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A SEARCH FOR DEVELOPMENTAL GENE SEQUENCES IN THE GENOMES OF FILAMENTOUS FUNGI

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There is now a sufficient number of filamentous fungal genomes in the public databases to warrant at least initial comparisons with animal and plant genomes. Our interest lies in the control of multicellular morphogenesis, which is a feature of filamentous ascomycetes and basidiomycetes. Search of a representative collection of filamentous fungal genomes with gene sequences generally considered to be essential and highly conserved components of normal development in animals failed to reveal any homologies. We conclude that fungal and animal lineages diverged from their common opisthokont line well before the emergence of any multicellular arrangement, and that the unique cell biology of filamentous fungi has caused control of multicellular development in fungi to evolve in a radically different fashion from that in animals and plants.

1. INTRODUCTION

Fungi form a large, arguably the largest, eukaryotic Kingdom that includes yeasts, moulds and mushrooms and probably comprises more than 1.5 million species (Hawksworth, 1991, 2001). Fungi have spread through all habitats. Many live parasitically or symbiotically with animals or plants, but they are characteristically saprotrophs that are able to produce externalized enzymes capable of digesting an enormous variety of organic and mineral materials in their immediate environment and absorbing the nutrients so released.

The small genome size and extensive classic genetic analysis of yeasts made these fungi especially suitable for pioneering genome analysis in the mid-1990s. A breakthrough in genome research was the determination of the first complete eukaryotic genome sequence of the well-studied budding yeast *Saccharomyces cerevisiae,* which was finished in 1996 (Goffeau *et al.*, 1996). However, the yeast growth form is highly specialized and the small genome of yeasts is not adequately representative of the genetic, biochemical and behavioural diversity of the bulk of the fungal kingdom, which are filamentous hyphal organisms (Bennett, 1997). More recently, the collection of fungal genomes available has become more representative with the sequencing of the genomes of several ascomycetes, including the classic genetic model organism *Neurospora crassa* (Galagan *et al.*, 2003; Schulte *et al.*, 2002). Then, in mid-2003, the Whitehead Institute at MIT announced the release of the first mushroom genome, that of the small ink-cap mushroom *Coprinus* (= *Coprinopsis*) *cinereus* (Anon, 2003a). Since then, full or partial genomes of several

other filamentous fungi have been sequenced and the genomic data deposited in fungal sequence databases. There is now a sufficiently representative collection of higher fungal genomes in the public databases to warrant at least initial enquiries that address questions of comparison with animal and plant genomes. Our interest lies in the control of multicellular morphogenesis which is a feature of filamentous ascomycetes and basidiomycetes. Unless a mycologist is speaking, fungi are never included in discussions of developmental biology. An example in point was published in the millennium combined issue of TCB, TIBS and TIG (Meyerowitz, 1999), an otherwise excellent paper which discussed evolution of the originally common genetic toolkit of major eukaryote clades without using the word fungus! Nevertheless, we heartily subscribe to the author's view that 'comparison of the genes used to serve similar functions shows how organisms can use different genes for similar ends and thereby reveals the principles of development' (Meyerowitz, 1999). In this chapter we report some initial searches for fungal sequences that are similar to those known to be both highly conserved and crucially important to the multicellular development of animals.

There is no obvious reason to exclude the third great Kingdom of eukaryotes from such comparisons, because there is plenty of evidence that fungi undertake developmental processes every bit as sophisticated as those seen in animals or plants. This is true for the majority of the Ascomycota that produce small fruiting structures, but reaches a pinnacle of expression with the mushrooms and toadstools of the Basidiomycota. That sliced mushroom on your dinner plate clearly shows regular tissue formation with specialisation of cell and tissue function, and the briefest after-dinner amble outdoors will reveal such a diversity of fungal fruit bodies that it should be no surprise to find that form and structure are just as important in fungal taxonomy as they are in animal and plant systematics. Yet there is every reason to be cautious about assuming comparability too readily, because of the potential effect of the considerable differences in cell biology that exist between the three major Kingdoms of eukaryotes.

The fundamental aspect of cell biology that distinguishes fungi from other major kingdoms is the apical growth of hyphae. Extension growth of the hypha is limited to the apex, but there is more to development than growth at hyphal tips can achieve alone. The vegetative fungal mycelium is an exploratory, invasive organism. Its component hyphae are regulated to grow outwards into new territory and consequently possess controls that ensure that hyphae normally grow away from one another to form the typical 'colony' with an outwardly-migrating growing front. Tissue development requires that different hyphae cooperate in an organised way. For tissue to be formed the invasive outward growth pattern of the vegetative mycelium must be modified so that independent hyphal apices grow towards each other, allowing their hyphae to branch and differentiate in a cooperative fashion. Kinetic analysis has shown clearly that fungal filamentous growth can be interpreted on the basis of a regular cell cycle (Prosser, 1995), and (Griffin, Timberlake and Cheney, 1974), pointed out that in mycelial fungi, branch formation (by increasing the number of growing points) is the equivalent of cell division in animals, plants and protests. Common to normal morphogenesis in animals and plants alike is the concept of cellular polarity and the developmental consequences of precise positioning of the plane of cleavage (in animals) or wall formation at the cell plate or phragmoplast in plants (e.g. (Samuels, Giddings and Staehelin, 1995). The classic examples of embryology in both groups of organisms include instances of asymmetric divisions partitioning 'stem' cells in ways that result in the daughter cells expressing some sort

of differentiation relative to one another, though the differentiation is not necessarily expressed immediately.

