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The genetic diversity of arbuscular mycorrhizal fungi in natural ecosystems – a key to understanding the ecology and functioning of the mycorrhizal symbiosis

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SUMMARY

The mycorrhizal symbiosis formed between plant roots and the arbuscular mycorrhizal (AM) fungi or Glomales is of great interest to ecologists because of its potential influence on ecosystem processes, its role in determining plant diversity in natural communities and the ability of the fungi to induce a wide variety of growth responses in coexisting plant species. Little attention, however, has been paid to the ecological role of diversity of AM fungi. Difficulties in identification, the inability to grow the fungi in pure culture, problems of taxonomic classification, and a lack of basic information on the life histories of AM fungi hinder studies of the ecological significance of diversity of AM fungi. Nucleic acid based techniques have the potential to fill this gap in our knowledge by offering better means of identification and the opportunity to study links between the genetic diversity of AM fungi and functional and morphological diversity. The application of genus-specific molecular markers has shown that different genera of AM fungi coexist in plant roots and that this is a common occurrence. Molecular techniques have also shown that natural AM fungal populations exhibit unexpectedly high genetic diversity, despite the assumption that diversity in these seemingly asexual fungi should be low. The high diversity occurs in multicopy ribosomal genes and their internal transcribed spacers, which are normally well conserved and homogeneous within an individual organism. The results show that sequence heterogeneity of the ribosomal genes can occur even in single spores of AM fungi, and we discuss how genetic diversity may be promoted and maintained. Contrasting results, indicating that genetic diversity among replicate spores from pot-cultured material is low (even though they contain within spore sequence heterogeneity), suggest that there are mechanisms which promote high genetic diversity of AM fungi in natural ecosystems.

We propose that AM fungi could be heterokaryotic as a result of the exchange of nuclei following hyphal fusion with other individuals but that other mechanisms, such as gene turnover and molecular drive, might also explain the generation of high genetic diversity without any exchange of genetic material among individuals. The high diversity in ribosomal gene sequences in AM fungi might cause problems in their use as molecular markers in field studies. A better understanding of the levels of genetic diversity of ribosomal genes within spores, among spores of the same morphology, and among spores of differing morphology is essential to the development of sound molecular markers for field studies and to the development of a phylogenetic classification.

We conclude that an understanding of the mechanisms which promote and maintain genetic diversity in the AM fungi is crucial, not only to further advances in ecological and evolutionary studies but also to studies of the molecular basis of the regulation of the symbiosis. Moreover, we predict that while observational investigations on AM fungal ecology and diversity using molecular techniques are of high value they will not give an understanding of the role of AM fungi in natural ecosystems and that further studies should also aim to fill the gaps in current knowledge of links between genetic diversity and distribution of AM fungi in natural ecosystems, and their functional diversity.

Key words: Ribosomal genes, internal transcribed spacer, molecular drive, gene turnover, Glomales.

INTRODUCTION

The distribution, abundance and life history of many plants and animals can easily be observed without using any specialized or sophisticated techniques. In addition, the taxonomy and classification of many plant and animal groups is well established to the species level. This information forms a sound

basis from which to pursue experimental studies of their ecology, evolution and role within natural ecosystems. In the case of some organisms, particularly those below ground, the difficulty of the task of describing their taxonomy, distribution, abundance and life history hinders meaningful experimental investigations of their role in natural ecosystems. One such group of organisms is the Zygomycete fungi of the order Glomales, which are also commonly known as arbuscular mycorrhizal (AM) fungi. The AM fungi form the arbuscular mycorrhizal symbiosis with the roots of plants, and in many cases this association is beneficial for both partners (Harley & Smith, 1983). Despite the lack of basic ecological and biological information about the AM fungi, they are of particular interest to ecologists (Fitter, 1990).

The lack of basic ecological information about the AM fungi stems from a few fundamental difficulties encountered in their identification, culture and taxonomy, coupled with the great difficulty in manipulating AM fungi in natural ecosystems without greatly modifying their environment in other ways. Some of the problems of identification, and possibly of taxonomy, can be overcome by using molecular techniques, and these techniques could provide us with an insight into the structure of natural communities of AM fungi. In this review we outline briefly why the understanding of AM fungal ecology is important to the understanding of the functioning of ecosystems, why the study of AM fungal diversity warrants attention, precisely what the gaps in our knowledge are, what molecular techniques are available to fill in the gaps and how the application of molecular techniques has already contributing to our understanding of AM fungal diversity and of the role of AM fungi in ecology. The techniques available have only recently been modified for specific use with AM fungi, and we stress that the results that we present (including results from our own complementary research programmes in York and Basle) are preliminary, but have already yielded interesting and, indeed, surprising information. We also discuss their implications in the understanding of the molecular mechanisms which promote and maintain diversity of AM fungi in natural ecosystems.

