approach, because uncorrelated measurables would be preferable for the reconstruction of multiple signals.

Second, isotope abundance can be measured for specific intramolecular positions of a metabolite (isotopomer distribution; see the glucose formula in Fig. 1). For example, it has been shown that the abundance of ^{18}O and D varies among the intramolecular positions of the glucose units of cellulose (Betson *et al.*, 2006; Sternberg *et al.*, 2006). For ^{13}C, isotopomer abundance variation is very likely for cellulose, as it has been observed in plant glucose (Schmidt, 2003). Isotopomer variation reflects the fact that each intramolecular position of a metabolite has a distinct biochemical history; therefore, each isotopomer abundance can, in principle, carry an independent signal. For example, the oxygen and hydrogen atoms bound to carbon 2 of glucose exchange strongly with source water during cellulose synthesis (Sternberg *et al.*, 2006; Augusti *et al.*, 2006). Therefore, the respective isotopomer abundances can be expected to be independent of all leaf-level processes and to depend exclusively on source water isotope abundance. This may open a way to reconstruct the source water isotope abundance, allowing temperature reconstruction and resolving the complication of overlapping environmental and physiological influences. With the source water isotope abundance reconstructed, the work of Gessler *et al.* might then be used to detect a humidity signal. Along this strategy, several signals may be reconstructed using isotopomer abundances in tree rings. This strategy may allow the detection of adaptations of trees to environmental changes on time scales of centuries, a largely unaddressed question in global change research.

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Key words: evaporative enrichment, isotope effect, isotopomer, reconstruction, stable isotopes, tree ring.

Rhynie chert: a window into a lost world of complex plant–fungus interactions

Using material from a block of 400-million-year-old Rhynie chert about half as big as a brick, Krings *et al.* (this issue; pp. 648–657) studied fungi populating 250 rhizomes from *Nothia aphylla*, an early vascular plant. As Taylor *et al.* (2004) pointed out, most palaeobotanists choose to study beautifully preserved plant material. Krings *et al.* have made one of the first studies where researchers instead examined a large number of creeping, underground rhizomes in various stages of fungus-induced distortion or degradation.

Modern plants distinguish friends from foes and respond differently to each. Krings *et al.* sought evidence of active plant responses to fungi in the silicified cellular anatomy of colonized *N. aphylla* rhizomes. Even in the Devonian, early in land plant evolution, *N. aphylla* cells responded differently to each of three different fungal colonists. Depending on the fungus, cells in the *N. aphylla* rhizomes increased the thickness of, and pigmentation in, their cell walls, increased their cell size, or encased fungal hyphae in plant cell wall material. All these morphological responses fall within the range of those shown by modern plants to invasion (Beckman, 1980).

'Some difficult puzzles are posed by this Rhynie material, such as, how were the spores from fungi dispersed when they were packed into host cells?'

Phylogenetic analysis and divergence time estimates

Before returning to Devonian fungus–plant interactions, we will consider which lineages of extant terrestrial organisms were already present in the Devonian, based on fossils and on phylogenetic evidence; our conclusions are summarized in Fig. 1. For the fungi and protist outgroups, we estimated ages using sequences from James *et al.*'s (2006) multigene, 214-taxon phylogeny and four calibration points. As calibration points for the fungi, we assumed that the common ancestor of the Glomeromycota, the Ascomycota and the Basidiomycota originated after the origin of terrestrial plants, ~500 Ma based on dispersed spores (Gray, 1985). We assumed that the Glomeromycota stem lineage was at least as old as 460 Myr based on Glomeromycota-like spores from the Ordovician (Redecker *et al.*, 2000). We fixed the time of the divergence of monocots from dicot plants at 168 Ma (Magallón & Sanderson, 2005) and that of the first divergences among the multicellular animals at 695 Ma (Douzery *et al.*, 2004).

The James *et al.* (2006) alignment (available at http://www.aftol.org/alignments/comb_prot_ex.nex) consisted of 2108 amino acid positions from protein-coding genes (elongation factor 1-alpha and the largest and second largest subunits of DNA-dependent RNA polymerase II) and 2244 nucleotide positions (ribosomal DNA large, small, and 5.8S subunits). We reduced James *et al.*'s (2006) original 214 taxa to 25. To infer a phylogeny with branch lengths, we used MrBayes 3.1.2 (Huelsenbeck *et al.*, 2006), allowing the program to estimate the proportion of invariant sites and the gamma shape parameter separately for the DNA and amino acid regions, and to select an appropriate model of amino acid substitution for the protein portion of the alignment. We used two independent runs of 500 000 generations each, sampling trees every 1000 generations. To estimate posterior probabilities for a consensus topology with branch lengths, we used a set of 500 trees, 250 per run, sampled after 250 000 generations, at which time the log likelihoods for each run had converged and reached a plateau.

