Journal of Nematology 35(2):194–197. 2003.

Evolution of Parasitism in Nematode-Trapping Fungi[1]

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Abstract: We are studying the evolution of parasitism in a group of soil-living ascomycetes that can grow as saprophytes as well as parasites by forming special morphological structures called traps. Analyses of 18S ribosomal DNA sequences have shown that these fungi form a monophyletic and isolated clade among the ascomycetes. The phylogenetic patterns within this clade are concordant with the morphology of the traps and separate species having adhesive traps (nets, knobs, and branches) from those having constricting rings. This suggests that these nematode-trapping fungi have a common ancestor, and that the ability to capture nematodes has been an important trait for further speciation and diversification within the clade. To obtain information on the genomic basis for this pattern, we recently started a large-scale sequencing project of the nematode-trapping fungus *Monacrosporium haptotylum*. This will allow the identification of genes uniquely expressed during the development of traps, and elucidate the molecular evolution of such genes within the nematode-trapping fungi clade.

Key words: expressed sequence tags, functional genomics, phylogeny.

Nematode-trapping fungi: The nematode-trapping fungi are a group of soil-living hyphomycetes that capture nematodes using specialized structures called traps. The morphological structure of the traps differs depending on the species and can be divided into four major categories: adhesive nets, adhesive knobs, adhesive branches, and constricting rings (Fig. 1). The species with adhesive cells capture nematodes using extracellular polymers that accumulate at the site of attachment between the fungus and the nematode (Tunlid et al., 1992). Such polymers are not found on the cell surface on the constructing rings. These cells can rapidly swell when a nematode is passing through the ring and thereby physically ensnare the nematode.

The dramatic ways in which the nematophagous fungi can infect nematodes have intrigued mycologists for centuries. They serve as an excellent model system for understanding fungal host interactions and parasitism because they are easy to grow in the laboratory and transformation protocols are available (Tunlid et al., 1999). In addition, the nematode *Caenorhabditis elegans* can be used as a host for many nematode-trapping fungi, which makes it possible to examine the molecular mechanisms of infection in detail. Finally, interest in studying nematode-trapping fungi also comes from their potential use as biological control agents against plant and animal parasitic nematodes (Kerry, 2000; Larsen, 2000).

Phylogenetic analysis: The phylogenetic relationships between the different nematode-trapping species needs to be known to understand the evolution of parasitism in nematode-trapping fungi. A phylogenetic tree of nematode-trapping fungi was reconstructed using 18S rDNA sequences (Fig. 1; Ahren et al., 1998). The tree is concordant with the morphology of traps rather than the morphology of conidia and coniciophores. Thus, the taxonomy within this group of fungi needs reevaluation (Hagedorn and Scholler, 1999). In addition, the phylogenetic tree shows a topology separating the species having adhesive traps from those having constricting rings. The fact that the non-parasitic *Dactylella* species divide the two parasitic clades suggests that the evolution of the nematode-trapping fungi may have evolved as two separate events—one leading to the constricting rings and one leading to the parasitic ability of the adhesive trap forming fungi. That scenario might suggest that the two *Dactylella* species have never had the ability to parasitize on nematodes. The other possibility is that the parasitism evolved once and the *Dactylellas* subsequently lost its parasitic ability. One way to distinguish between these two alternatives would be to identify unique virulence genes in the nematode-trapping species and examine whether the *Dactylellas* have these genes, or at least pseudogenes of them.

