation has occurred at this evolutionary node. In *Eurotiales* a unique two-component regulatory system for pentose release and metabolism has evolved, while the regulatory system was lost in the *Onygenales*. The observed evolutionary changes (in *Eurotiomycetidae*) mainly affect the regulatory system as in contrast, homologues for most genes of the L-arabinose/D-xylose catabolic pathway are present in all the filamentous fungi, irrespective of the presence of XlnR and/or AraR.

INTRODUCTION

The order *Eurotiales* consists of the families *Trichocomaceae* and *Elaphomycetaceae*. Most species belonging to the *Trichocomaceae* are saprobic filamentous ascomycetes, which in nature grow predominantly in soil or on decaying plant material. The *Elaphomycetaceae* entails a family of underground, saprobic or mycorrhiza-forming fungi. The family *Trichocomaceae* includes the well-known genera of *Penicillium* and *Aspergillus*. Aspergilli are found throughout the world in almost all ecosystems and are well-known for their ability to degrade different complex plant polymers. Despite the fact that some *Aspergillus* species have evolved additional lifestyles, for example as human or plant pathogens, there seems to be no restriction to a specific niche concerning their saprobic lifestyle.

Decaying plant material consists for a major part of plant cell wall polysaccharides which can be split into three major groups: cellulose, hemicellulose and pectin. L-arabinose and/or D-xylose are the main components of the hemicelluloses arabinoxylan and xyloglucan, and of pectin. Release of these sugars from polysaccharides as well as metabolic conversion of them through the pentose catabolic pathway (PCP) has been studied for many years, particularly in *Aspergillus* and the genus *Trichoderma* belonging to the order *Hypocreales* [reviewed in (de Vries & Visser 2001, de Vries 2003, Stricker *et al.* 2008)]. The PCP was first described in *Aspergillus niger* (Witteveen *et al.* 1989) and shown to consist of a series of reversible reductase/dehydrogenase steps followed by phosphorylation to D-xylulose-5-phosphate, which enters the pentose phosphate pathway (PPP). In *A. niger,* the gene encoding D-xylose reductase (*xyrA*) (Hasper *et al.* 2000), D-xylulokinase (*xkiA*) (vanKuyk *et al.* 2001), L-arabitol dehydrogenase (*ladA*) and xylitol dehydrogenase (*xdhA*) (Seiboth *et al.* 2003, de Groot *et al.* 2007) have been characterised. For *Trichoderma reesei,* genes encoding L-arabitol dehydrogenase (*lad1*) (Richard *et al.* 2001) and xylitol dehydrogenase (*xdh1*) (Seiboth *et al.* 2003) have been described. In *A. niger*, induction of pentose release and the PCP occurs in the presence of L-arabinose and/or D-xylose (Witteveen *et al.* 1989). In the presence of D-xylose, the xylanolytic transcriptional activator XlnR (van Peij *et al.* 1998b) regulates the expression of genes encoding extracellular polysaccharide degrading enzymes, as well as the expression of *xyrA* [reviewed in (de Vries 2003)]. L-arabinose induction of the PCP is not mediated via XlnR. The genes of the L-arabinose catabolic pathway are co-regulated with the genes encoding extracellular arabinanolytic enzymes (α-L-arabinofuranosidase and endoarabinanase) (Flipphi *et al.* 1994, de Vries *et al.* 1994) and L-arabitol is most likely the inducer (de Vries *et al.* 1994, vanKuyk *et al.* 2001). Analysis of *A. niger* arabinanolytic regulatory mutants, *araA* and *araB*, demonstrated an antagonistic effect between XlnR and the L-arabinose/L-arabitol responsive regulation (de Groot *et al.* 2003).

In this study, we report the identification and characterisation of the L-arabinose catabolic pathway specific regulator (AraR) in *A. niger* and demonstrate that this regulator is only present in the order *Eurotiales*. These fungi have evolved a fine-tuned two-regulator activating system for pentose release and catabolism compared to other filamentous ascomycetes that only contain XlnR or have neither of the regulators.

MATERIALS AND METHODS

Strains, media and growth conditions

The *A. niger* strains used in this study are listed in Table 1 and are all derived from *A. niger* CBS 120.49. *Aspergillus niger* strains were grown in Minimal Medium (MM) or Complete Medium (CM) with addition of a carbon source at 30 °C. MM contained (per liter): 6

Table 1. Strains used in this study.

Strain	Genotype	Reference
N402	*cspA1*	Bos *et al.* (1988)
N572	*cspA1, fwnA1, pyrA6, xkiA1, nicA1*	vanKuyk *et al.* (2001)
NW249	*cspA1, ΔargB, pyrA6, nicA1, leuA1*	Jalving *et al.* (2000)
UU-A049.1	*cspA1, pyrA6, nicA1, leuA1, ΔargB::pIM2101(argB+)*	This study
UU-A033.21	*cspA1, pyrA6, nicA1, leuA1, ΔargB:: pIM2101(argB+), ΔaraR*	This study
UU-A054.4	*cspA1, pyrA6, nicA1, leuA1, ΔargB:: pIM2101(argB+), ΔaraR::araR*	This study
UU-A062.10	*cspA1, ΔargB, nicA1, leuA, pyrA6:: A. oryzae pyrA,ΔxlnR*	This study
UU-A063.22	*cspA1, nicA1, leuA1, ΔargB:: pIM2101(argB+), ΔaraR, pyrA6::A. oryzae pyrA, ΔxlnR*	This study

g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCL, 0.5 g $MgSO_4 \cdot 7\ H_2O$ and 200 µl trace elements solution (Vishniac & Santer 1957), pH 6.0. CM = MM supplemented with (/L): 2 g peptone, 1 g casamino acids, 1 g yeast extract and 0.5 g yeast ribonucleic acids, pH 6.0. For growth on solid media, 1.5 % agar was added to the medium. When necessary, the medium was supplemented with 0.2 g/L arginine, 0.2 g/L leucine, 0.2 g/L uridine and/or 1 mg/L nicotinamide.

In transfer experiments, all the strains were pre-grown in CM containing 2 % D-fructose. After 16 h of incubation, the mycelium was harvested without suction over a filter, washed twice with MM without a carbon source and transferred to 50 mL MM containing the appropriate carbon source and supplements. The mycelium was harvested with suction over a filter and culture samples were taken after 2 and 4 h of incubation. The mycelium samples were dried between tissue paper and directly frozen in liquid nitrogen.

Molecular biology methods

Molecular biology methods were performed according to standard procedures (Sambrook *et al.* 1989), unless stated otherwise. All PCR reactions were performed using Accutaq™ LA DNA Polymerase (Sigma-Aldrich) according to the manufacturer's instruction. The flanking regions of the *araR* gene were amplified with 5'primers and 3'-primers (see online Supplemental Table 1) by PCR to generate the 5' flank with the *Hind*III/*Sph*I site and 3' flank with a *Kpn*I/*BamH*I site, respectively, to enable deletion of the complete coding region of *araR* by replacing it with the *argB* selection marker. The functional construct was obtained using PCR with the extreme 5'- end 3'-primers (see online Supplemental Table 1) for complementation of *araR*. The *araR* disruption cassette (containing the *argB* gene for selection for arginine prototrophy) was transformed to the *A. niger* strain NW249 (*pyrA6*, *leuA1*, *nicA1*, Δ*argB*). The *xlnR* gene was amplified with the extreme 5'-primer and 3'-primer by PCR (see online Supplemental Table 1) The PCR fragment was ligated into pGEM-T-easy (Promega) from which the *Nsi*I/*Pst*I restriction sites were removed. The construct was digested with *Sal*I/*Eco*RI to remove most of the coding region including the DNA binding domain and ligated with the *A. oryzae pyrA* gene that was digested with *BamH*I (made blunt with Klenow fragment) and *SalI*. The *xlnR* disruption cassette was transformed to *A. niger* strains NW249 (*pyrA6*, *leuA1*, *nicA1*, Δ*argB*) and UU-A033.21 (*pyrA6*, *leuA1*, *nicA1*, Δ*araR*). All *A. niger* transformations were carried out as described previously (Kusters-van Someren *et al.* 1991).