However, cross-walls in fungal hyphae are almost always formed at right angles to the long axis of the hypha. Except in cases of injury or in hyphal tips already differentiated to form sporing structures, hyphal tip cells are ***not*** subdivided by oblique cross-walls, nor by longitudinally oriented ones. Even in fission yeast cells forced to produce irregular septation by experimental manipulation, the plane of the septum was always perpendicular to the plane including the longest axis of the cell (Miyata, Miyata and Johnson, 1986). In general, then, the characteristic fungal response to the need to convert the one-dimensional hypha into a two-dimensional plate or three-dimensional mass cannot depend on a different geometrical arrangement of the septum. The only solution open to the fungal hypha is the formation of branches and even casual observation shows that branching patterns alter greatly during fungal differentiation and tissue development. Consequently, placement of the hyphal branch (that is, its position of emergence and subsequent direction of growth) is the fungal equivalent of the determination of morphogenetic growth by orientation of the plane of division and the new cross-wall as is seen in plants, and the morphogenetic cell migrations that contribute to development of body form and structure in animals (Moore, 1998).

Viewed in this light, therefore, Kingdom Fungi is seen as employing processes during morphogenesis that have affinities with both of the other major eukaryotic kingdoms. The presumption that the three kingdoms have evolved their multicellular organisation independently, but using much the same set of genes inherited from their common unicellular ancestor, is the main reason for interest in making sequence comparisons between the genomes.

Currently, it is generally thought that eukaryotes emerged from prokaryotic ancestry a little less than 2 billion years ago (Gupta and Golding, 1996; Knoll, 1992), and in the present day about 60 lineages of eukaryotes can be distinguished on the basis of their cellular organization (Patterson, 1999). Most of these are traditionally classified as protists, but one lineage comprises green algae and the land plants, and another animals and fungi. Understanding of eukaryote phylogenetic relationships is not yet complete (Cavalier-Smith, 1993; Katz, 1998, 1999; Knoll, 1992; Kuma *et al.*, 1995; Kumar and Rzhetsky, 1996; Roger, 1999; Sogin *et al.*, 1996; Sogin and Silberman, 1998). The plant comparison is compelling because recent discussion has stressed the importance of the symbiotic partnership between phototrophs and fungi in early colonization of the land, protein sequence comparisons indicating that major fungal and algal lineages were present one billion years ago (Heckman *et al.*, 2001). Animals and fungi are more directly related, though. It is generally agreed that the Metazoa and choanoflagellates (collar-flagellates) are sister groups, and that these, together with the fungi and chytrids form a single lineage called the opisthokonts. This name opisthokont (Copeland, 1956) refers to the posterior (opistho) location of the flagellum (kont) in swimming cells. The term was applied to the (animals + fungi) clade (Cavalier-Smith and Chao, 1995) because comparative molecular analysis has indicated that fungi and animals are each other's closest relatives (Baldauf and Palmer, 1993; Baldauf, 1999; Patterson and Sogin, 2000; Sogin and Silberman, 1998; Wainright *et al.*, 1993).

So, accepting the opinion that fungi are more closely related to animals than to plants, we decided to search the genomic databases of several tissue-making filamentous fungi for sequences homologous to conserved genes involved in animal development biology. Tissue-making filamentous fungi, that is ascomycetes and

basidiomycetes known to form macroscopic fruit bodies, were chosen as it was felt that the cellular mechanics of their development pathways might be similar those of animals.

Several gene networks controlling animal development processes were identified as potential search targets. These were predominantly conserved sequences involved in controlling multicellular structure such as: transmembrane molecules used for cell adhesion and cell signalling; gene regulatory DNA-binding proteins that coordinate expression of other genes; and sequences involved in the processes of movement and/or targeting of moving cells.

We searched for homologues of *Caenorhabditis elegans* sequences involved in apoptosis and in the signalling mechanisms Notch, TGF-β, Wnt (including the MOM and POP genes) and also a Hedgehog sequence from *Drosophila melanogaster* (as *C. elegans* lacks a Hedgehog homologue). As an additional comparison, the genome of the plant *Arabidopsis thaliana* (Bevan *et al.*, 2001) at The Institute for Genome Research (TIGR) was searched as a control for each sequence search. These species were chosen as they represent good model organisms of animals and plants respectively and their complete DNA sequences are known and well annotated. The Wnt, Hedgehog, Notch and TGF-β signalling mechanisms are all absent in *Arabidopsis* but this plant has its own highly developed signalling pathways (Herve *et al.*, 1999; Katz *et al.*, 2004; Reyes and Grossniklaus, 2003; Thummler *et al.*, 1995; Walker, 1994; Yu and Tang, 2004). Filamentous fungal genomes were also explored for sequences from one of these, the ethylene hormone signalling pathway (Chang, 1996; Hua and Meyerowitz, 1998), to investigate whether fungal signalling shows any similarities to that plant system. *Arabidopsis* also lacks direct homologues of the caspases, enzymes normally involved in apoptosis in animals, but plants do possess distantly related sequences termed metacaspases, which perform a similar function (Watanebe and Lam, 2004). As caspase-like activity has recently been associated with entry into the stationary phase by cultures of *Aspergillus fumigatus* (Mousavi and Robson, 2003), and because of other observations discussed below that suggest programmed cell death occurs in several fungi, we included caspase sequences in our searches.

The filamentous fungal species selected for genome analysis were: the basidiomycetes *Coprinus cinereus* (syn. *Coprinopsis* (Redhead *et al.*, 2001)) and *Ustilago maydis* (Anon, 2003a, b) at the Whitehead Institute's Centre for Genome Research; *Cryptococcus neoformans* (last updated 29/4/03) at TIGR; *Phanerochaete chrysosporium* at the Department of Energy's (DOE) Joint Genome Institute; and the ascomycetes *Aspergillus nidulans* (Anon, 2003c) and *Neurospora crassa* (Galagan *et al.*, 2003), also at the Whitehead Institute's Centre for Genome Research; and *Aspergillus fumigatus* at TIGR (URLs are detailed in Table 1). These form a representative and accessible collection of tissue-making ascomycete and basidiomycete filamentous fungal genomic databases that are finished or nearing completion.