THE ECOLOGICAL SIGNIFICANCE OF AM FUNGAL DIVERSITY

The potential impact of AM fungi on the functioning of ecosystems is not insignificant. Arbuscular mycorrhiza are ubiquitous, with *c.* 90% of terrestrial plant species forming the AM symbiosis. The AM fungi can induce large growth benefits in the host plant. In many cases, the growth benefit has been attributed to improved plant phosphorus nutrition resulting from an increased efficiency in phosphorus acquisition from the soil by the extraradicle AM fungal hyphae (Koide, 1991). In return, the plants donate carbohydrates to the fungal partner (Smith & Smith, 1990) and this carbohydrate can move from one plant to another through hyphae (Grime *et al.*, 1987). Thus, at the ecosystem level the AM fungi have the potential to influence both the carbon and phosphorus cycles.

Recently, great emphasis has been placed on understanding how ecosystems function and, in particular, the role that diversity (or biodiversity) plays in ecosystem; the impetus being the concern over both global and local losses in biodiversity and the effects that these might have on the environment. AM fungi have been shown to play a significant role in the floristic diversity and structure of annual and perennial plant communities (Grime *et al.*, 1987; Gange, Brown & Farmer, 1990; Sanders & Koide, 1994). However, in most of these ecological investigations, little or no attention has been paid to the diversity of AM fungi themselves. This is because experiments conducted in pots have indicated that AM fungi show little host specificity. Consequently, it was thought that few selection pressures that would favour extensive divergence arise in mutualistic symbioses (Law, 1985). In support of this, the morphological diversity of AM fungi is, indeed, low (*c.* 152 species in six genera; Walker & Trappe, 1993). However, Jakobsen, Abbott & Robson (1992) have shown that different isolates of AM fungi can result in different effects on plant growth. Unfortunately, few of the experiments demonstrating different effects of AM fungal isolates on plant growth have been conducted in an ecological context; the fungi used have been isolated from different soils and environmental conditions but have then been compared on the basis of their functionality with plants with which they might never naturally grow.

Studies conducted in Basle on the Swiss Biodiversity Programme have used a collection of fungi which were all isolated from the same plant community (a species-rich calcareous grassland called Nenzlinger Weide). Their functionality with several plant species from the grassland has been assessed; functionality is defined as an effect on the overall growth, growth habit, vegetative and reproductive fitness of the plant and of the fungus. The results have shown that isolates indeed differ in their effects on plant growth but that these effects differ according to plant species. An extreme case is seen in the two dominant coexisting grasses at Nenzlinger Weide, *Bromus erectus* and *Festuca ovina*. One fungal isolate from the field site induced faster growth in *Festuca ovina* and a reduced growth rate (similar to that of the non-mycorrhizal situation) of *Bromus erectus*; another isolate from the same site had exactly the opposite effect (M. van der Heijden, pers. comm.). Other experiments on the functionality of these fungal isolates with *Prunella vulgaris* and *P. grandi-*

flora have indicated that AM fungal diversity is likely to affect the plant's reproductive strategy and, in particular, whether reproduction is likely to be sexual or asexual (clonal) (Streitwolf-Engel *et al.*, unpublished). These studies outline the potential importance of diversity of AM fungi in natural communities.

Understanding the real, rather than the potential, significance of AM fungal diversity in natural communities has posed a greater problem. For a given plant community we need to know how diverse the AM fungal community is, which plant roots are colonized by which AM fungi, whether there is seasonality in the patterns of colonization, whether any specificity between plants and AM fungi occurs and what the effect of those AM fungi will be on plants and the ecosystem. However, the difficulty of identifying AM fungi in the roots of plants has always been an obstacle to their study in natural communities. Although identification of AM fungi in roots based on morphological observations has been successful in pot experiments (Abbott, 1982; J. Merryweather, pers. comm.) the usefulness of this technique for field investigations must be limited because the hyphal morphology of one AM fungal isolate is likely to vary with host species (Gallaud, 1905; Gerdemann, 1965; Lackie *et al.*, 1987). Molecular or nucleic acid techniques have been used successfully for *in planta* identification of ectomycorrhizal fungi (Gardes *et al.*, 1991; Bruns & Gardes, 1993; Gardes & Bruns, 1993) and endophytic fungi such as *Epichloë* sp. (Groppe *et al.*, 1995) and the techniques available for identification of AM fungi in plant roots are described in 'Molecular Techniques Available for Studying AM Fungal Ecology' (below).

TAXONOMY AND DIVERSIFICATION OF AM FUNGI

The arbuscular mycorrhizal symbiosis is ancient; the fossil record suggests that it occurred in the first land plants (Pyrozynski & Dalpé, 1989; Remy *et al.*, 1994). Morphological diversification is low, with only 152 recognized species in six genera (Walker & Trappe, 1993). Law (1985) pointed out that considering the age of the symbiosis this diversification is low, and he suggests that this is typical of mutualistic symbioses, as there are few external pressures which would favour the selection of new traits. However, the low divergence of AM fungi seems at odds with their functional diversity. Information regarding the genetic diversity of AM fungi can also be obtained using molecular techniques and the results of such investigations can, and have, helped to construct a phylogenetic classification of AM fungi (Simon, Lévesque & Lalonde, 1993).