The primers used to generate the probes for Southern and Northern analysis are listed in online Supplemental Table 1. The probes were DIG-labelled using the PCR DIG Probe Syntheses Kit (Roche Applied Science) according to the supplier's instructions. A cDNA library (de Groot *et al.* 2007) or genomic DNA (obtained from N402) was used as a template in the PCR reactions for synthesis of the probes.

Expression analysis

Total RNA was isolated from mycelium that was ground in a microdismembrator (B Braun) using a standard RNA isolation method with the TRIzol Reagent (Invitrogen). In the Northern analysis, 3 µg total RNA was transferred to a Hybond-N^+ membrane (Amersham Biosciences). The Minifold II slot blot apparatus (Schleicher & Schuell) was used for Slot blot analysis. Equal loading was determined by soaking the blot for 5 min in 0.04 % methylene blue, 0.5 M acetate pH 5.2 solution.

Hybridisation of the DIG-labeled probes to the blot was performed according to the DIG user's manual (www.roche-applied-science.com). All the blots were incubated overnight at 50 °C. The blots were exposed for 25 min up to 24 h to a Lumi-Film Chemiluminescent Detection Film (Roche Applied Science). Micro array analysis was performed as described previously (Levin *et al.* 2007).

Phylogenetic analysis

The amino acid sequences of AraR, XlnR, LadA, XdhA, XyrA and XkiA were used as queries in a local Blast against the protein files of 38 fungal genomes (see online Supplemental Table 2) with a expect value cut-off of 1E-10. The resulting ORFs were aligned using ClustalX and a Maximum Parsimony tree (1 000 bootstraps) was produced using MEGA (v. 4.0).

Enzyme assays

Extracellular enzyme activity was measured using 0.01 % *p*-nitrophenol linked substrates, 10 µL of the culture samples, 25 mM sodium acetate pH 5.0 in a total volume of 100 µL. Samples were incubated in microtiter plates for 120 min at 30 °C. Reactions were stopped by addition of 100 µL 0.25 M Na_2CO_3. Absorbance was measured at 405 nm in a microtiter platereader (Biorad Model 550). The extracellular enzyme activity was calculated using a standard curve ranging from 0 to 80 nmol *p*-nitrophenol per assay volume.

To measure intracellular enzyme activity, cell free extract was prepared by adding 1 mL extraction buffer (50 mM K_2HPO_4, 5 mM

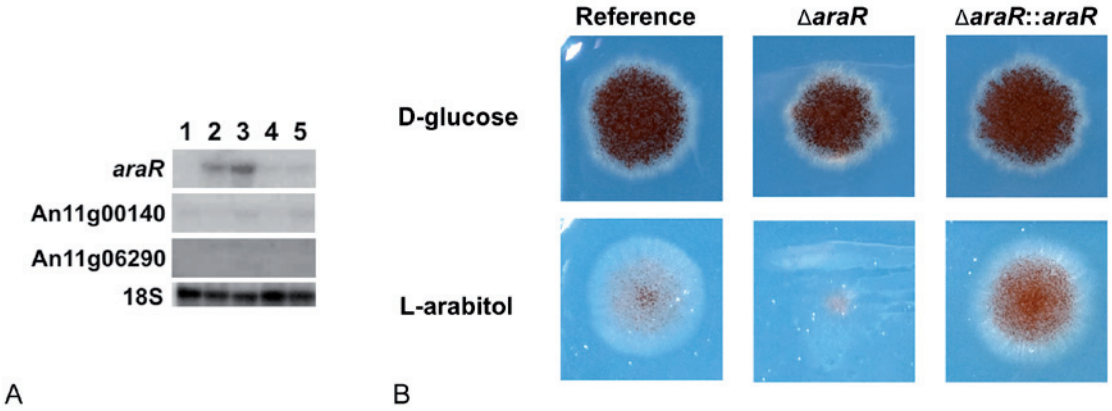

Fig. 1. A. Expression analysis of the three XlnR homologues (An04g08600 (*araR*), An11g00140 and An11g06290) on D-fructose (1), L-arabinose (2), L-arabitol (3), D-xylose (4) and xylitol (5). **B.** Growth of the reference (UU-A049.1), Δ*araR* (UU-A033.21) and Δ*araR::araR* (UU-A054.4) on D-glucose and L-arabitol.

MgCl2, 5 mM 2-mercaptoethanol, 0.5 mM EDTA) to powdered mycelium. The mixtures were centrifuged for 10 min at 12000 RPM at 4 °C. The L-arabitol and xylitol dehydrogenase activities were determined using 100 mM glycine pH 9.6, 0.4 mM NAD^+ and 1 M L-arabitol or xylitol, respectively. L-arabinose reductase and D-xylose reductase activities were determined using 50 mM Tris-HCL pH 7.8, 0.2 mM NADPH and 1 M L-arabinose or D-xylose, respectively. L-arabinose reductase (ArdA) and D-xylose reductase (XyrA) both convert D-xylose to xylitol and L-arabinose to L-arabitol, but have a higher activity on their primary substrate (de Groot *et al.* 2003). As a result, the measured activity is the sum of the two enzymes. To be able to discriminate between the two enzymes, the ratio of the activity on L-arabinose and on D-xylose was calculated that allows us to extrapolate the relative activities of ArdA and XyrA. An increase in the ratio indicates a relative increase in ArdA or decrease in XyrA, while a reduction in the ratio indicates a relative increase in XyrA or decrease in ArdA.

Absorbance changes were measured at 340 nm using a spectrometer (Spectronic Unicam UV1). L-arabinose and D-xylose reductase activity and L-arabitol and D-xylitol dehydrogenase activity was calculated using the molar coefficient for NADPH and NADH (both ε = 6.22 mM-1cm-1) and the following formula:

$$\text{Activity (U/mL)} = [(\text{A/min-Abl/min}) * d * v] / (l * a * \varepsilon).$$

Abl/min = decrease absorbance per minute before adding substrate. A/min = decrease absorbance per minute after adding substrate. a = sample volume (mL). d = sample dilution. v= total volume cuvet. l = lightpath (cm). Protein concentrations of intracellular and extracellular samples were determined using a BCA protein assay kit (Pierce).

RESULTS

Identification and analysis of araR

Blast analysis of XlnR against the *A. niger* genome (Pel *et al.* 2007) revealed 3 homologues with expect values smaller than e^{-30} (An04g08600, An11g00140, An11g06290). Expression analysis of these genes revealed that the closest *xlnR* homologue (An04g08600) was specifically induced in the presence of L-arabinose or L-arabitol, while only low constitutive expression was observed for An11g06290 and no expression for An11g00140 (Fig. 1A). In order to study its possible role in L-arabinose utilisation, a disruption strain for An04g08600 (referred to as *araR*) was constructed and verified by Southern analysis (data not shown). The disruption strain showed poor growth on L-arabitol, whereas complementation with *araR* restored growth again (Fig. 1B).