2. METHODS & MATERIALS

Default values were used throughout unless otherwise stated.

2.1 Locating Query Sequences

GenBank (Benson *et al.*, 2003), maintained by the National Centre for Biotechnology Information (NCBI) and the Nucleotide Sequence Database (Kulikova *et al.*, 2004), maintained by the European Molecular Biology Laboratory (EMBL),

were used to locate DNA sequences. Swiss-Prot, release 42.12 of 15-Mar-2004, TrEMBL Release 25.12 of 15-Mar-2004 (Bairoch and Apweiler, 2000), and GenBank (Benson *et al*., 2003) text searches were used to retrieve protein sequences.

2.2 Similarity Search

An e-value of 1e = -1 was used for each BLAST search. BLASTN (version 2.2.8, last updated Jan-05-2004) and BLASTP (version 2.2.8, last updated Jan-05-2004) at the NCBI (Altschul *et al.*, 1997) were used to search both nucleotide and protein sequences for similar sequences. The organism default parameter was altered so that only fugal sequences were searched.

WU BLASTN nucleotide-nucleotide and WU BLASTP protein-protein searches (both version 2.0, last updated Mar-3-2004; (Gish, 1996-2004) were performed with the gene and protein sequences respectively at the TIGR *Arabidopsis thaliana* (Bevan *et al.*, 2001) and *Cryptococcus neoformans* sites (see Table 1).

Table 1. Table displaying all query organisms used (one plant and seven filamentous fungi); where their genomic databases were accessed and their URLs

Organism	Source	URL
Arabidopsis thaliana	TIGR	http://www.tigr.org/tdb/e2k1/ath1/
Aspergillus fumigatus	TIGR	http://www.tigr.org/tdb/e2k1/afu1/
Cryptococcus neoformans	TIGR	http://www.tigr.org/tdb/e2k1/cna1/
Neurospora crassa	Whitehead Institute	http://www.broad.mit.edu/annotation/fungi/neurospora/
Ustilago maydis	Whitehead Institute	http://www.broad.mit.edu/annotation/fungi/ustilago_maydis/
Coprinus cinereus	Whitehead Institute	http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/index.html
Aspergillus nidulans	Whitehead Institute	http://www.broad.mit.edu/annotation/fungi/aspergillus/
Phanerochaete chrysosporium	DOE Joint Genome Institute	http://genome.jgi-psf.org/whiterot1/whiterot1.home.html

In the case of the TIGR *Aspergillus fumigatus* site (see Table 1), WU BLASTN nucleotide-nucleotide and WU tBLASTn protein-translated nucleotide searches were performed as the WU BLASTp function was not available.

BLASTN nucleotide-nucleotide and BLASTP protein-protein searches (both version 2.2.1, last updated Aug-1-2001; (Altschul *et al.*, 1997) were performed with the gene and protein sequences respectively at the Whitehead Institute's *Neurospora crassa* (update of 24/4/03) and *Aspergillus nidulans* (update of 31/10/03) sites (see Table 1).

BLASTN nucleotide-nucleotide and tBLASTn protein-translated nucleotide searches were performed at the Whitehead Institute's *Ustilago maydis* (28/5/03 update) and *Coprinus* (= *Coprinopsis*) *cinereus* (25/6/03 update) sites and also at the Department of Energy's (DOE) Joint Genome Institute *Phanerochaete chrysosporium*

site (v. 1.0, updated 16/2/02), as there was no BLASTp function available (see Table 1).

2.3 Further Analysis

The genome locating services provided by the websites in Table 1 were used to retrieve the protein and/or DNA sequence of a potential hit, and Internet sites listed in Table 2 were used for further analysis of protein and DNA sequences.

BLOCKS version 14.0, last updated October 2003 (Henikoff and Henikoff, 1994), and PFAM version 12.0, last updated January 2004 (Bateman *et al.*, 2004) were used to query DNA and protein sequences and to ascertain their domain families.

Interpro [13] release 7.0, last updated 27/11/2003 (Mulder *et al.*, 2003) and PFAM keyword searches were used to look up information on protein domains and families.

CLUSTAL-W version 1.82, (Higgins *et al.*, 1994) at the EMBL-EBI was used to construct two automatic multiple sequence alignments of metacaspases from the DNA and protein sequences respectively of a variety of organisms. The CLUSTAL-W phylogram showing tree distances were used to visualize phylogenetic differences between the sequences. A phylogram is a branching diagram (tree) assumed to be an estimate of phylogeny. In these trees, the branch lengths are proportional to the amount of inferred evolutionary change. The CLUSTAL-W output format default was then altered to give PHYLIP output and these results were analyzed using PAUP 4.0*, Beta 10 (Swafford, 1998). Phylogenetic analysis was performed using the distance method, and the trees produced were unrooted. In distance-based analysis, neighbour-joining searches (Saitou and Nei, 1987) were used. Analysis was performed using the uncorrected ("p") distance measure.

Table 2. Names and URLs of sites used for location and further analysis of protein and DNA sequences.