Until recently, methods for studying AM fungal diversity and the ecology of the symbiosis have been wholly reliant on the morphology of the spore phase. Spores of AM fungi are relatively large, easy to extract from soil, and have several morphological characters that allow species identity to be determined by experienced personnel. However, the use of spore data alone for the assessment of ecosystem diversity and ecology has long been recognized as unsatisfactory (Walker, Mize & McNabb, 1982; Klironomos *et al.*, 1993; Rolden-Fajardo, 1994). Situations can be envisaged which could give rise to misleading conclusions, such as the presence of non-sporulating fungi, spore types that are isomorphic but are in fact distinct, or the presence of spores in soil in differing numbers being assumed to reflect the situation within the root. In addition, data derived from observation of spore numbers reflect past events in the symbiosis; how this information relates to the contemporary situation is at best uncertain. The study of spores alone, therefore, is a suspect method for the study of the ecology of the symbiosis and is especially likely to lead to a low estimation of diversity.

The taxonomy of AM fungi has also been based on the morphology of spores and, therefore, ecological studies have also been reliant on the taxonomic classification. The ecological worth of constructing data on AM fungal community structure which are based on morphological characteristics is questionable, since the relationship between the morphological diversity of AM fungi and their genetic and functional diversity has not been established. According to Walker (1992) many described taxa are 'workable' in that they are recognizable and are found repeatedly in different parts of the world, albeit in diverse environments. For example, several species of AM fungi which were reported in a semi-arid site in Australia (McGee, 1989) also occurred in a species-rich meadow in the north of England (Sanders, 1993), both ecosystems comprising completely different soils, vegetation and climate. This indicates that AM fungi are very plastic in their environmental adaptability. There are many other examples in the literature of the enormous plasticity of AM fungal species which is reflected by their distribution in extremely diverse environments. The lack of host specificity in the mycorrhizal symbiosis also indicates that the AM fungi are very plastic with respect to host range. How this enormous plasticity comes about in AM fungi is not yet explained.

Molecular techniques can give information about the genetic diversity of AM fungal species which might help to explain these phenomena but the latter must only be adequately resolved if individual clones of AM fungi can be identified and recognized so that genetic characteristics can be used to identify the morphological, functional and ecological differences between AM fungi (Walker, 1992). However, it is uncertain whether AM fungal clones exist. The

fungi are coenocytic organisms where single spores contain thousands of nuclei (Burggraaf & Beringer, 1989; Bianciotto & Bonfante, 1992). Although AM fungi are thought to be asexual, it is not certain whether their spores are homokaryotic or heterokaryotic (Walker, 1992). The likelihood of the existence of heterokaryote AM fungal spores is discussed in 'Implications for the Biology of the AM Fungi', below.

MOLECULAR TECHNIQUES AVAILABLE FOR STUDYING AM FUNGAL ECOLOGY

Techniques for studying genetic diversity

It is not our aim to describe all the molecular techniques available for ecological studies but to outline those which have been used, or modified specifically, for studying AM fungi, and those which are likely to be useful for future studies. For a description of the use of molecular techniques in ecology we refer the reader to Bachmann (1994). For many organisms, a DNA fingerprint for identification or for the estimation of genetic diversity can be achieved by using restriction fragment length polymorphism (RFLP) analysis. The technique can only be used where a large amount of DNA can be obtained from individual organisms. Because AM fungi cannot be maintained in pure culture, obtaining sufficient DNA for RFLP is difficult. Consequently, all the nucleic acid techniques employed for identifying AM fungi or for studying their genetic diversity use the polymerase chain reaction (PCR), which amplifies relatively small amounts of DNA.

A universal molecular phylogeny has been based largely on sequences of the small subunit ribosomal RNA gene (18S in eukaryotes and 16S in prokaryotes) and other regions of ribosomal DNA (Winker & Woese, 1991). For elucidating the phylogenetic relationships of fungi, the 18S and 5.8S rRNA genes, along with flanking regions known as internal transcribed spacers (ITS), can be amplified and sequenced (White *et al.*, 1990). The 18S and 5.8S genes evolve relatively slowly and are useful for studies of distantly-related organisms. The ITS region evolves faster than the 18S and 5.8S genes and can differ between species. The advantage of using these regions of DNA is that multiple copies of the genes are present in all organisms. It is no surprise, therefore, that the first report of DNA amplified from AM fungi using the polymerase chain reaction (PCR) and sequenced was from the 18S rRNA gene (Simon, Lalonde & Bruns, 1992). The DNA was amplified from small numbers of spores using so-called universal primers; short length oligonucleotides which are complementary to highly conserved sequences and which therefore cause the amplification of DNA from a wide variety of different organisms. Simon found that the sequences of the 18S rRNA gene differed between species of AM fungi and, following the design of a Glomales specific primer (VANS1) was able to amplify and sequence the 18S rRNA gene from several different species of AM fungi which represented all the genera of the Glomales (Simon *et al.*, 1993*a*).