We estimated divergence times by the penalized likelihood method with a truncated Newton algorithm, using the computer package r8s, version 1.70 (available from M. Sanderson at http://ginger.ucdavis.edu/r8s/) with the consensus topology and branch lengths from the Bayesian input tree. As an outgroup, we chose *Toxoplasma gondii*, a taxon that does not appear within the plant, animal and fungal lineages. Following Bayesian analysis, we removed *T. gondii* from the resulting tree, leaving a tree with 24 taxa, and a basal trichotomy with branch lengths, as required by the r8s computer programs. We tested for possible alternative equally likely estimates for node ages with the 'Checkgradient' option of the penalized likelihood program and set the analysis for 10 replicates under different starting conditions, using 'set num_time_guesses'. Penalized likelihood can compensate for substitution rate variation among lineages if 'smoothing' is optimized to take advantage of statistical correlation of rates within lineages. We optimized 'smoothing' at 5600 to correct for autocorrelation (r8s, version 1.70, user's manual, available from M. Sanderson at http://ginger.ucdavis.edu/r8s/). This degree of smoothing was high and it indicated a large amount of substitution rate variation across lineages. Such variation was a reminder that estimates for ages of fungal groups have a large and unknown amount of error resulting from inconsistent substitution rates as well as from possible errors in calibration or in estimation of phylogeny (Taylor & Berbee, in press). The resulting phylogeny (Fig. 1) was consistent with the tree from the complete 214 taxa from James *et al.* (2006) in showing the Glomeromycota, zygomycetes and Chytridiomycota as paraphyletic to one another, but the Bayesian support for this branching order was not overwhelming. Alternative trees with reasonably high likelihoods showed a monophyletic clade that included the mycorrhizal Glomeromycota as well as saprobic species such as *Rhizopus oryzae* and commensals such as *Smittium culisetae*, a grouping also found by Liu *et al.* (2006). Whether these fungi were paraphyletic or monophyletic, phylogenetics and fossil evidence supported their presence in the Devonian, early in the history of vascular plants (Taylor *et al.*, 2004).

Candidate groups of fungi or fungus-like heterotrophs; which were possible relatives of *N. aphylla* fungi?

With an estimate of fungal divergences in time (Fig. 1), we can see that the extant fungi most similar to the *N. aphylla* fungi are from basal lineages (Fig. 1). These include zygomycetes, which share aseptate hyphae with some of the fossils, and Chytridiomycota water molds, which, like others of the fossils, have spherical thalli. However, fungi are not the only plant pathogens with a long evolutionary history. Oomycetes and plasmodiophoromycetes are nonfungal protists that were likely present in the Devonian (Fig. 1) and could account for some of the *N. aphylla* 'fungal' remains. The oomycetes originated from a photosynthetic ancestor also common to brown algae and diatoms; oomycetes convergently evolved fungus-like hyphae and an absorptive mode of nutrition. Oomycetes cause disease in many land plants (late blight of potatoes is one such example) and, like the *N. aphylla* fungi and living zygomycetes, their hyphae lack regular septa. Because of the lack of available sequences, plasmodiophoromycetes were not included in Fig. 1, but they are biotrophic pathogens related to foraminifera (Archibald & Keeling, 2004) that infect a variety of hosts, including oomycete fungi and vascular plants. In plant cells, plasmodiophoromycetes form zoosporangia as well as thick-walled resting spores which are released when the plant roots disintegrate. They, like Chytridiomycota, could be responsible for spherical thalli within plant material.