The araR gene consists of 2552 bp interrupted by a single intron of 53 bp. Within the 1000 bp promoter region of *araR* putative six binding sites for the carbon catabolite repressor protein CreA (Kulmburg *et al.* 1993) and two binding sites for the xylanolytic regulator XlnR (van Peij *et al.* 1998b, de Vries *et al.* 2002) can be found. The AraR protein contains a Zn(2)Cys(6) binuclear cluster domain (amino acids 36-73, Pfam00172) and a Fungal specific transcription factor domain (amino acids 386-532, Pfam04082). An amino acid motif Arg-Arg-Thr-Leu-Trp-Trp is found at position 493 to 498. This motif differs in only one amino acid from a conserved motif of unknown function found in Zn(2)Cys(6) family members (Arg-Arg-Arg-Leu-Trp-Trp), first described in the UaY regulator in *Aspergillus nidulans* (Suarez *et al.* 1995). AraR shows 32 % identity to XlnR, with the highest homology in the C-terminal part of the proteins. The sequence between the 2nd and the 3rd Cysteine in the Zn(2)Cys(6) region was previously shown to be important in DNA binding specificity of this class of regulators (Marmorstein *et al.* 1992, Marmorstein & Harrison 1994), but differs significantly between AraR (C_2HSRRVRC_3) and XlnR (C_2NQLRTKC_3). Between the third and the fourth Cysteine, the Proline residue can be found that is essential for correct folding of the DNA binding domain (Marmorstein *et al.* 1992) and is highly conserved in all the fungal zinc binuclear transcriptional regulators.

The presence of AraR in the genome is restricted to *Eurotiales* and possibly to *Trichocomaceae*

BlastP analysis of both AraR and XlnR against 38 fungal genome sequences (see online Supplemental Table 2) identified homologues for both proteins in all 11 analysed species of the family *Trichocomaceae* of the order *Eurotiales* (*Aspergillus clavatus*, *A. flavus*, *A. fumigates*, *A. nidulans*, *A. niger*, *A. oryzae*, *A. terreus*, *Neosartorya fischeri*, *Penicillium chrysogenum*, *P. marneffei*, *Talaromyces stipitatus*), but neither of them was found in three representatives of *Onygenales* (*Coccidioides immitis*, *Histoplasma capsulatum*, *Uncinocarpus reesei*) (Fig. 2). XlnR was also found in the genomes of all other filamentous ascomycetes used in this study. No XlnR and AraR homologues were found in ascomycete yeasts, basidiomycetes or zygomycetes.

In addition, a BlastP analysis was performed with the amino acid sequence of four genes of the *A. niger* pentose catabolic

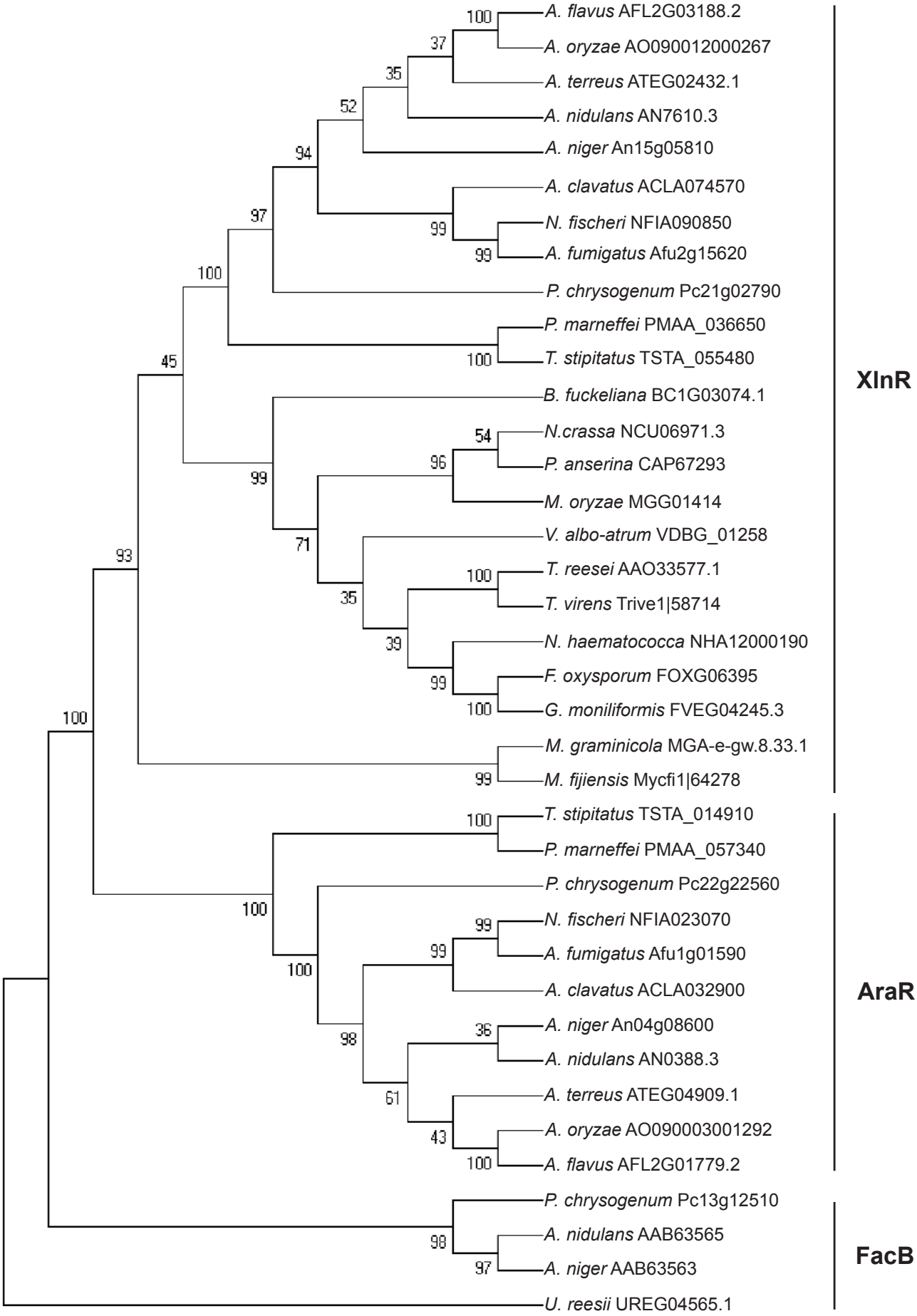

Fig. 2. Bootstrapped (1000 bs) Maximum Parsimony tree of of putative homologues of XlnR and AraR in fungi. Homologues of the *A. nidulans* acetate regulatory protein (FacB) were used as an outgroup.

pathway (*ladA*, *xyrA*, *xdhA* and *xkiA*) against the genomes of the fungal species that contain XlnR and/or AraR as well as the three *Onygenales* genomes used in this study (see online Supplemental Fig. 1). Phylogenetic analysis showed that all genomes contain homologues of three genes of the pentose catabolic pathway. Homologues for the 4th gene (*ladA*) were found in all species except for *Onygenales*.