The studies of Simon *et al.* (1992) demonstrate that if sequences of common genes differ between species or isolates of AMF it is possible to use these for identification purposes and for studies of genetic diversity. The techniques have been applied to AM fungal spores, and the ITS region (which is thought to be less conserved than the 18S) has been amplified from single spores of several isolates (Sanders *et al.*, 1995). Amplification of DNA from single spores permits studies of AM fungal diversity in natural populations, and Sanders *et al.* (1995) used it for this purpose. ITS regions from single spores of different known AM fungal isolates were amplified using the universal primers ITS1 and ITS4. By cutting the ITS fragment with restriction enzymes, different fingerprints could be obtained for the different isolates. The technique, known as PCR-RFLP, was used for the rapid identification of genetically diverse spores. The method is, however, severely limited unless the ITS regions are actually sequenced. A difference in banding pattern between two amplified products from two different spores confirms that the latter are genetically different. However, if no differences are found between the two fingerprints the result does not mean that the two ITS regions are identical, but might mean that the correct restriction enzyme for finding those differences was not used. Sequencing of the ITS regions is an obvious way to overcome this. However, in field investigations, the genetic diversity of AM fungal spores was so great that PCR-RFLP was sensitive enough to detect genetic diversity in the community without the need to sequence the amplified products (Sanders *et al.*, 1995).

Another technique, known as random amplified polymorphic DNA (RAPD-PCR) is also available for studies of genetic diversity. The technique is potentially more sensitive in detecting genetic differences between individuals, and by screening large numbers of arbitrary primers, the desired sensitivity can be found for detecting genus, species or individual genotype differences. Wyss & Bonfante (1993) successfully used RAPD-PCR to identify spores at the level of species and to differentiate between isolates of the same species. So far the technique has not been used for studying the diversity of natural AM fungal communities.

Techniques for identifying AM fungi in roots

If the sequence of a known region of DNA differs between species or isolates it is possible to develop specific primers for their identification and quanti-

fication. Once primers are developed they can be used to identify AM fungi from spore, hyphal or colonized root material. As many ecological studies of AM fungi are aimed at determining the relationship between the host and colonist the most desirable site from which to obtain valid information is clearly the root itself.

Initial uses of molecular methods involved isozymes. Hepper, Sen & Maskall (1986) found that it was possible to detect the presence of single and mixed species of *Glomus* in pot cultures of maize by observing allozyme patterns. This work was extended into the first assessment of AM fungi in a semi-natural grassland by Rosendahl, Rosendahl & Søchting (1990) and showed that several distinct fungi could be isolated from a single host plant, indicating that not only could more than one genetic type exist in a single plant but also that the fungal diversity might be greater than was expected from information derived from spore abundance. Immunological methods have also been developed for detection of AM fungi in roots but have not been used for field investigations (Wright & Morton, 1989; Sanders *et al.*, 1992; Hahn, Horn & Hock, 1995). Currently, however the most favoured techniques available use the extremely high definition given by DNA sequence information for the identification of AM fungal spores and endophytic hyphae. All these methods use the polymerase chain reaction (PCR) or its derivatives.

Simon *et al.* (1992) used universal fungal primers to sequence the 18S ribosomal RNA gene from spores of *Glomus intraradices* and *Gigaspora margarita*. The sequence information obtained was used to design a primer that was specific to the Glomales, VANS1. They showed that this primer, when used in conjunction with the universal primer NS21, was able to amplify AM fungal DNA directly from leek roots infected with *Glomus vesiculiferum* and this has been repeated in four other plants (Di Bonito, Elliott & Des Jardin, 1995). Following the development of family-specific primers (Simon, Lévesque & Lalonde, 1993*b*) this work was extended to identify different genera of AM fungi in roots. Simon *et al.* (1993*b*) also exploited single strand conformational polymorphism (SSCP) rapidly to screen PCR-amplified DNA sequences for variation. SSCP has been reported to be able to detect single base substitutions between homologous DNA sequences (Ainsworth, Surh & Coulter-Mackie, 1991; Makino *et al.*, 1992). These differences are detected by electrophoresing the samples on a high-resolution polyacrylamide gel under conditions where the two strands of the amplified product run as two independent molecules, their rate of progress being directly affected by their conformation within the matrix of the gel. Simon *et al.* (1993*b*) showed that amplified fragments differing by only a few bases could be detected and selected for sequencing. This technique could prove to be a useful screening method before resorting to lengthy sequencing projects for the assessment of AM fungal diversity.