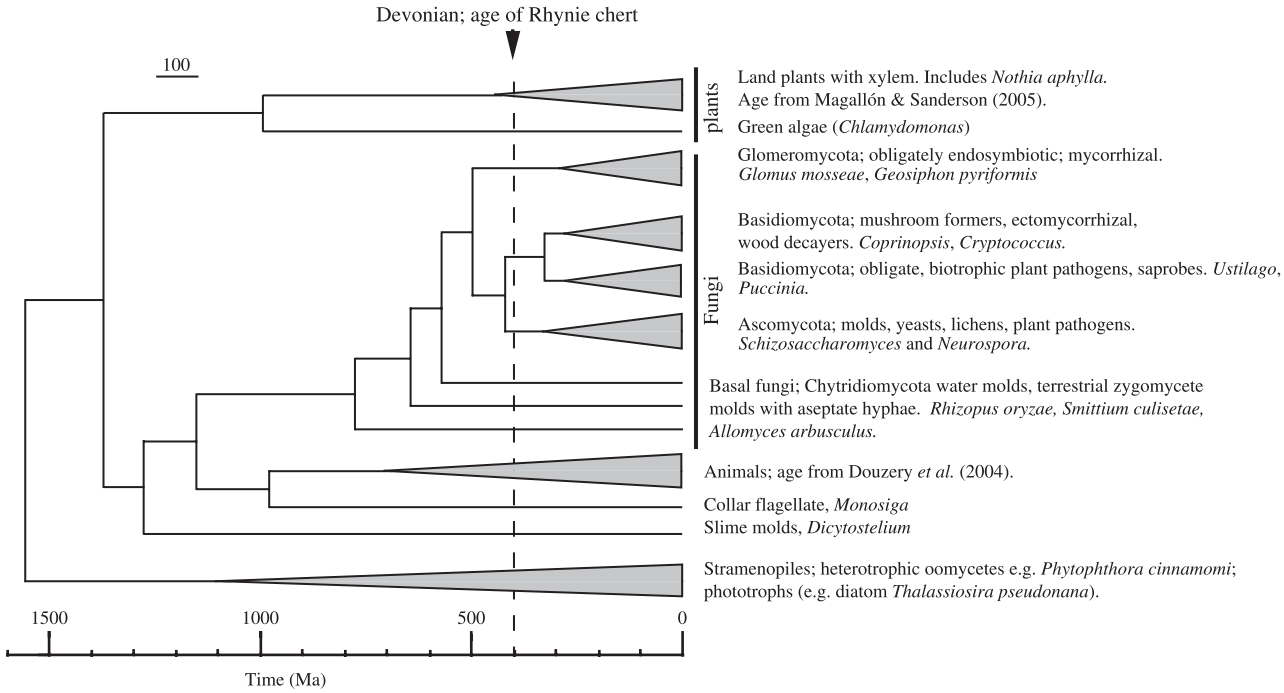

Fig. 1 Relative ages of fungi and plants. A land plant age estimate is from Magallón & Sanderson (2005); the animal age is from Douzery *et al*. (2004), and the fungal and outgroup ages were estimated for this Commentary using the James *et al*. (2006) six-gene data set and the molecular clock inference program r8s. Triangles represent groups that included the species listed and appeared monophyletic in a Bayesian analysis.

Comparison of Devonian and modern fungi in plants

A strength of the Krings *et al*.'s paper is that each of the three fungi was found many times. In the absence of identifying characters, Krings *et al.* referred to each kind of fossil fungus by number. '*Nothia aphylla* fungus no. 1' consisted of narrow, aseptate hyphae and wider 'spores' and, although this was the least frequent of the fungi, it was found in 15% of the axes, or 37 of the 250 examined. The fungus caused damage in plant tissue, judging from the disintegrated host cells lying below the region of cells filled with fungal hyphae and spores. Evidence that the host was alive at the time of infection is provided by a layer of thicker, darkened cell walls that separated the zones with and without fungus no. 1 in the infected *N. aphylla* rhizome. The formation of thickened cell walls and darkening of tissues are common responses of living plants to fungal invaders. Other evidence that the host was alive at the time of fungal colonization is provided by a layer of granular material covering hyphae found in conducting cells and a thickened and twisted rhizoid that was colonized by fungus.