Several other phylogenetic trees on nematode-trapping fungi have been published that are based on the analysis of ribosomal genes (Hagedorn and Scholler, 1999; Liou and Tzean, 1997). The conclusions from these studies, which are slightly different from that of Ahren et al. (1998), are briefly discussed here. The topology of the reconstructed tree presented by Liou and Tzean (1997) is similar to the tree by Ahren et al. (1998) (Fig. 1), but the rooting of the trees differ. Tzean and Liou used the nematode-trapping fungi *Monacrosporium phymatopagum* as an outgroup, which, however, might not be a basal taxon. The choice of *M. phymatopagum* as an outgroup was based on a tree proposed by Rubner (1996), where a simple form of trap (like trapping nematodes directly on hyphae) was assumed to be "primitive" compared to a more complex trap (such as an adhesive net). However, there are difficulties in assuming that this "primitive" state is closer to the ancestral state. Loss of traits might be common among parasitic fungi because an efficient parasite can lose its ability to be a competitive saprophyte. In our reconstruction several different taxa (orders) of ascomycetes were used as outgroups to make sure that the rooting of the tree was correct.

Hagedorn and Scholler (1999) used the 3′ end of

Received for publication 24 June 2002.

[1] Symposium paper presented at the 40th Annual Meeting of The Society of Nematologists, 25–29 August 2001, Salt Lake City, UT.

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This paper was edited by B. C. Hyman.