There have, as yet, been few studies in which the use of nucleic acid information has been extended into a non-laboratory environment. Clapp *et al.* (1995) found difficulty with the VANS1 primer when used for the PCR amplification of AM fungal DNA from field-collected bluebell roots (*Hyacinthoides non-scripta*). This apparently stemmed from interference by the host plant DNA. When dilution of the template DNA was found to have no effect, the host-plant DNA was removed by subtractive hybridization, the overall process being named SEAD (selective enrichment of amplified DNA), whereafter amplifications using VANS1 were successful. Parallel control pot experiments, with onion roots experimentally infected with *Glomus mosseae*, were found to be easy to amplify without resorting to SEAD.

A different approach to the development of species-specific primers was taken by Lanfranco *et al.* (1995) who compared banding profiles generated by RAPD-PCR to identify unique bands in different AM fungi. These bands were cloned and sequenced, and the information used to design species-specific primers. There is difficulty, however, in directly applying RAPD-PCR based techniques to the endophytic stage as the primers will almost certainly produce PCR products derived from the host plant DNA. It might, however, be possible to utilize a SEAD-based procedure to allow the identification of single infections. The identification of multiple colonizations by RAPD-PCR might prove more difficult.

Nucleic acid based technology can potentially be used for the quantification of AM fungal colonization and is essential for making estimations of the relative abundance of different AM fungi and allowing an assessment to be made of co-existence of AM fungi in roots. Simon, Lévesque & Lalonde (1992) have developed a quantitative PCR technique for the estimation of AM fungal colonization. The PCR amplification of a fragment from an AM fungus and the deletion of a small number of bases allows the production of an amplification standard that when cloned and introduced into the early stages of a DNA extraction will subsequently co-amplify with the root-derived AM fungal template. A comparison of the ratio of brightness of the two bands produced by staining with ethidium bromide on an agarose gel (with a small correction for the lower size of the standard) reflects the initial concentration of the template with respect to the known concentration of the standard, provided that the conditions of the PCR are identical. This technique is being used extensively at the University of York with the primers P0 and M3 which were found to be more reliable than VANS1 for the quantification of

colonization of *Glomus mosseae* under experimental conditions (S. E. Edwards, pers. comm.). Problems with the formation of hybrid molecules between the fungal product and the standard, represented as a third band on the gel, have been largely overcome by the use of totally unrelated template DNA as template for the amplification. This is isolated by PCR amplification of DNA from an unrelated organism using the same primers at low annealing temperature (Uberla *et al.*, 1991), resulting in a molecule of similar size to, but with no sequence homology with, the AM fungal template sequence.

Population genetics

The methods described are of limited use for studies of population genetics of AM fungi. A traditional approach in population genetics is to use isozyme analysis but this can be expensive and can require large amounts of material. In the case of non-culturable organisms such as AM fungi, where the smallest individual unit is a spore, sufficient material might not always be readily available. An alternative is to use microsatellite markers. Microsatellite DNA (also known as short tandem repeats or simple sequence length polymorphisms) are stretches of tandem mono-, di-, tri- and tetranucleotide repeats of various lengths. The loci containing microsatellites act as heritable, but selectively neutral markers (they are usually non-coding regions of DNA), which often differ in length between individuals. Their use for studies of genetic diversity, gene flow in populations, maternity and paternity analysis and relatedness, and genetic distance between individuals, species and populations, is reviewed by Bachmann (1994). Although, to our knowledge, microsatellite markers have not yet been found for the Glomales there is no reason to suppose that they do not exist. Regions of microsatellite DNA have been found in the *Epichloë* endophyte (Groppe *et al.*, 1995) and the length of the region differs between genetically different individuals of the fungus. Development of specific primers to amplify a region of *Epichloë* DNA containing the microsatellite region, for use in studies of diversity in populations of these fungi, has been successful. By reviewing the GenBank and EMBL sequence databases, Groppe *et al.* (1995) have also shown that microsatellite DNA is common in many fungi and therefore there is no reason to expect that it does not occur in the Glomales.

Linking genetic diversity and functionality of AM fungi in natural ecosystems

The methods described above are all for the identification of AMF or the detection of genetic diversity among AM fungi. In each case, the targeted regions of DNA which are used for characterization of AM fungi are either genes which bear little relation to the symbiosis-specific functions of AM fungi, e.g. the ribosomal genes or, in the case of RAPD-PCR and microsatellite DNA, are frequently non-coding regions. Thus, obtaining markers for studying the functional ecology of AM fungi is unlikely to advance with the use of these techniques. It is possible that certain genes are symbiosis-specific and that their expression indicates particular functions occurring in the symbiosis. These could be valuable for field studies if their expression could be detected and quantified.