Influence of AraR and XlnR on growth of *A. niger* on monomeric and polymeric carbon sources

In addition to the *araR* disruptant (UU-A033.21), an *xlnR* disruptant (UU-A062.10) and an *araR/xlnR* double disruptant (UU-A063.22) were generated, as described in Materials and Methods. The utilisation of several monomeric and polymeric carbon sources was analysed in all strains (including the reference) to determine the effect of the single disruption of the *araR* gene and the double disruption of *araR* and *xlnR* (Fig. 3). Polymeric sugars containing L-arabinose residues (arabinan, Arabic gum, arabinogalactan and apple pectin) and D-xylose residues (birchwood xylan) were included in the analysis. Guar gum was used as a control; it is a galactomannan and contains no L-arabinose or D-xylose residues.

Disruption of *araR* resulted in reduced growth on L-arabinose, xylitol, arabinan, Arabic gum, arabinogalactan and apple pectin and poor growth on L-arabitol (Fig. 3). Disruption of *xlnR* resulted in reduced growth on birchwood xylan, while growth was unaffected on D-xylose, xylitol and the other carbon sources. Disruption of both regulators resulted in a similar phenotype as disruption of *araR* for L-arabitol, Arabic gum, arabinan and arabinogalactan and a similar phenotype as disruption of *xlnR* for birchwood xylan. In contrast to the single disruptants, no growth was observed on D-xylose for the double disruptant, only residual growth on L-arabitol and L-arabinose, and reduced growth on xylitol.

AraR and XlnR control L-arabinose and D-xylose release and catabolism

The reference, Δ*araR*, Δ*xlnR* and Δ*araR*/Δ*xlnR* strains were pre-grown in complete medium containing D-fructose. After 16 h of growth, equal amounts of mycelium were transferred for 2 and 4 h to minimal medium containing 25 mM D-fructose, 25 mM L-arabinose or 25 mM D-xylose. Extracellular α-L-arabinofuranosidase (Abf) and intracellular PCP enzyme activities (Ard, Xyr, Lad, Xdh) were analysed. Activity of α-L-arabinofuranosidase (Abf), L-arabitol dehydrogenase (Lad) and xylitol dehydrogenase (Xdh) was strongly reduced in the Δ*araR* and Δ*araR*/Δ*xlnR* strain compared to the reference strain when grown on L-arabinose (Fig. 4A). On D-xylose, Lad and Xdh activity was reduced in Δ*araR* and Δ*araR*/Δ*xlnR*. For L-arabinose reductase (ArdA) and D-xylose reductase (XyrA), the ratio of the activity on L-arabinose and on D-xylose was calculated that allowed extrapolation of the relative activities of ArdA and XyrA (see Materials and Methods). The ratio in the Δ*araR* strain became less than 1.0 after 4 h growth in the presence of L-arabinose, while the ratio of the reference strain was around 1.5, which suggests that the ArdA activity was reduced in the Δ*araR* strain (Fig. 4A). The Ard/Xyr ratio in the wild type and Δ*araR* disruptant grown on D-xylose were both around 1. In the absence of both regulators, no Ard and Xyr activities were detected (data not shown). Xylitol dehydrogenase activity (Xdh) was reduced in the Δ*araR* strain on L-arabinose and to a lesser extent on D-xylose compared to the reference strain (Fig. 4). All the measured activities after 2 h of growth on L-arabinose and D-xylose in the Δ*xlnR* are similar to those published previously (de Groot *et al.* 2003). After 4 h, the difference in activity between the reference and Δ*xlnR* is similar to that observed after 2 h of growth, except for Xdh and Abf. Xdh activity in the Δ*xlnR* became similar to that in the reference strain after 4 h on D-xylose, whereas the Abf activity increased at this point. No activity for any of the enzymes was detected during growth of D-fructose.

In addition, expression levels were determined using micro array analysis for genes involved in release (*abfA, abfB*) and catabolism (*ladA, xdhA, xyrA, xkiA*) of L-arabinose and D-xylose. No gene expression was observed for any of the genes discussed in this section during growth on 25 mM D-fructose (data not shown). Expression profiles of all the genes in Table 2, except for *araR* and *xlnR,* were confirmed by Northern analysis (see online Supplemental Fig. 1). Expression of *araR* and *xlnR* was below detection levels for Northern analysis in these samples. Disruption of *araR* resulted in 74, 6, 10, 2 and 13-fold reduced expression levels of *abfA*, *abfB*, *ladA*, *xdhA* and *xkiA*, respectively, after 2 h of growth on L-arabinose (Table 2). Disruption of *xlnR* did not significantly reduce expression levels of any of the tested genes, except for *xyrA* for which expression reduced 2-fold after 2 h of growth on D-xylose. Disruption of *araR* did not affect *xdhA*, *xkiA* and *xyrA* expression on D-xylose, while none of the genes were affected on L-arabinose by disruption of *xlnR* (see online Supplemental Fig. 1). None of the tested genes were expressed in the Δ*araR*/Δ*xlnR* strain, except for *abfB* (see online Supplemental Fig. 1). Expression of *xlnR* was not affected in the Δ*araR* on L-arabinose, whereas *araR* expression showed a 3-fold increase in the Δ*xlnR* on D-xylose compared to the reference.

DISCUSSION

Previously, it has been shown that the pentose catabolic pathway is under control of the D-xylose specific transcriptional activator (XlnR) and a second, unidentified L-arabinose specific transcriptional activator regulator (de Groot *et al.* 2007). In this study, we identified the gene encoding the L-arabinose responsive regulator, AraR, and confirmed its role in the release and catabolism of L-arabinose and D-xylose. AraR is a member of the Zn(2)Cys(6) family of transcriptional regulators and a close homologue of the xylanolytic transcriptional activator XlnR from *A. niger*. Functional analysis of AraR and XlnR as described in this study confirm the previously published antagonistic relation of the two regulatory systems involved in pentose catabolism (de Groot *et al.* 2003).

Expression levels of *abfA*, *abfB*, *ladA* as well as the corresponding enzyme activities (Abf and Lad) were strongly reduced in the Δ*araR* strain on L-arabinose, indicating that they are only controlled by AraR. Gene expression levels of *xdhA* and *xkiA* are reduced in the Δ*araR* strain after 2 h of growth on L-arabinose. On D-xylose, *xdhA* expression is up-regulated in the Δ*xlnR* strain compared to the reference strain, which confirms data published previously (de Groot *et al.* 2007). An increase in *xkiA* expression was observed in the Δ*xlnR* strain on L-arabinose. These results indicate that both AraR and XlnR are involved in regulating the expression of *xdhA* and *xkiA*. The stronger effect in the Δ*araR* strain, suggests that AraR has a larger influence on *xdhA* and *xkiA* expression than XlnR.