Some difficult puzzles are posed by this Rhynie material, such as, how were the spores from fungi no. 1 and no. 2 dispersed when they were packed into host cells and, in the case of fungus no. 1, attached to hyphae? In modern ecosystems, spores of Ascomycota, Basidiomycota and zygomycete fungi, and also of oomycetes, form on the outside of their plant substrate, often responding to environmental cues such as light, surface features, and oxygen availability. In these fungi, dispersal is effected by wind, water or animal movement. In the Devonian samples, large numbers of spherical spore-like structures were packed within cells of decaying tissues. Modern fungi immersed in underground plant tissue release motile spores capable of moving through viscous decaying matter, or they release spores that swim away once the plant material has been lost to decay. Could spores in the *N. aphylla* rhizomes have germinated into crawling plasmodia, as in some stages in the plasmodiophoromycetes? Or, might they have directly released flagellated zoospores like modern oomycetes or Chytridiomycota, or released zoospores after the further disintegration of surrounding tissue, like modern plasmodiophoromycetes?

Comparison with fungi associated with sporophytes of extant plants provides little help in identifying fungi no. 1 and no. 2. The closest taxonomic relationships of *N. aphylla* may be with the Zosterophyllophyta (Krings *et al*., 2007), in which case, its closest living relatives may be lycophytes, or club mosses. Unlike the Devonian fossil fungi, most fungi recorded from modern lycophytes have regularly septate hyphae and are members of Ascomycota and Basidiomycota (Farr *et al*., 1989). The oomycete reported from lycophyte sporophytes, *Phytophthora cinnamomi* (Farr *et al*., 1989), has aseptate hyphae but no other characters in common with the *N. aphylla* fossils.

However, the gametophytes of several early diverging land plants have mycorrhizal, Glomeromycotan fungi that resemble fungus no. 3 in the *N. aphylla* rhizomes. Glomeromycota has

the best continuous fossil record of any group of fungi, beginning with the 460 Ma Ordovician spores that serve in Fig. 1 as a calibration point marking the minimum age for the Glomeromycota stem lineage (Redecker *et al.*, 2000). By the Devonian, fossil evidence for the Glomeromycota included structures that modern vesicular mycorrhizal fungi form in their hosts, such as arbuscules (the highly branched fungal hyphae within host plant cells that are perhaps the main site of plant–fungal nutritional exchange), vesicles, aseptate hyphae, and spores (Remy *et al.*, 1994). As might be expected if it were a mutualistic mycorrhizal partner, fungus no. 3 was common, colonizing more than 95% of rhizome segments. As in modern fungal partners of lycophytes (Schmid & Oberwinkler, 1993) and other nonflowering plants such as the fern ally *Psilotum nudum* (Duckett & Ligrone, 2005) and the liverwort *Marchantia* (Russell & Bulman, 2005), the hyphae did not produce arbuscules. However, the hyphae of the fungus did produce vesicles or give rise to thick-walled spores.

Somewhat surprisingly, hyphae from fungus no. 3 were encased with what Krings *et al.* interpreted as plant cell wall material. Encasement is a common response of a plant to invasion of its cells by a plant pathogen, for example, by the haustorium of a rust fungus or powdery mildew. An encasement layer may form around the hypha of a mycorrhizal fungus as it enters a cell, but hyphae of extant vesicular–arbuscular mycorrhizal fungi are not usually surrounded by a regular sheath of plant cell material (Schmid & Oberwinkler, 1993; Duckett & Ligrone, 2005). In extant nonflowering plants, mycorrhizal fungi usually produce coils of thin hyphae within host cells, but hyphal coils were missing from fungus no. 3 in *N. aphylla*. Possibly, the hyphal coils were missing because the Devonian fungi, although representing Glomeromycota, differed just as much from their closest living relatives as *N. aphylla* does from any living lycopod.

Not only have plants changed since the Devonian, but fungi must also have changed, and not necessarily in faithful coevolutionary lock step with plants. If perfect coevolution were the rule, then the fungus that was the most recent common ancestor of one Glomeromycota species from a liverwort and a second Glomeromycota species from a flowering plant would date back to the Silurian origins of its host lineages. A sample of fungi from divergent hosts would then serve as a good sample of the fungi originally associated with ancient plants. Unfortunately, however, the *Glomus* species from the liverwort *Marchantia* are relatively recent and are nested among *Glomus* species from flowering plants (Russell & Bulman, 2005). Figure 1 suggests that the first division of the Glomeromycota into surviving lineages may have occurred well after early land plant radiations. Not only the Glomeromycota but also the Ascomycota and Basidiomycota coalesce to one Devonian species each. Although the diversity of Devonian fungi may have been less than what is presently found, it had to be greater than three species. The small sample size of surviving lineages from the larger pool of Devonian fungi limits what phylogenetic inference from living fungi can tell us about the ecological relationships of their ancestors. In light of Devonian diversity that surely has been lost, fungal fossils from Rhynie chert provide a remarkable window, in fact, the only window, into a lost world of complex fungus–plant interactions. Krings *et al.* have taken a great stride in beginning to interpret the view from that window.