A possible method for the detection of symbiosis-specific genes would be the technique of differentially expressed mRNA display (Liang, Averboukh & Pardee, 1993). The procedure works by extracting mRNA from two individuals, one of which has undergone a specific treatment; in the case of studies of AM fungi this might be from both colonized and uncolonized roots. A reverse transcription PCR reaction (RT-PCR) converts mRNA into cDNA which is then amplified (Bauer *et al.*, 1993). The differential display of mRNA, which is a representation of differential gene expression between the two treatments, can then easily be observed on a gel. The result is that bands which are specific to the mycorrhizal symbiosis can be identified, cloned and sequenced. Finding out whether sequenced regions are of fungal or plant origin would require subsequent verification by Southern hybridization analysis. The technique would allow an experimental approach to understanding how AM fungi interact with environmental variables by manipulating environmental conditions, and by observing differential gene expression in the symbiosis. Such an approach could also lead to the development of genetic markers for symbiosis-specific functions for use in field studies. This would give an insight into the genetic regulation of the AM symbiosis under manipulated environmental conditions. In addition, carrying out differential display techniques on plants colonized by different AM fungal isolates would allow the identification of genes which may be specific to a particular plant/AM fungal isolate interaction, the first step to finding a link between genetic and functional diversity among AM fungi. To our knowledge, the use of differential display for the purposes of understanding AM fungal interactions with the environment has not yet been performed.

A second approach to isolating specifically expressed mRNAs involved in the interaction between the AM fungal colonist and its host plant could be achieved by subtraction methodology. Such procedures combine cDNA products resulting from RT-PCR of, for example, AM fungal colonized and uncolonized plants in such a way that cDNA fragments common to both, and therefore not relevant to the interaction, would be lost. The only cDNAs that are retained are those that were induced in the host plant by the presence of the fungus. As in the case of

differentially expressed mRNA, the plant or fungal origin of resultant products would have to be verified by Southern hybridization, but this approach has the advantage that far fewer cDNA products will be involved in the final screening, allowing easier interpretation. This technique has been successfully used to isolate differentially expressed genes induced by salt stress (Gulick & Dvorák, 1990) and low temperatures (Aguan *et al.*, 1991). There are many more examples of its use for the isolation of differentially expressed mRNAs and there is no reason why it cannot be used to isolate genes induced by AM fungal colonization.

MOLECULAR EVIDENCE FOR THE DIVERSITY OF AM FUNGI

Diversity of AM fungi in laboratory-cultured material

On the basis of the 18S ribosomal RNA gene sequences, Simon *et al.* (1993 *a*) and Simon (1996) have produced a phylogeny for the Glomales which they compared to the major periods of divergence of terrestrial plants. The numbers of base differences among species and genera of the Glomales were used to estimate the likely periods of divergence of the different genera. The data of Simon *et al.* (1993 *a*) corroborate the fossil and taxonomic evidence of the divergence of the Glomales, based on spore morphology, and confirm that AMF are ancient and that there has been little diversification (Pyrozynski & Dalpé, 1989; Remy *et al.*, 1994).

Evidence from other molecular studies suggests that the genetic diversity of mycorrhizal fungi is not quite so simple. Using RAPD-PCR, Wyss & Bonfante (1993) developed fingerprints from different isolates of *Glomus mosseae*. Some of the isolates originated from the same culture but were then maintained in different laboratories for several years. Slightly different fingerprints were found between those isolates, which could indicate that genetic differences in the Glomales can arise over relatively short periods of time (*c.* 12 yr).

Information from the sequencing of the ITS region has revealed surprising information about the possible genetic diversity of the Glomales. Sanders *et al.* (1995) were able to obtain reproducible specific fingerprints from ITS regions of AMF using PCR-RFLP. The DNA came from single spores originating from single spore isolates. Some fingerprints gave multiple banding patterns which, when summed, did not add up to the original size of the ITS region. This suggested that different sequences of the ITS region were amplified from single spores but in a reproducible manner (other spores from the same culture yielding the same pattern). Cloning and sequencing of the amplified ITS region from single spores of *Glomus mosseae* revealed that different sequences of the ITS region are, undoubtedly, present in single spores. Two sequences obtained from one spore were sufficiently similar to suggest that they are closely related. Comparison with another, similar, ITS sequence obtained following phage cloning, confirmed that the sequences were from *Glomus mosseae* (Franken & Gianinazzi-Pearson, 1996). The implications of these findings for the diversity of the arbuscular mycorrhizal symbiosis are discussed in 'Implications for the Biology of the AM Fungi', below.

The diversity of AM fungi in natural ecosystems

There is very little information on the diversity of AM fungi in natural ecosystems, although evidence is emerging that the diversity is greater than has been inferred from morphological studies of spores. Typical estimates of species diversity in natural ecosystems on the basis of spore counts range between approx. 5–20 different species in a community. Using the PCR-RFLP method to analyse the genetic diversity in natural AM fungal populations, Sanders *et al.* (1995) found that the ITS region of ten morphologically identical spores of the genus *Glomus* were genetically different. This result indicates an unexpectedly high heterogeneity in the ITS region in natural populations of AM fungi.