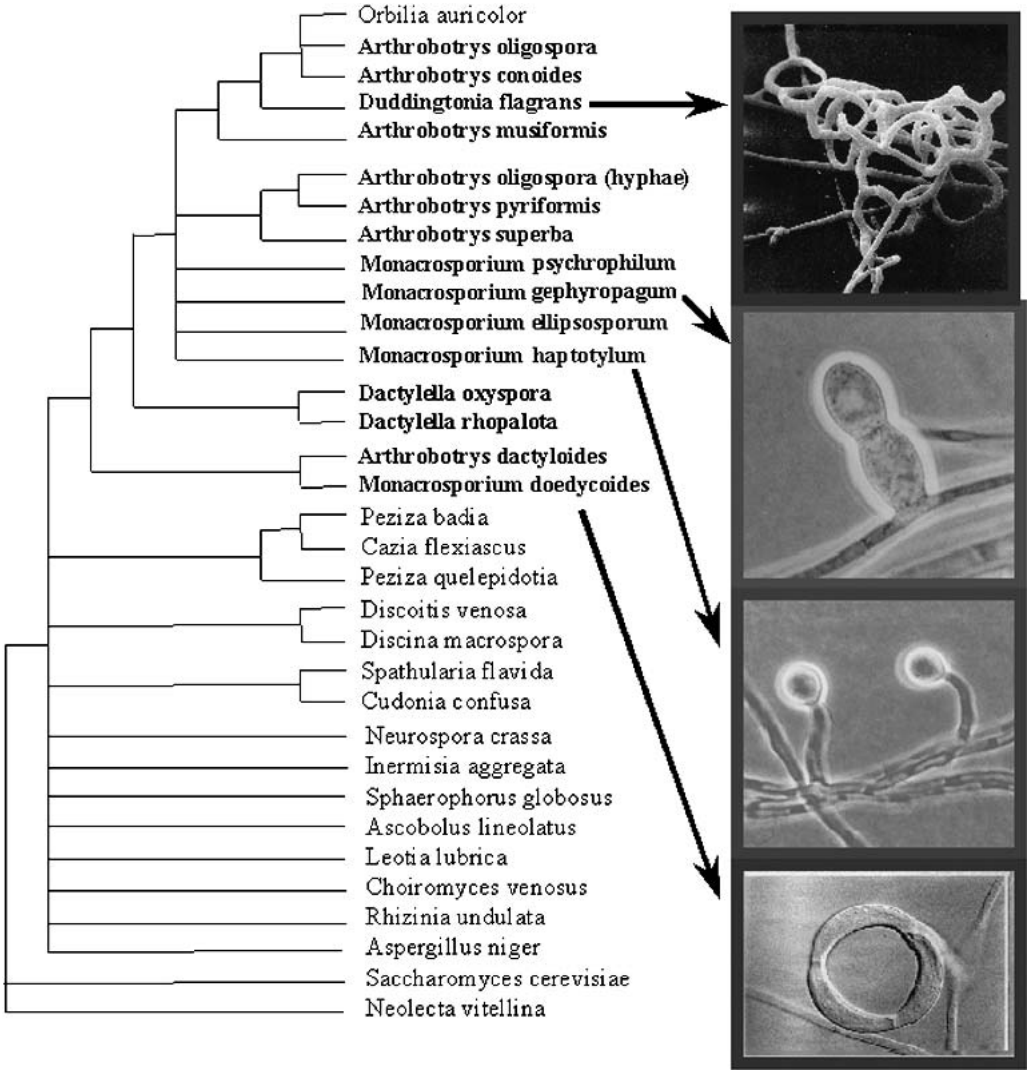

FIG. 1. Redrawn from Ahren et al. (1998). A reconstructed phylogenetic tree of 15 nematode-trapping species and 17 other ascomycetes using 18S rDNA sequences. Only bootstrap values above 50% are shown. Pictures reproduced from Nordbring-Hertz et al. (1995), courtesy of IWF, Göttingen.

18S rDNA, and the ITS1 and ITS2 sequences for phylogenetic analyses. The trees obtained were very similar to the result obtained by the phylogenetic reconstruction based on the full 18S rDNA sequences (Fig. 1). However, Hagedorn and Scholler could not confirm the position of the non-predacious *Dactylellas* between the nematode-trapping fungi that have adhesive traps and those having constricting rings. Our findings give some indication, but with low bootstrap values. To verify the position of the *Dactylellas*, more genes need to be sequenced and analyzed to give better resolution. Work is under way using β-tubulin and calmodulin, as well as *PII*, a serine protease (Åhman et al., 1996), and initial results indicate that the resolution will be significantly improved. A second advantage in using several regions of the genome is that it minimizes the risk of ending up with a gene tree instead of a species tree. The above studies suggest that these nematode-trapping fungi have a common ancestor and that the ability to capture nematodes has been an important trait for further speciation and diversification within the clade. At the genetic level there are basically three mutually non-exclusive explanations that can account for the origin of parasitism in nematode-trapping fungi:

(i) Parasitism of nematodes is a result of selection on regulatory genes. Thus, closely related parasitic and non-parasitic species have basically the same set of genes, and adaptation to the nematode parasitic habit is due to differences in regulation of gene expression.
(ii) Parasitism is due to the presence of novel genes. Novel genes can be acquired through gene duplications or horizontal gene transfer (HGT).
(iii) Parasitism is due to the loss of specific genes.

To test which of the alternatives of hypothesis are

responsible for evolution of nematode parasitism in fungi, an extensive knowledge of the genetic basis of fungal infections of nematodes is needed. For example, regulatory genes controlling the development of infection structures and the colonization of host tissues need to be identified. Genome sequences of closely related fungal species, varying in pathogenicity, must be compared to identify novel gene and possible gene losses. At present, this type of information is not available for any nematode-trapping fungi.

Genomics of nematode-trapping fungi: The rapid development of methods within genomics and functional genomics has opened up new possibilities for obtaining information on the genetic basis of interactions between various organisms such as parasitic fungi and their hosts (Tunlid, 1999). The methods include large-scale sequencing, mutagenesis, and molecular phylogenetic analyses.

In the absence of a sequenced genome, an expressed sequence tag (EST) approach can be a comparably inexpensive and fast alternative to obtain sequence information of coding regions in an organism. As with all large-scale sequencing projects, bioinformatics tools have to be applied to analyze the large amounts of data generated. To make it possible to compare sequences from various tissues (e.g., vegetative mycelium, traps) and species, we have developed a Web-based visualization tool, PHOREST, for comparative EST projects (Ahren et al., unpubl.).