Expression of the AraR regulated genes on D-xylose and reduction of the expression in the *araR* disruptant can be explained by the presence of a small amount of L-arabinose in the D-xylose

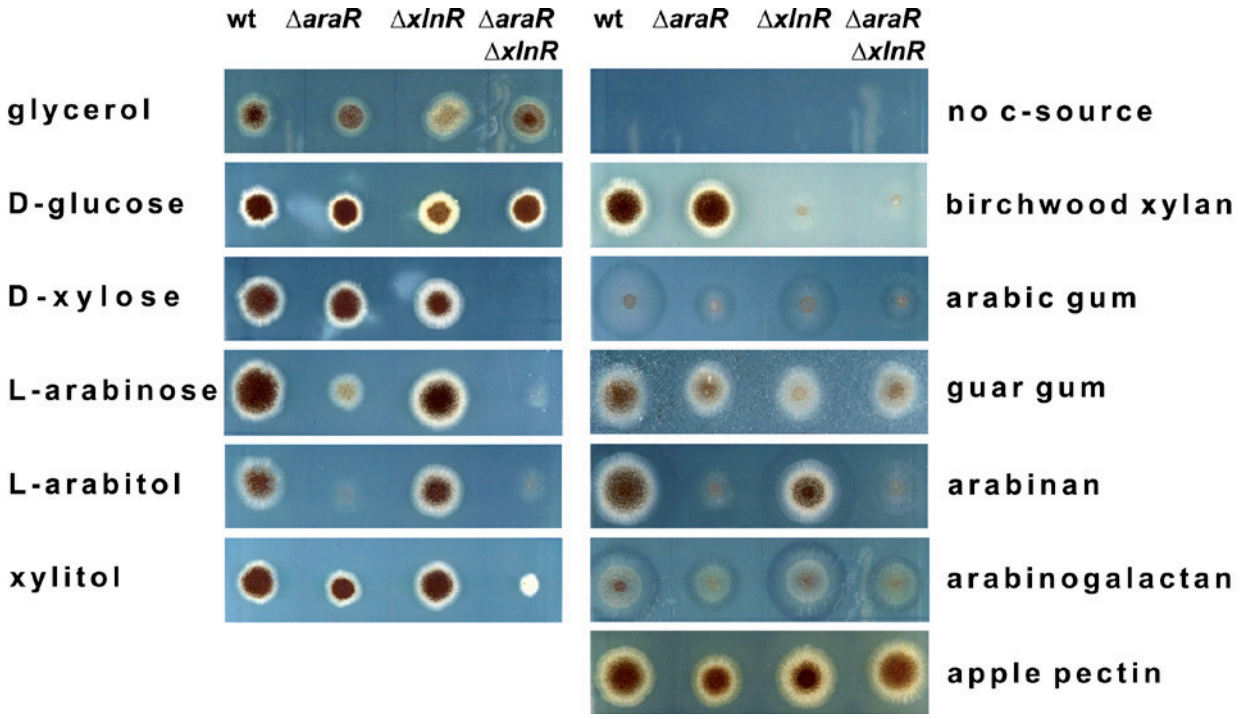

Fig. 3. Growth of the reference strain (Ref., UU-A049.1), and the Δ*araR* (UU-A033.21), Δ*xlnR* (UU-A062.10) and Δ*araR*/Δ*xlnR* (UU-A063.22) strains on a selection of mono- and polysaccharides. Concentrations of the substrates were 25 mM for D-glucose, D-xylose, L-arabinose, L-arabitol, xylitol and glycerol, and 1 % for birchwood xylan, Arabic gum, guar gum, arabinan, arabinogalactan and apple pectin.

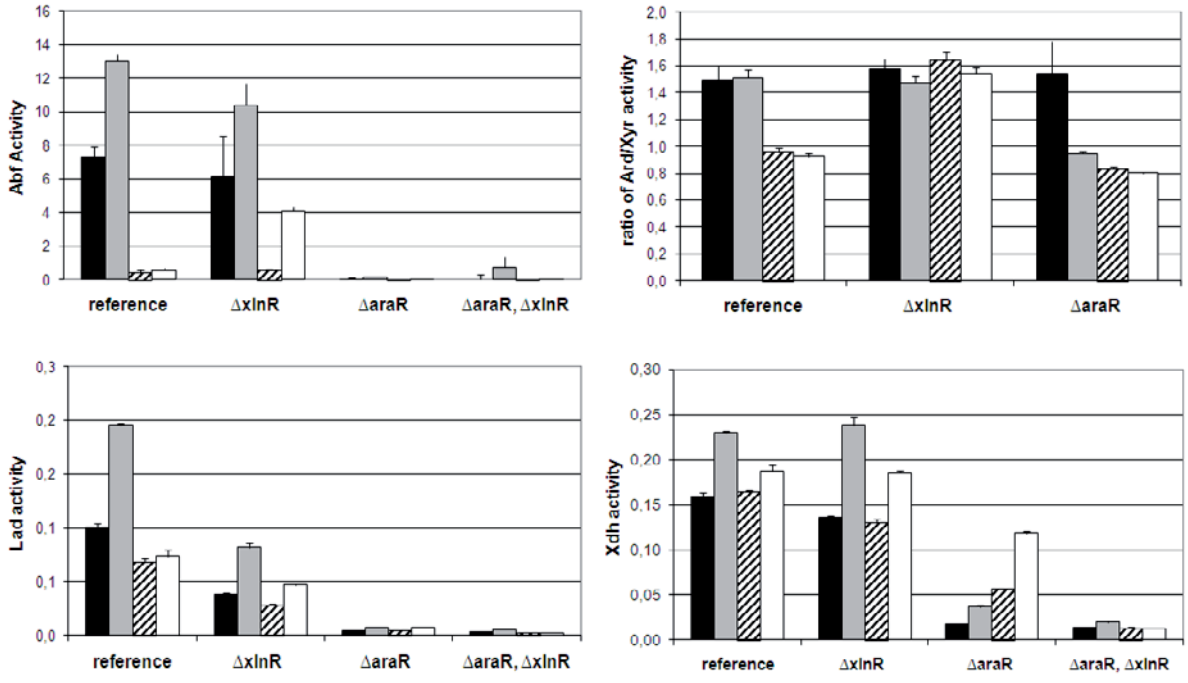

Fig. 4. Comparison of intracellular and extracellular enzyme activities in reference and disruption strains. The reference strains (UU-A049.1), Δ*araR* (UU-A033.21), Δ*xlnR* (UU-A062.10) and Δ*araR*/Δ*xlnR* (UU-A063.22) were transferred for 2 and 4 h on 25 mM L-arabinose or 25 mM D-xylose. Extracellular α-L-arabinofuranosidase (Abf), the ratio of intracellular L-arabinose reductase (ArdA) and D-xylose reductase (XyrA) activity, and the intracellular activities of xylitol dehydrogenase (Xdh) and L-arabitol dehydrogenase (Lad). Black bars: L-arabinose, 2 h; grey bars: L-arabinose, 4 h; dashed bars: D-xylose, 2 h; white bars: D-xylose, 4 h.

preparation from SIGMA (R.P. de Vries, unpubl. data). This is supported by a reduction in the expression of these genes on D-xylose at 4 h compared to 2 h.

The discrepancies between some of the expression and activity data can be explained by the substrate specificities of the enzymes. The L-arabinose and D-xylose reductases are both active on both pentoses, so under conditions where both are expressed, the measured activity is the result of the combined activity of the two enzymes. Although xylitol dehydrogenase is (almost) not active on L-arabitol, the L-arabitol dehydrogenase is active on xylitol (de Groot *et al.* 2007), indicating that the measured xylitol dehydrogenase can also consist of two components depending on the condition used.