Site and/or tool	URL
GenBank	http://www.ncbi.nlm.nih.gov/Genbank/index.html
EMBL Nucleotide Sequence Database	http://www.ebi.ac.uk/embl/
Swiss-Prot & TrEMBL	http://us.expasy.org/sprot/
BLOCKS	http://www.blocks.fhcrc.org/
PFAM	http://www.sanger.ac.uk/Software/Pfam/
Interpro	http://www.ebi.ac.uk/interpro/
CLUSTAL-W	http://www.ebi.ac.uk/clustalw
PAUP*	http://paup.csit.fsu.edu/

3. RESULTS

3.1 Apoptosis

The seven filamentous fungal genomic databases, along with that of the plant *A. thaliana*, were first searched for sequences displaying similarity to the sequences of three genes involved in apoptosis in the *Caenorhabditis elegans*: ced-3, ced-4 and egl-1, which are homologues of a caspase, Apaf-1 and Bad respectively. Both the DNA and protein sequences of the above genes were used to search each of the query databases. Many low-quality results were returned but none of them were good enough to warrant further investigation.

The DNA and protein sequences of the yeast metacaspase YOR197w (also known as YCA1) were then used to search each of the query databases. There were no hits returned from the *C. cinereus, P. chrysosporium* and *U. maydis* databases. However, two hits with good e-values were found in each of the *A. nidulans, N. crassa, C. neoformans* and *A. fumigatus* databases. Also hits to several latex-abundant family protein/caspase family protein/metacaspases (AMC) were returned from the *A. thaliana* database.

3.1.1 Metacaspase Multiple Sequence Alignment

Using CLUSTAL-W, two metacaspase multiple sequence alignments, were performed using protein and DNA sequences respectively. Each multiple sequence alignment consisted of: the putative filamentous fungal metacaspases, two yeast metacaspases (one *S. cerevisiae* and one *S. pombe*) and three plant metacaspases (AMC1, 2 and 8 from *A. thaliana*).

The phylogram trees with distances displayed from CLUSTAL-W were used to visualize the phylogenetic distances between each sequence (Figs 1 & 2). The aligned sequences were then analyzed using PAUP 4.0* Beta-10 and unrooted neighbour-joining trees were returned and analyzed (data not shown).

The DNA metacaspase multiple sequence alignment differs from that of the protein in that it contains two extra *A. fumigatus* sequences. The TIGR *A. fumigatus* site was incomplete at the time of writing, and attaining gene and protein sequences proved very tough. Attempts to translate the *A. fumigatus* sequences were unsuccessful as the sequences acquired from the TIGR database were not complete genes and therefore contained many stop codons.

3.2 Signalling Mechanisms

The gene sequences investigated in this category were all from *C. elegans*, except for the Hedgehog gene from the *Drosophila melanogaster* (as the *C. elegans* lacks a Hedgehog homologue).

3.2.1 MOM & POP

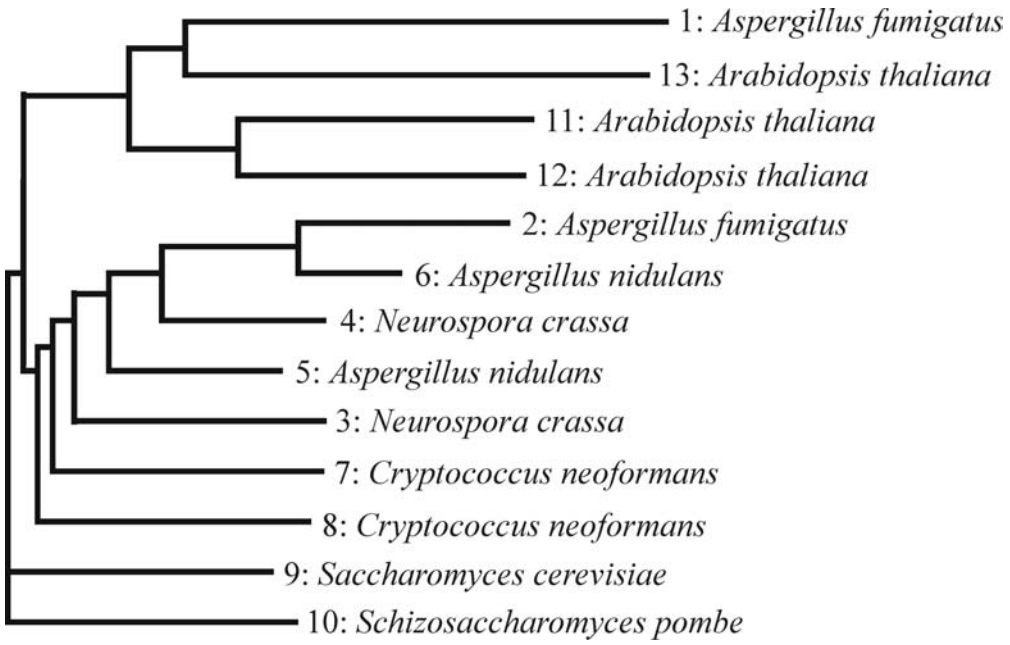
The MOM and POP gene sequences investigated here were the MOM-1, 2 and 5 and the POP-1 genes. The normal function of POP-1 is to prevent endoderm formation, thus the Wnt signal is required to prevent POP-1 function (Rocheleau *et al.*, 1997). BLAST searches of the genomic databases of the seven filamentous fungal species and the plant *A. thaliana* were performed for each MOM-1, 2 and 5 and pop-1. Many matches to small fragments were located but no significant matches were returned.

3.2.2 Wnt Signalling Pathway

Here we searched the seven filamentous fungal and the *A. thaliana* genomic databases for homologues of the Wnt-1, Wnt-2, Egl-20 and Lin-44 sequences. Their nucleotide and protein sequences were located and used to search the query databases, but again no quality hits were returned.