We have started an EST sequencing project to investigate the genomic diversity of the nematode-trapping fungi *Monacrosporium haptotylum* that captures nematodes using adhesive knobs. When grown in liquid cultures with aeration, the knobs detach from the vegetative mycelium and can be collected as a separate fraction after filtration (Friman, 1993). The infection structures are fully functional and can capture and infect a nematode. Three separate cDNA libraries have been constructed: one containing transcripts from the vegetative mycelia, a second from the isolated infection structures (knobs), and a third library with transcripts from knobs infecting a nematode ("Infection Library"). *Caenorhabditis elegans* was chosen as the host. Because the genome of *C. elegans* is sequenced (The *C. elegans* Sequencing Consortium, 1998), it is possible to differentiate between fungal and nematode sequences in the "Infection Library." The "Infection Library" was harvested 24 hours after infection, and then at least 95% of the nematodes were completely immobilized and considered to be killed by the fungus.

The gene discovery process was conducted using PHOREST (Ahren et al., unpubl.). So far, a total of 4,767 clones have been sequenced from the three cDNA libraries (Table 1). As EST sequences were added to the database, the sequencing was conducted so that the level of redundancy was kept similar between the libraries. Redundancy is one way to estimate the likelihood of a sequence being unique. Because the complexity of the transcriptome was much higher for the "Infection Library," more than 2.5 times as many sequences were needed to give approximately the same redundancy. When comparing the different cDNA libraries, very few clusters contained sequences from more than one library. A large percentage (92.4%) of the transcripts in this study was unique for one of the stages investigated. This clearly shows that most of the genes expressed in the infection structures (knobs), as well as during infection of the nematode, are different when compared to each other and to the vegetative mycelium. Assembling the 4,767 transcripts using CAP (Huang, 1992) gave a total of 2,006 clusters. These clusters are considered to represent putatively unique transcripts. Out of these 2,006 clusters, a large fraction showed no sequence similarity (FASTA score <1) against the non-redundant protein database (nr) from NCBI. A larger proportion of these sequences, denoted orphans, were found in the "Infection Library."

Sequences with a FASTA score above 100 were annotated using 11 different functional categories according to the yeast genome classification of MIPS (Mewes et al., 1997). The annotated sequences were 120 (26.6%) in the Mycelium, 79 (26.1%) in the Knobs, and 147 (11.7%) in the "Infection Library." In the "Mycelium Library," the largest functional categories were metabolism, protein destination, and protein synthesis. In the "Knob Library," transcripts belonging to the protein synthesis group dominated. The "Infection Library" was more complex, with several well-represented categories, such as metabolism, energy, cell growth, and protein synthesis. When comparing the three libraries,

TABLE 1. Data on clusters and sequence similarities of the analyzed ESTs from *M. haptotylum*.

	ESTs			Sequence homology (FASTA/GenBank) (Scores)			
Library		ESTs		High (>299)	Moderate (100–299)	Low (1–99)	Orphans (<1)
Mycelium	1,067	498	321 (64%)	10%	27%	13%	50%
Knobs	1,004	372	265 (71%)	13%	19%	11%	57%
Infection	2,696	1,328	948 (71%)	5%	21%	13%	61%
Total	4,767	2,198					

only a small fraction had clusters in more than one library, indicating that different sets of genes are expressed during development of traps as well as during infection when compared to the vegetatively growing mycelium.

Conclusion

Phylogenetic analyses of ribosomal sequences have shown that the nematode-trapping ascomycetes have a common ancestor and are closely related. We are, however, far from understanding the genomic basis for the evolution of parasitism within this group of fungi. Using large-scale sequencing in combination with microarray analyses should allow identification of regulatory genes involved in trap formation, as well as presence of novel genes and possible gene losses in various species of nematode-trapping fungi. In addition, the obtained information also can be used to enhance the biocontrol activity of nematode-trapping fungi. For example, novel probes can be developed to screen for more virulent strains of fungi. Enhanced strains also could be obtained by genetic engineering. Alternatively, pathogen genes encoding enzymes, toxins, etc. could be transferred into other organisms that are present in the habitat of the parasitic nematodes (e.g., a host plant) (Tunlid and Ahren, 2001).

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