Previously, it has been shown that the expression of *xyrA* was only reduced and not absent in the Δ*xlnR* on D-xylose (de Groot *et al.* 2003) and it was suggested that in addition to XlnR another unknown inducing factor is involved. Our results confirm this observation. The reason why there is no reduction in growth of the Δ*xlnR* strain on D-xylose can be explained by the fact

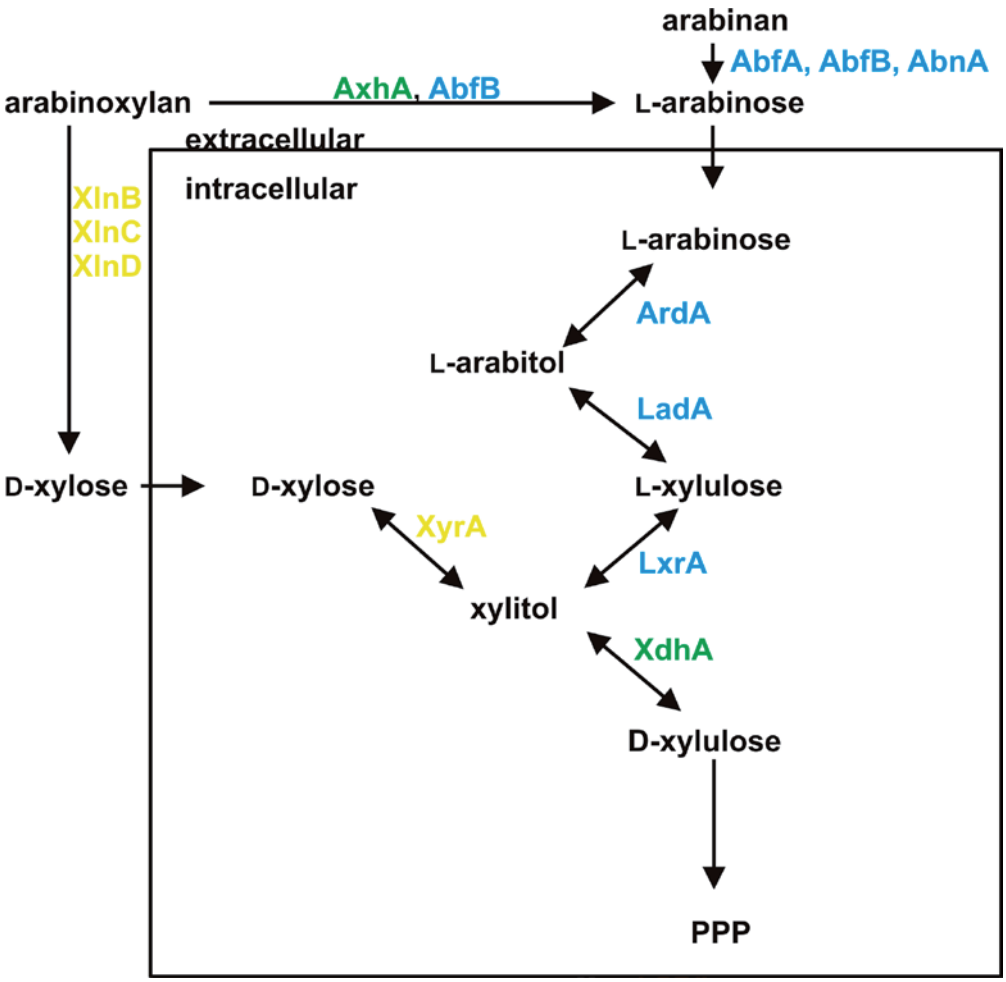

Fig. 5. Regulatory model for release and utilisation of D-xylose and L-arabinose in *A. niger*. ArdA = L-arabinose reductase; LadA = L-arabitol dehydrogenase; LxrA = L-xylulose reductase; XdhA = xylitol dehydrogenase; XyrA = D-xylose reductase; XkiA = D-xylulose kinase; AbfA, AbfB = α-L-arabinofuranosidase A and B; AbnA= endo-1,5-alpha-L-arabinanase; AxhA = arabinoxylan arabinofuranohydrolase; XlnB, XlnC = endoxylanases B and C; XlnD = β-xylosidase. The square depicts the fungal cell wall. AraR regulated genes are in blue. XlnR regulated genes are in yellow. Genes regulated by AraR and XlnR are in green. Inclusion of *axhA*, *abnA*, *xlnB*, *xlnC*, *xlnD* was based on co-regulation with the other genes as reported previously (Gielkens *et al.* 1997, van Peij *et al.* 1998a, de Groot *et al.* 2003).

that *xyrA* expression/activity was not absent combined with the compensatory regulation by AraR for *xkiA* and *xdhA* expression. No growth was observed for the double disruptant on D-xylose, suggesting both regulators are necessary for growth on D-xylose. The strong growth reduction of the Δ*xlnR* strain on xylan, similar to growth of the double disruptant, indicates that D-xylose release is mainly dependent on XlnR.

Only residual growth was observed for the double disruptant on L-arabinose and L-arabitol, demonstrating the importance of AraR and XlnR for growth on these substrates. Strongly reduced growth was observed for the Δ*araR* strain and the Δ*araR*/Δ*xlnR* strain on arabinan, indicating that release of L-arabinose residues depends only on AraR.

The absence of AraR orthologues in fungal genomes except for those of the aspergilli and penicillia and its similarity to XlnR suggests that this regulator has originated by a gene duplication of *xlnR* after *Eurotiales* split from the other filamentous ascomycetes. All genomes available from *Eurotiales* are of the family *Trichocomaceae*, while currently none are available for the other family of this order, *Elaphomycetaceae*. At this point we can therefore not determine whether this gene duplication may have occurred even later, when *Elaphomycetaceae* and *Trichocomaceae* split into two different families.

The regulatory system controlling pentose release and utilisation in this group of fungi likely evolved to become a highly interactive two-regulator system. Whether this implies that in the other ascomycete fungi XlnR is responsible for L-arabinose and D-xylose induced expression remains to be studied. It suggests there are large evolutionary differences in regulation of the pentose catabolic pathway. Afterthe *Onygenales* split from *Eurotiales* it seems to have lost both XlnR and AraR regulators. Homologues for three of the *A. niger* genes of the pentose catabolic pathway (*xdhA*, *xyrA* and *xkiA*) are present in the other fungal genomes. The L-arabitol dehydrogenase encoding gene (*ladA*) appears to have been lost in *Onygenales*, but is present in all species that contain XlnR. This may suggest that loss of L-arabinose utilisation has proceded further in *Onygenales* than just loss of the regulatory systems.

Data from our study was combined with the previously reported data on XlnR (van Peij *et al.* 1998a, de Groot *et al.* 2003) to construct a regulatory model for release and utilisation of L-arabinose and D-xylose in the *A. niger* (Fig. 5). This model correlates not only well with the expression profiles of the pentose-related genes but also with the growth comparison of the disruptant strains and the reference. It indicates that XlnR and AraR control distinct sets of genes in response to the presence of D-xylose and L-arabinose, respectively. However, in the absence of one of the regulators the other can partially compensate for this loss. Although the data supporting this model comes from *A. niger*, we postulate that this model applies to all *Eurotiales*, since we have demonstrated in this study that the presence of AraR is conserved among all species of *Eurotiales* studied so far.

Table 2. Expression analysis of genes encoding extracellular L-arabinose releasing enzymes and PCP enzymes. *abfA*, *abfB* = α -L-arabinofuranosidase A and B, *ladA* = L-arabitol dehydrogenase, *xdhA* = xylitol dehydrogenase, *xyrA* = D-xylose reductase, *xkiA* = D-xylulose kinase, *araR* = arabinanolytic regulator, *xlnR* = xylanolytic regulator. The expression levels are mean values of duplicate samples. The ratio was calculated of the expression levels of the reference strain and disruption strain.