3.2.3 Hedgehog Signalling Pathway

The sonic hedgehog gene and protein sequences from the only *Drosophila melanogaster* hedgehog sequence were used to search the query databases. No quality hits were returned.



Seq. 1: *Aspergillus fumigatus*	chr_0|TIGR.5170|54
Seq. 2: *Aspergillus fumigatus*	chr_0|TIGR.5237|59
Seq. 3: *Neurospora crassa*	AABX01000434
Seq. 4: *Neurospora crassa*	AABX01000362
Seq. 5: *Aspergillus nidulans*	AF528964
Seq. 6: *Aspergillus nidulans*	AACD01000042
Seq. 7: *Cryptococcus neoformans*	185.m02420
Seq. 8: *Cryptococcus neoformans*	186.m04030
Seq. 9: *Saccharomyces cerevisiae*	NC_001147
Seq. 10: *Schizosaccharomyces pombe*	AF316601
Seq. 11: *Arabidopsis thaliana* (AMC1)	NM_100097
Seq. 12: *Arabidopsis thaliana* (AMC2)	NM_118643
Seq. 13: *Arabidopsis thaliana* (AMC8)	NM_101508

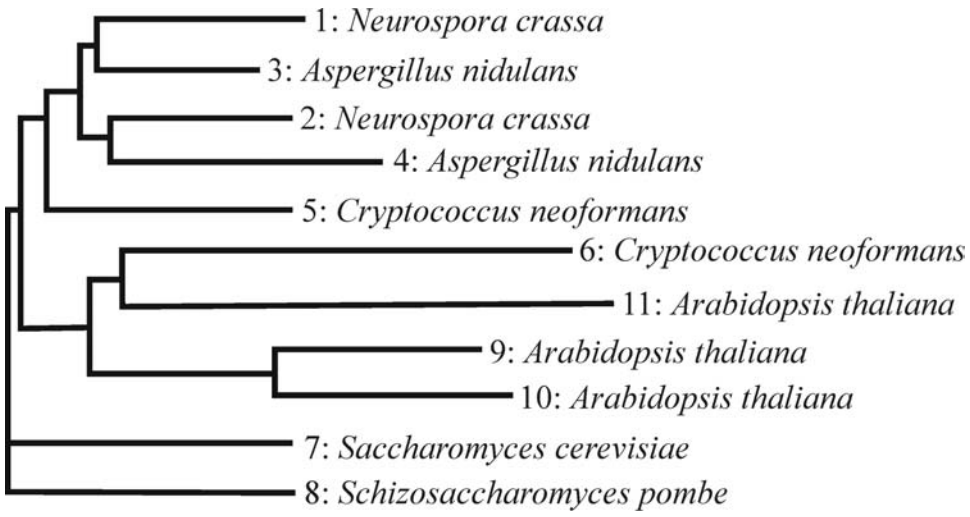
Fig. 1. CLUSTAL-W metacaspase nucleotide sequence phylogram showing tree distances. The top clade contains the three plant (*A. thaliana*) sequences and one of the *A. fumigatus* sequences (sequence number 1). The middle clade contains the rest of the filamentous fungal sequences. Sequences 9 and 10, which are the two yeast metacaspase sequences, essentially form an outgroup as the bottom clade which is quite distinct from the others. Branch lengths are proportional to the amount of inferred evolutionary change.

3.2.4 Notch Signalling Pathway

The notch sequences Glp-1 and Lin-12 were used to search the query databases. There were some low-quality hits. However, upon further investigation it was found that theses hits were all against an ankyrin repeat located in the protein sequences. There were no other quality hits returned.

3.2.5 TGF-β Signalling Pathway

Here the *C. elegans* TGF-β sequences of Daf-4, Daf-7, Dbl-1, Unc-129 and Cet-1 were used to search the query databases. No quality hits were returned.



Seq. 1: *Neurospora crassa*	EAA29413
Seq. 2. *Neurospora crassa*	EAA30484
Seq. 3: *Aspergillus nidulans*	AA013381
Seq. 4: *Aspergillus nidulans*	EAA63988
Seq. 5: *Cryptococcus neoformans*	185.m02420
Seq. 6: *Cryptococcus neoformans*	186.m04030
Seq. 7: *Saccharomyces cerevisiae*	YOR197
Seq. 8: *Schizosaccharomyces pombe*	CAA20127
Seq. 9: *Arabidopsis thaliana* (AMC1)	AAP84706
Seq. 10: *Arabidopsis thaliana* (AMC2)	AAP84707
Seq. 11: *Arabidopsis thaliana* (AMC8)	AAP84711

Fig. 2. CLUSTAL-W metacaspase protein sequence phylogram showing tree distances. Again the sequences divide into three main clades. One comprises the three plant proteins (sequences 9, 10 & 11), together with a *C. neoformans* (sequence 6) protein sequence. The two plant metacaspases AMC 1 and 2 (sequences 9 &10) seem to be quite distinct from the other plant metacaspase AMC 8 (sequence 11), which is the one that shows similarity to the *Cryptococcus* sequence. The rest of the filamentous fungal sequences (sequences 1, 2, 3, 4 & 5) fall into a distinct clade, leaving the two yeast sequences (sequences 7 & 8) isolated again, virtually as an outgroup. Branch lengths are proportional to the amount of inferred evolutionary change.

3.2.6 Plant Ethylene Signalling Pathway

The *A. thaliana* sequences of ESR1 and ETR1, which are receptors involved in the plant ethylene hormone signalling pathway, were used to search for homologues in the query fungal databases; but again no quality hits were returned.