Investigations of the diversity of natural *Scutellospora* populations in an oak woodland in the north of England highlight the diversity that can occur among and within spores in natural populations of the Glomales. In order to confirm that the VANS1 primer site was conserved in the *Scutellospora* species at the study site, DNA was extracted from single, field-collected spores and PCR-amplified using SS38 (universal primer) and VAGIGA (family-specific primer) primers (designed by Simon *et al.* 1993 *b*). These primers amplify a region of DNA which should contain the VANS1 primer site. The PCR products were cloned and several clones were sequenced from each spore. More than one 18S ribosomal RNA gene sequence occurred in a single spore, confirming the evidence from the ITS sequences of the genetic diversity of single spores. More surprisingly the sequences contained different variants of the sequence corresponding to the VANS1 primers, usually varying by three bases over its 21 bp length, indicating that the VANS1 site is not conserved (Clapp *et al.*, unpublished). It is conceivable that these sequence differences could reduce the efficiency of annealing of the VANS1 primer and explain the difficulties that have previously been encountered using this primer to amplify from field samples (Clapp *et al.*, 1995). The results raise questions regarding the validity of using VANS1 sequences for the phylogenetic classification of the Glomales (see 'Implications for the Biology of the AM Fungi' below).

Despite the confusing and overwhelming genetic diversity seen in natural populations and communities of the Glomales, studies using ribosomal gene sequences have given the first indications of distribution of AM fungi in the roots of a natural plant community. Studies at the University of York have concentrated on the *Hyacinthoides non-scriptal*/AM fungi symbiosis which has been described in relation to the life history and phosphorus nutrition of *H. non-scripta* (Merryweather & Fitter, 1995*a*, *b*). To this end, amplified DNA from both spores and endophytic hyphae have been sequenced to associate the rhizosphere species with the endophytic stages and to characterize the distribution of AM fungi in time and space in the roots of these plants. The work has concentrated on the 5′ end of the 18S rRNA gene and the results obtained so far appear to imply that the situation is more complex than at first envisaged. Initial studies, using SEAD (Clapp *et al.*, 1995), found that more than one fungal genus could be represented in a single root of bluebell and that this seems to be the normal situation rather than an exception. Up to three different genera of AM fungi (*Acaulospora*, *Glomus* and *Scutellospora*) are represented in the roots of the bluebell population. The presence of *Acaulospora* and *Scutellospora* fungi in the roots was expected on the basis of spore counts made from the soil at the field site, although *Glomus* sequences within the roots were not expected, as spores from this genus had been found only infrequently in the soil. This observation indicates that it is very likely that many more species are present in roots than can be found by solely surveying spores in the rhizosphere.

Direct sequencing of PCR products derived from the family-specific primers (Simon *et al.*, 1993*b*) and VANS1 in these roots, however, was not successful as more than one sequence was present. To overcome this difficulty, the DNAs were cloned and two colonies were sequenced from each family-specific amplification. In every case the sequences from each genus were found to be different (Clapp *et al.*, 1995). This apparent diversity is undergoing further investigation which concentrates on the genus *Scutellospora* from the same field site. Similar studies on field-collected roots have also yielded several sequences, but whether these can be assigned to different species or to multiple ribosomal gene sequences within single species, is as yet impossible to say (Clapp *et al.*, unpublished).

To date, it appears that molecular information obtained from colonized roots in natural ecosystems is likely to identify the presence of many species or genotypes of AM fungi that are as yet undetected. It may be possible to counter the difficulties in obtaining fungal sequence data from natural ecosystems (possibly due to the variation within the VANS1 site) by the use of degenerate primers which might then allow direct amplification of sequences from roots without the need to resort to more complicated procedures such as SEAD.

IMPLICATIONS FOR THE BIOLOGY OF THE AM FUNGI

Implications for the maintenance of genetic variation in AM fungi

Molecular studies of sequences of ribosomal genes in the Glomales reveal unexpectedly wide genetic diversity, especially in organisms which are thought to be asexual. We are not aware of other organisms in which different ribosomal genes occur within a single individual. A major question now is whether the variation is a result of the presence of distinct genotypes in different nuclei in the same spore (heterokaryotic) or gene heterogeneity within single nuclei. With our current lack of knowledge of biology of AM fungi either or both scenarios are possible.