	Reference	**Δ*araR***	**Ratio**	**Reference**	**Δ*xlnR***	**Ratio**
	2 h L-ara	**2 h L-ara**	**ref/ Δ*araR***	**2 h D-xyl**	**2 h D-xyl**	**ref/ Δ*xlnR***
abfA	6622 ± 919	89 ± 9	74.4	5827 ± 545	7578 ± 748	0.8
abfB	4985 ± 516	901 ± 143	5.5	869 ± 4	2176 ± 150	0.4
ladA	4224 ± 417	414 ± 12	10.1	2229 ± 24	3482 ± 8	0.6
xdhA	5013 ± 661	2281 ± 417	2.2	4344 ± 315	6567 ± 377	0.7
xyrA	4808 ± 641	4048 ± 685	1.2	6248 ± 587	3655 ± 66	1.7
xkiA	2690 ± 402	211 ± 18	12.7	2588 ± 34	1843 ± 102	1.4
araR	100 ± 28	1 ± 0	100	20 ± 1	65 ± 5	0.3
xlnR	145 ± 21	185 ± 41	0.8	157 ± 9	3 ± 0	52.3

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SUPPLEMENTARY INFORMATION

Table 1. Primers used in this study.

	Primer 5' to 3'	
gene	**dw**	**up**
***araR* 5' flank**	GGTACCCTTTGATGTTAGTTG	GGATCCATCGCGGGGAAAC
***araR* 3' flank**	GCATGCTTAAATTATCTTCCGCC	AAGCTTTCAATTTTTGTGTCTGGAG
xlnR	CTTGGTTGGTCTCCGTCTG	GGGAAGTGCGGAGGGAGTG
abfA	AGGGTGGCAACTCATCCAG	GCCAGCACCGTCAACTTG
abfB	ACCCGCGCCCTATACAGC	CTGCTTCGTGCCATCGTTG
ladA	AGATCTCTACCGCAACTGTTCTCG	CTGCAGTTTAAATCTTCTGACCAG
xdhA	AGATCTGCACCCAGAACACCAACG	CTGCAGAATTCTATGAATCGACACC
xyrA	AACAGCGGCTACGACATGC	TCTGCTTCAACCGCTGAGG
xkiA	CATCGGCTTCGACCTCTC	CAGTGCTTCCCTTCCTGG

Table 2. Fungal genomes used for the phylogenetic analysis.

Species anamorph	Species teleomorph	website	(sub)phylum	order
Aspergillus clavatus		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus fischerianus	*Neosartorya fischeri*	http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus flavus		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus fumigatus		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus nidulans	*Emericella nidulans*	http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus niger JGI		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus oryzae		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus terreus		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Botrytis cinerea	*Botryotinia fuckeliana*	http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html	Ascomycota	*Helotiales*
Candida albicans		http://www.broad.mit.edu/annotation/genome/candida_group/MultiHome.html	Ascomycota	*Saccharomycetales*
Candida robusta	*Saccharomyces cerevisiae*	http://www.broad.mit.edu/annotation/genome/saccharomyces_cerevisiae/Home.html	Ascomycota	*Saccharomycetales*
Coccidioides immitis		http://www.broad.mit.edu/annotation/genome/coccidioides_group/MultiHome.html	Ascomycota	*Onygenales*
Coprinus cinereus	*Coprinopsis cinerea*	http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Home.html	Basidiomycota	*Agaricales*
Cryptococcus neoformans	*Filobasidiella neoformans*	http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans/Home.html	Basidiomycota	*Tremellales*
Fusarium graminearum	*Gibberella zeae*	http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html	Ascomycota	*Hypocreales*
Fusarium oxysporum		http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html	Ascomycota	*Hypocreales*
Fusarium verticillioides	*Gibberella moniliformis*	http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html	Ascomycota	*Hypocreales*
Histoplasma capsulatum	*Ajellomyces capsulatus*	http://www.broad.mit.edu/annotation/genome/histoplasma_capsulatum/Home.html	Ascomycota	*Onygenales*
Laccaria bicolor		http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html	Basidiomycota	*Agaricales*
Mucor circinelloides		http://genome.jgi-psf.org/Mucci1/Mucci1.home.html	Mucormycotina	*Mucorales*
Nectria haematococca		http://genome.jgi-psf.org/Necha1/Necha1.home.html	Ascomycota	*Hypocreales*
Neurospora crassa		http://www.broad.mit.edu/annotation/genome/neurospora/Home.html	Ascomycota	*Sordariales*
Paracercospora fijiensis var. *difformis*	*Mycosphaerella fijiensis*	http://genome.jgi-psf.org/Mycfi1/Mycfi1.home.html	Ascomycota	*Capnodiales*
Penicillium chrysogenum		http://www.ncbi.nlm.nih.gov/genomeprj	Ascomycota	*Eurotiales*
Penicillium marneffei		http://www.ncbi.nlm.nih.gov/genomeprj	Ascomycota	*Eurotiales*
Talaromyces stipitatus		http://www.ncbi.nlm.nih.gov/genomeprj	Ascomycota	*Eurotiales*
Phycomyces blakesleeanus		http://genome.jgi-psf.org/Phybl1/Phybl1.home.html	Mucormycotina	*Mucorales*
Podospora anserina		http://podospora.igmors.u-psud.fr/	Ascomycota	*Sordariales*
Postia placenta		http://genome.jgi-psf.org/Pospl1/Pospl1.home.html	Basidiomycota	*Polyporales*
Pyricularia oryzae	*Magnaporthe oryzae*	http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/Home.html	Ascomycota	*Magnaporthales*
Rhizopus oryzae		http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/Home.html	Mucormycotina	*Mucorales*
Schizosaccharomyces japonicus		http://www.broad.mit.edu/annotation/genome/schizosaccharomyces_japonicus	Ascomycota	*Schizosaccharomycetales*

Table 2. (Continued).

Species anamorph	Species teleomorph	website	(sub)phylum	order
Septoria tritici	*Mycosphaerella graminicola*	http://genome.jgi-psf.org/Mycgr1/Mycgr1.home.html	Ascomycota	*Capnodiales*
Sporotrichum pruinosum	*Phanerochaete chrysosporium*	http://genome.jgi-psf.org/Phchr1/Phchr1.home.html	Basidiomycota	*Corticiales*
Trichoderma reesei	*Hypocrea jecorina*	http://genome.jgi-psf.org/Trire2/Trire2.home.html	Ascomycota	*Hypocreales*
Trichoderma virens	*Hypocrea virens*	http://genome.jgi-psf.org/Trive1/Trive1.home.html	Ascomycota	*Hypocreales*
Uncinocarpus reesii		http://www.broad.mit.edu/annotation/genome/uncinocarpus_reesii/Home.html	Ascomycota	*Onygenales*
Ustilago maydis		http://www.broad.mit.edu/annotation/genome/ustilago_maydis/Home.html	Basidiomycota	*Ustilaginales*
Verticillium albo-atrum		http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html	Ascomycota	*Hypocreales*