4. DISCUSSION

The obvious motive for this sort of survey is to estimate how the different eukaryotic kingdoms have made use of their common genetic heritage since the divergence of the major eukaryotic clades, animals, fungi and plants, about 1×10^9 years ago. There is an essential underlying logic to morphogenesis and it is this that justifies sequence comparisons between organisms that are so very different today. Since their last common ancestor was unicellular, complex multicellular development must have arisen independently in the three kingdoms, so comparison of the way similar functions are controlled can reveal whether and how different cellular mechanisms have been used to solve common developmental demands (Meyerowitz, 1999). However, bearing in mind the considerable differences that exist in the basic

cell biology of animals, fungi and plants, similarities between the mechanisms used in their developmental biologies may be hard to find.

In this initial survey we have attempted to establish whether fungal multicellular development shows any closer relationship to that of animals than to that of plants by searching filamentous fungal genomic databases for sequences demonstrating similarity to developmental gene sequences. The phylogenetic logic of this approach is that the plant lineage was the first to diverge, leaving animals and fungi in a common clade (the opisthokonts) for potentially several hundred million years before diverging into the distinct kingdoms we know today. It is not unreasonable to argue that the opisthokonts evolved basic strategies for dealing with cellular interactions prior to their divergence and that evidence of this might be found in present day genomes in the form of similarities between sequences devoted to tasks that can be defined broadly as 'developmental'. On the other hand, as 41% of the predicted proteins of the *Neurospora crassa* genome lack significant matches to known proteins from public databases (Galagan *et al.*, 2003) it is not at all impossible that fungi have their own unique development processes using genes found exclusively in fungi.

4.1 Programmed cell death

Programmed cell death (apoptosis) is one of the central cellular processes in multicellular eukaryote development (Hentgartner, 2000), and there are many observations (discussed below) that imply that programmed sacrifice of hyphal segments is important in fungal development. However, no positive hits were recorded in the query databases to the sequences of the three genes involved in apoptosis in *Caenorhabditis elegans*. This result may be expected as no caspase homologue has yet been found in fungi (Thrane *et al.*, 2004), though sequences characteristic of the distantly related metacaspases have been found in fungi, plants and protozoa (Wu *et al.*, 2003). With these findings in mind, we chose the yeast metacaspase YOR197w (also known as YCA1), which is involved in regulation of apoptosis in *Saccharomyces cerevisiae* (Madeo *et al.*, 2002), to search the filamentous fungal query databases.

C. cinereus, P. chrysosporium and *U. maydis* did not contain a metacaspase homologue but *A. nidulans, N. crassa, C. neoformans* and *A. fumigatus* all contained two. The two *N. crassa* hits corresponded to the two hypothetical metacaspases previously deduced (GenBank Accession No. XP-331176 and XP-330804); and one of the *A. nidulans* hits corresponded to the recently cloned and described casA metacaspase (GenBank Accession No. AAO13381) (Cheng *et al.*, 2003). The many hits encountered in *Arabidopsis thaliana* were to be expected as it is known that plants possess metacaspases.

4.1.1 Metacaspase Multiple Sequence Alignment

In the DNA (Fig. 1) and protein (Fig 2) multiple sequence alignments of the metacaspase sequences two main regions showing high similarity were observed, which are assumed to correspond with metacaspase apoptotic domains. The trees returned by CLUSTAL-W and PAUP were very similar, comprising clades that contained essentially the same groups of sequences, which were at similar distances from each other.

The DNA sequence phylogram tree returned by CLUSTAL-W (Fig. 1) showed three main clades: one containing the two yeast sequences; one containing the three plant sequences along with one of the *Aspergillus fumigatus* sequences; and the other containing the rest of the filamentous fungal sequences. The two yeast metacaspases

from *S. cerevisiae* and *S. pombe* formed an outgroup. In the earlier BLAST searches the *A. fumigatus* sequence that associated with the *Arabidopsis thaliana* metacaspases returned the inferior e-value and is obviously more similar to the plant metacaspases than to those of the other filamentous fungi. However, the plant metacaspase to which it seems most closely related (AMC 8) is somewhat distant from the other two (AMC 1 and AMC 2).

The protein multiple sequence alignment tree displayed similar results. Fig. 2 shows three main clades: with the two yeast metacaspases (sequences 7 and 8) forming an outgroup and the plant metacaspases (sequences 9, 10 and 11), being associated with a filamentous fungal sequence (sequence 6). In this case the fungal sequence found within the plant clade is one of the *C. neoformans* sequences. It is worth noting that the *C. neoformans* sequence that clusters with *Arabidopsis* in Fig. 2 is the most distantly related to the other filamentous fungal sequences in the DNA tree shown in Fig. 1. The main clade in Fig. 2 is comprised of the rest of the filamentous fungal sequences (sequences 1, 2, 3, 4 and 5). Evidently, several fungal genomes contain metacaspase homologues, there being one in ascomycetous yeasts, and two in several filamentous fungi.

In some cases it is likely that such sequences will prove to be responsible for the caspase-like activities that have been associated with sporulation (Thrane *et al.*, 2004) and the onset of stationary phase lysis (Mousavi and Robson, 2003) in *Aspergillus* species. There are many other reports that suggest dependence on a programmed cell death (PCD) in fungi. Interestingly, some of these observations relate to *Coprinus* (= *Coprinopsis*) *cinereus* despite the fact that we have been unable to find any caspase or metacaspase homologues in this genome. Recently, for example, (Lu, Gallo and Kües, 2003) have presented elegant cytological evidence for 'apoptotic' DNA degradation in basidia of meiotic mutants of the mushroom *C. cinereus*; a compelling comparison with animal apoptosis where specific DNA fragmentation is an essential component. Hasty comparison with vertebrates in which avoidance of antigen release as the cell dies is a primary 'design requirement' to protect the animal against autoimmunity may be a mistake. Protection against autoimmunity is not a consideration in fungi; rather such a process in a mushroom may well be a matter of resource conservation (the *Coprinopsis* mutants cannot complete meiosis so there is presumably some value in recycling the contents of the defective basidia)(Money, 2003).