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Key words: Devonian fungi, fossil fungi, fungal ecology, fungal evolution, molecular clock, mycorrhizas, plant–microbe interactions, plant pathogens.

Meetings

Small RNAs hit the big time

Gene silencing: the biology of small RNAs and the epigenome – 24th Symposium in Plant Biology, Riverside, California, USA, January 2007

Small RNAs have exploded on the scene as ubiquitous, versatile repressors of gene expression in plants, animals and many fungi. These small RNAs (21–26 nt) are cleaved from a double-stranded (ds) RNA and induce effects through homologous sequence interactions. Small RNAs come in various forms: microRNAs (miRNAs), heterochromatizing RNAs, tiny noncoding RNAs, short interfering (si) RNAs, natural antisense short interfering RNAs (nat-siRNAs) and small temporal (st) RNAs. They control mRNA stability or translation, or target epigenetic modifications of DNA or histones at specific regions of the genome. Small RNAs have established a new paradigm for understanding eukaryotic gene regulation. The pivotal discovery of dsRNA and its role in RNA silencing in the cell was acknowledged by the award of the Nobel Prize in Medicine or Physiology in 2006 to Andrew Fire (Standford University, CA, USA) and Craig Mello (University of Massachusetts, Worcester, MA, USA), 8 yr after the seminal publication (Fire *et al.*, 1998). In January 2007, the University of California at Riverside hosted a meeting to discuss the recent advances in small RNAs and epigenetics within the plant science field. In this article, we present the highlights of that meeting, summarize the current status of the field in plants, and point out areas yet to be explored.

'… Arabidopsis currently has the largest data set of small RNAs of any species with 300 000 unique small RNAs …'

Small RNAs, the genetic dark matter of the cell

Appreciation of the number and complexity of small RNAs in plants and other organisms has been accelerated by revolutionary advances in high-throughput nanosequencing technology (Fig. 1). Pyrosequencing and sequencing by synthesis methods now allow 500 000 small RNA sequences to be generated in a single experiment (Margulies *et al.*, 2005). This is typified by Arabidopsis, which currently has the largest data set of small RNAs of any species, at 300 000 unique small RNAs and growing (Henderson *et al.*, 2006; Lu *et al.*, 2006; Rajagopalan *et al.*, 2006; Fahlgren *et al.*, 2007). These sequences, along with current small RNA sequencing projects in model organisms and agronomically important species such as *Physcomitrella patens*, *Chlamydomonas reinhardtii*, rice (*Oryza sativa*) and maize (*Zea mays*), will provide a valuable resource for the scientific community to exploit (Axtell *et al.*, 2006; Johnson *et al.*, 2006; Qi Yijun, National Institute of Biological Sciences, Beijing, China; David Baulcombe, The Sainsbury Laboratory (TSL), John Innes Centre (JIC), Norwich, UK; Pam Green, Delaware Biotechnology Institute (DBI), Newark, DE, USA). An

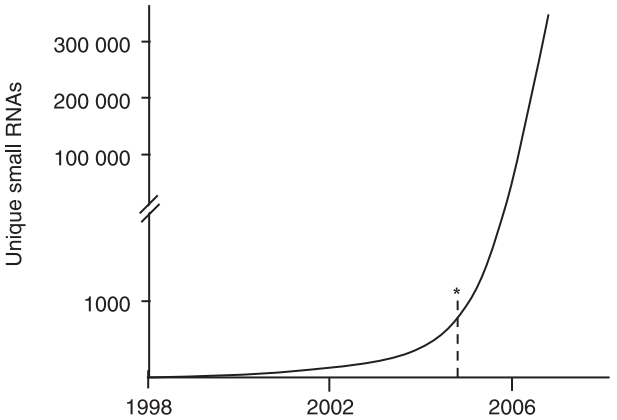

Fig. 1 The number of sequenced unique small RNAs from *Arabidopsis thaliana*. The asterisk indicates the first small RNA data set published using picolitre pyrosequencing technology.

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