SUPPLEMENTARY INFORMATION

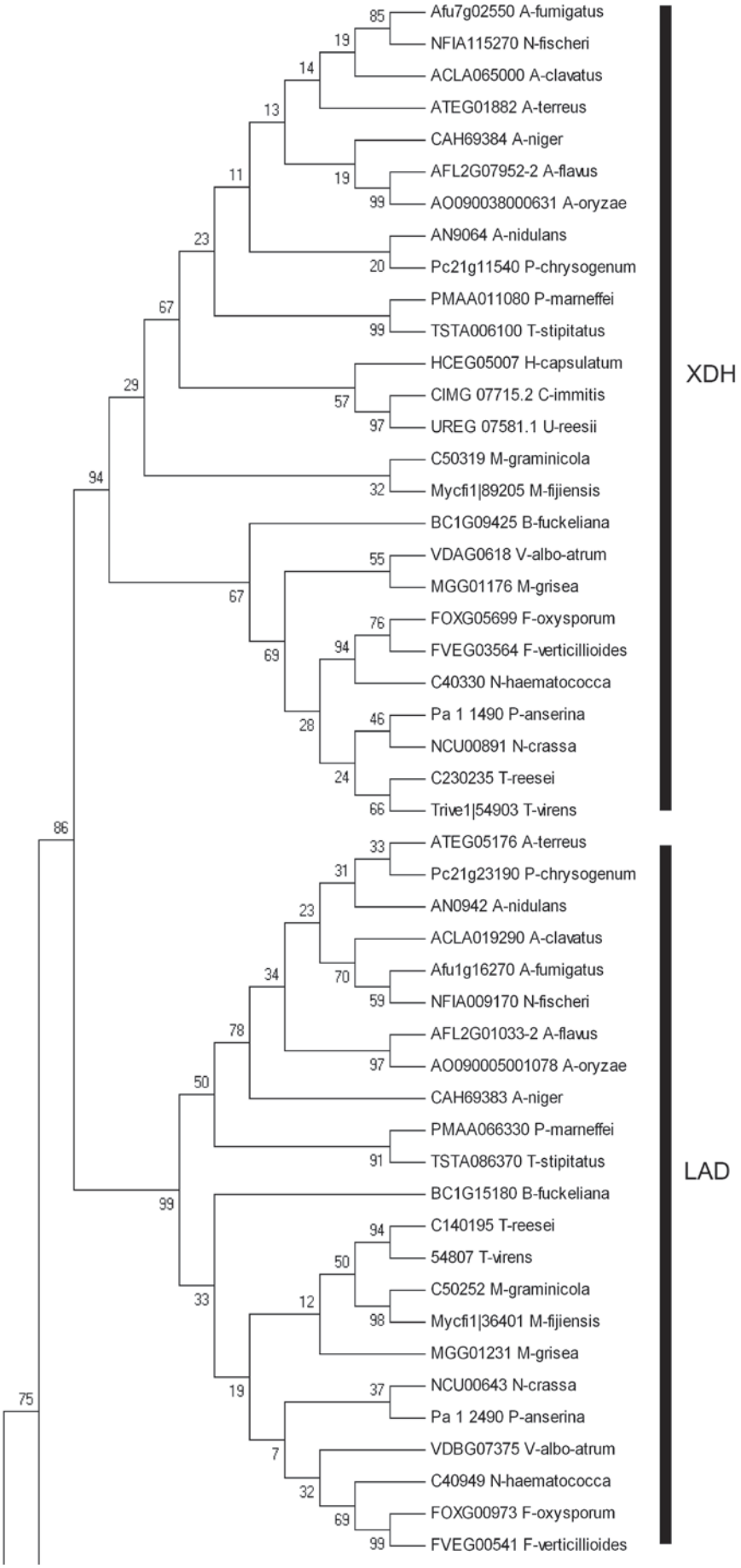

Fig. 1. Maximum Parsimony bootstrap tree (1000 bootstraps) of pentose catabolic pathway genes. XDH = xylitol dehydrogenase, LAD = L-arabitol dehydrogenase, XKI = D-xylulose kinase, XYR = D-xylose reductase.

SUPPLEMENTARY INFORMATION

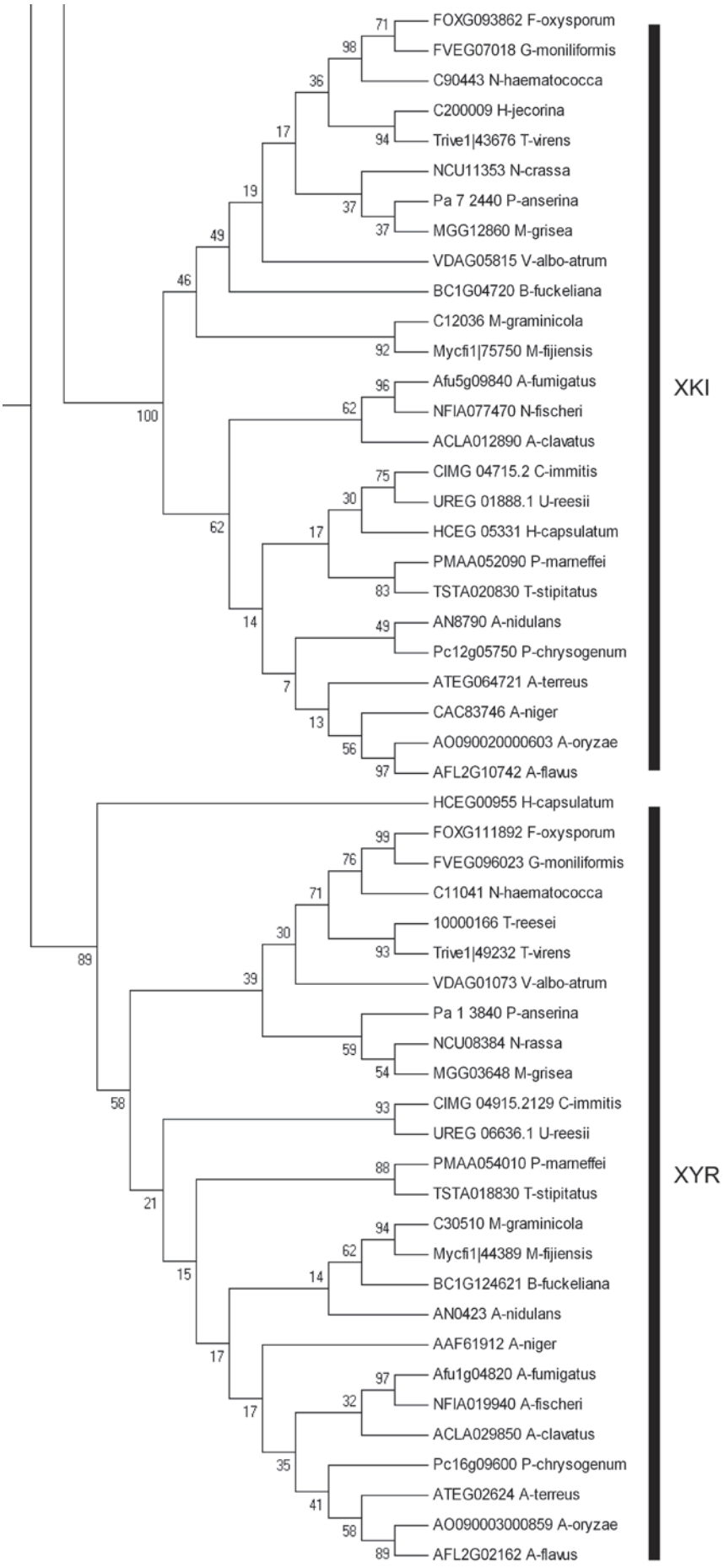

Fig. 1. (Continued).