There may well, in fact, be several different mechanisms, reflecting the several different roles, for PCD in fungi. Cell death is a common occurrence in various structures starting to differentiate, for example the formation of gill cavities in the cultivated mushroom *Agaricus bisporus* (Umar and van Griensven, 1997, 1998). These authors point out that specific timing and positioning imply that cell death is part of the differentiation process and that fungal PCD could play a role at many stages in development of many species. Individual hyphal compartments can be sacrificed to trim hyphae to create particular tissue shapes. PCD is used, therefore, to sculpture the shape of the fruit body from the raw material provided by the hyphal mass of the fruit body initial and primordium (Moore *et al.*, 1998). Several examples detailed by Umar & van Griensven (1998) feature a PCD programme that involves the sacrificed cells over-producing mucilaginous materials that are released by cell lysis. Probably the most obvious example of fungal PCD is the autolysis that occurs in the later stages of development of fruit bodies of ink-cap mushrooms (many species of *Coprinus*, *Coprinopsis* and *Coprinellus*) that involves specifically-timed production and organised release of a range of lytic enzymes (Iten, 1970; Iten and Matile, 1970). This autolysis in coprinoid fungi has been interpreted as a mechanism to remove gill

tissue from the bottom of the cap to avoid interference with discharge of spores from regions above (Buller, 1924, 1931). This interpretation of autolysis being part of the developmental programme of a fungal fruit body predates by 40 years the first use of the phrase programmed cell death (Lockshin, 1963; Lockshin, Zakeri and Tilly, 1998).

4.2 Signalling Mechanisms

Cell-cell signalling is essential for many biological processes ranging from developmental patterning to the regulation of cell proliferation and cell death. In addition, almost all cancer cells have mutations in genes involved in signalling. The animal signalling mechanisms investigated here were the Wnt, Hedgehog, Notch and TGF-β. It is known that they are all absent in *Arabidopsis,* but plants compensate with their own highly developed signalling pathways (Meyerowitz, 1999).

4.2.1 Wnt Signalling Pathway

The Wnt signalling pathway is highly conserved and functions during the development of many animals by regulating processes such as cell proliferation, cell polarity, mitotic alignment and the specification of cell fate. Inappropriate expression of the Wnt pathway is implicated in tumorigenesis (van Es, Barker and Clevers, 2003). The Wnt genes encode secreted glycoproteins that regulate a huge variety of developmental processes in vertebrates and invertebrates by inducing transcriptional or morphological changes in responding cells. The Wnt family comprises 18 members in humans, four members in *Drosophila* (the best-studied being Wingless), five in *Caenorhabditis elegans* and at least one in *Hydra* (Huelsken and Birchmeier, 2001; Kalderon, 2002; Seto and Bellen, 2004; Thorpe, Schlesinger and Bowerman, 2000).

The MOM and POP genes investigated here all play a role in the Wnt signal transduction pathway in *C. elegans*. The MOM-1, 2 and 5 and the POP-1 genes are all essential in providing polarity signals that distinguish endoderm from mesoderm in the early embryo (Thorpe *et al.*, 1997). POP-1 distinguishes the fates of anterior daughter cells from their posterior sisters throughout development as part of a MAP kinase-like signalling mechanism (Shin *et al.*, 1999). The MOM-2 gene encodes a member of the Wnt family of proteins, while MOM-5 encodes a protein that resembles the mammalian Wnt receptor (the Frizzled protein). The MOM-1 gene appears to encode a protein needed for the secretion of the Wnt protein. The normal function of POP-1 is to prevent endoderm formation, thus the Wnt signal is required to prevent POP-1 function (Rocheleau *et al.*, 1997). The lack of highly positive hits suggests that filamentous fungi lack the Wnt signal transduction pathway. This notion was further explored by searching for Wnt proteins, but again we had no success. Thus, the lack of hits to several components of the pathway adds weight to the conclusion that the Wnt signalling pathway is absent from fungi.

4.2.2 Hedgehog Signalling Pathway

Hedgehog proteins belong to a smaller family of secreted signal molecules that act as local transcriptional mediators serving as key organizers of tissue patterning in many developmental processes in animals. Although they are notably absent from some invertebrates, the prime example of this deficiency being *Caenorhabditis elegans*, signalling proteins of the Hedgehog family are generally considered to be essential for patterning and morphogenesis in most multicellular animals (Nybakken and Perrimon, 2002; Stark, 2002; Tabin and McMahon, 1997). There are similarities between the Wnt and Hedgehog pathways, and, like Wnt, misregulation of Hedgehog

leads to several disease states in humans (Kalderon, 2002; Mullor, Sanchez and i Altaba, 2002; Wetmore, 2003). No quality hits were returned from our search of the filamentous fungal genomes with Hedgehog gene and protein sequences from *Drosophila melanogaster*, suggesting that fungi also lack the Hedgehog signalling pathway.

4.2.3 Notch Signalling Pathway

Notch is a large cell-surface receptor that is activated by contact with membrane-bound ligands on neighbouring cells. Signalling through notch receptor proteins is probably the most widely used signalling pathway in animal development. It is involved in several developmental pathways with a widespread role in the determination of cell fates, including defining the two different cell fates that result from asymmetric cell divisions, and in signalling events that establish tissue boundaries (Fleming, Purcell and Artavanis-Tsakonas, 1997; Martinez Arias, Zecchini and Brennan, 2002; Weinmaster, 2000). Notch signalling also defines the future dorsal-ventral axis in nematode embryos, and is one of the systems implicated in the establishment of a segmental pattern within the vertebrate body plan (Bessho and Kageyama, 2003; Dubrulle and Pourquié, 2002; Pourquié, 1999).

A search for homologues of the *Caenorhabditis elegans* Notch sequence in genomes of filamentous fungi yielded hits only to the ankyrin repeat located within that sequence. According to InterPro, the ankyrin repeat is one of the most common protein-protein interaction motifs in nature and occurs in a large number of functionally diverse proteins from eukaryotes (Mulder *et al.*, 2003). Therefore, we conclude again that the lack of quality returns suggests that the Notch signalling pathway is also absent from filamentous fungi.

4.2.4 TGF-β Signalling Pathway

The transforming growth factor-β (TGF-β) superfamily consists of a large number of molecules that act either as cytokine hormones, or more commonly as local mediators. During development they regulate pattern formation and influence various cell behaviours, including proliferation, differentiation, extracellular matrix production and PCD (Massague, Hata and Liu, 1997; Souchelnytskyi, Moustakas and Heldin, 2002). Lack of quality hits to the *Caenorhabditis elegans* TGF-β sequence in a search of the genomes of filamentous fungi suggests that this signalling pathway is also absent from these fungi.

4.2.5 Plant Ethylene Signalling Pathway

From the results outlined above, we can conclude that all four of the Wnt, Hedgehog, Notch and TGF-β signalling pathways are absent from the seven query fungi; just as they are absent in plants. In compensation, plants have their own highly developed signalling pathways. In the ethylene signalling pathway the simple two-carbon gas ethylene functions as a plant hormone. It is important at many stages of a plant development including: germination, flower development, fruit ripening and responses to many environmental stimuli (Muller-Dieckmann, Grantz and Kim, 1999; Stearns and Glick, 2003; Zhao *et al.*, 2002). Ethylene perception and signal transduction into the cell are carried out by a family of membrane-bound receptors of which ETR1 and ESR1 are members. Again no substantive similarities were returned by a search of fungal genomes using the *Arabidopsis thaliana* ETR1 and ESR1 sequences, suggesting that fungi also lack the plant hormone ethylene signalling pathway.

4.3 General discussion

The sort of survey we present here is predicated on the assumption that there is a fundamental logic to development that is inescapably shared by the different eukaryotic kingdoms. As a unicellular system develops into a multicellular organism, a need for intercellular communication is assumed. As soon as two cells collaborate there will be a need for differential gene expression, for polarity, and for positional awareness. There will be a need to assign cell fates, and to control sequential and synchronous events. Numerous roles can be imagined for both intra- and intercellular trafficking, with a consequential need for mechanisms capable of signal creation, transmission, reception, and eventual action and these are represented among the animal sequences we have used here.

It is further expected that if two extant kingdoms shared a common ancestor that itself evolved at least some way towards multicellularity, the mechanisms then developed would survive through phylogenetic change as sequence homologies between the lineages. This expectation is fulfilled in all those aspects that characterise eukaryotic unicells. Interestingly, this does include some features that are important in morphogenesis. One example is the Armadillo sequence repeats that form a conserved three-dimensional protein structure functioning in intracellular signalling, membrane docking and cytoskeletal regulation (Coates, 2003; Wang *et al.*, 2001). Another example is the MADS-box domain, which is found in a diverse range of eukaryotes and reveals conservation of a transcriptional regulator (Alvarez-Buylla *et al.*, 2000; Krüger *et al.*, 1997).

Nevertheless, we have demonstrated here that several major components of animal cell interaction do not have homologies in fungal genomes. There seem to be three inferences that are not necessarily mutually exclusive. First, this circumstance may indicate that fungal and animal lineages diverged from their common opisthokont line well before the emergence of any multicellular arrangement and have consequently evolved all aspects of multicellular management independently.

Second, the cell biology of fungal cells may be so different from that of animals (as outlined in section 1) that responses to the basic ‘logical’ demands of multicellular development are equally different. In this respect it is significant that a new vector-based mathematical model of hyphal growth (the Neighbour-Sensing model) shows that fruit bodies can be simulated by applying the same regulatory functions to all of the growth points active in a structure at any specific time (Meškauskas, McNulty and Moore, 2004). Shape of the fruit body emerges from the concerted response of the entire population of hyphal tips, in the same way, to the same signals. This at least suggests the possibility that control of multicellular development in fungi is radically different from that in animals and plants, and may be indicating that fungal tissues can get by without much cell-to-cell communication. The implication could be that a multicellular system that depends exclusively on apical growth must comply with a far simpler set of rules, and the simplicity of the rule set is reflected in cell signalling mechanisms that tend to employ very basic metabolites rather than hormones or cytokines (Moore, 1998; Novak Frazer, 1996).

Third, it is possible that this initial survey has failed to identify homologies which exist because, perhaps, the animal gene sequences selected, or the fungal genomes examined, or both, were in some way unrepresentative.

Only further more comprehensive analysis will decide between these possibilities, but we believe that the first and second inferences combined make the most likely conclusion.

5. CONCLUSION

Searching what we believe to be a representative collection of filamentous fungal genomes with gene sequences generally considered to be essential and highly conserved components of normal development in animals failed to reveal any homologies. We conclude this indicates that fungal and animal lineages diverged from their common opisthokont line well before the emergence of any multicellular arrangement and that the unique cell biology of filamentous fungi has caused control of multicellular development in fungi to evolve in a radically different fashion from that in animals and plants. A more comprehensive analysis should confirm this.

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