The heterokaryote hypothesis proposes that different sequences of ribosomal genes occur in different nuclei in a single spore. This is highly pertinent, because the presence of different genotypes of nuclei occurring in single spores has strong implications for the biology, ecology and evolution of the mycorrhizal symbiosis. Heterokaryote spores could not be considered as genetic individuals but as populations. This raises questions of how the heterokaryote has arisen and whether there is any transfer of nuclei between hyphae of different AM fungi. Heterokaryotic mycelia occur frequently in higher fungi (Alexopoulos & Mims, 1979) and exchange of nuclei following anastomosis occurs in many filamentous fungi. A similar mechanism has been proposed for the diversification of *Epichloë* endophytes in *Festuca rubra* following the discovery of several different sequences of β-tubulin (*tub2*) gene copies in the fungus which can best be explained by the hybridization of vegetative hyphae (Tsai *et al.*, 1994).

The high genetic diversity that would be maintained in this way may help to explain how an asexual organism can exhibit such high plasticity both in environment and host range and why the Glomales seem to give remarkably inconsistent results in many scientific experiments. It is also tempting to believe the heterokaryote hypothesis when such high genetic diversity has been observed in both the York and Basle studies among and within morphologically identical spores in natural populations. In contrast, results from pot-cultured material from single spores with PCR-RFLP suggest genetic consistency among spores from the same culture (even though they are genetically diverse in a single spore). This indicates that some mechanism must be operative in natural populations of AM fungi which promotes and maintains genetic diversity. The presence of genetically diverse nuclei (heterokaryotic) does not necessarily demonstrate that the exchange of genetic

material occurs between individual fungi. Variation in ribosomal genes among or within nuclei of AM fungi could occur by other mechanisms, and those of gene turnover and molecular drive are addressed below.

Knowledge of gene turnover and molecular drive (Hoelzel & Dover, 1991) may explain the occurrence of highly variable ribosomal gene sequences. The multiple copies of ribosomal genes are normally homogeneous within a single genome. It is because of this homogeneity that ribosomal gene and ITS sequences are useful for studies of phylogeny. In sexual organisms, incomplete alignment and subsequent unequal crossing over can occur during recombination. These events are responsible for the generation of multicopy genes (Smith, 1976) and for maintaining homogeneity of the sequences (Dover, 1986; Hoelzel & Dover, 1991). In asexual organisms, mitotic recombination occurs very infrequently (Darnell, Lodish & Baltimore, 1990). As a consequence, the opportunity for unequal crossing over would also be infrequent. Therefore, perhaps a certain amount of heterogeneity among the copies could be expected, owing to mutations which are not frequently corrected by unequal crossing over. This could explain the high variability in the ribosomal gene sequences seen within single spores of the Glomales if the Glomales are asexual. Sexual reproduction events have not been observed in the AM fungi, although this does not rule out the possibility of their occurrence. If sexual reproduction were a regular occurrence in the AM fungi, then different sequences of ribosomal genes would be unlikely to occur within a single nucleus or between different nuclei (assuming that recombination were a regular event) because recombination would maintain homogeneity of the gene copies. Therefore, our results point strongly to AM fungi being asexual. If the lack of frequent recombination accounts for the genetic variability of ribosomal genes in single spores of the Glomales then the different sequences could, theoretically, occur both within and among nuclei in a spore. It would seem that the distribution of different ribosomal gene sequences in the spore can best be elucidated if single nuclei can be isolated by microdissection, then amplified by PCR. However, this might also not provide the conclusive evidence of the mechanisms controlling genetic variation in the Glomales.

Unequal crossing over is not the only mechanism which helps to maintain homogeneity among gene copies. Gene conversion can also be responsible for this homogeneity. The process of gene conversion normally occurs during recombination but can also occur in mitotic cells, although it is a much rarer event. Some gene conversion in mitotic cells probably occurs during DNA replication, where mismatch repair mechanisms are in operation (Kourilsky, 1986). This would allow some repair of mutated ribosomal gene copies. Whether the mechanism of gene conversion in the absence of recombination can also be responsible for the incorporation of new gene sequences throughout the gene family is unclear. In the case of the Glomales, the question is whether the rate of gene conversion proceeds at a rate fast enough to repair the ribosomal genes as the mutations occur. This would cause homogeneity of ribosomal genes within a nucleus, but heterogeneity of sequences among nuclei in the same spore. The other possibility is that mutations could occur at a much faster rate than gene conversion, therefore resulting in some heterogeneity of gene sequences within and among nuclei.

Implications for a phylogeny of AM fungi

The existence of rRNA sequence variation within single spores of AM fungi puts into question the validity of the use of these sequences for understanding the phylogeny of this fungal group. In view of the probable lack of recombination in the AMF and the unchecked mutations which could occur, the variation seen in the ITS region in spores is not surprising, because mutations are much more likely to persist in non-coding regions than in coding regions. This is because selection reduces the number of deleterious mutations persisting in a population, whereas mutation in non-coding regions would frequently be selectively neutral (Wilson, Ochman & Prager, 1987). However, the variation in the VANS1 region observed in a natural population of *Scutellospora* indicates that the suggested phylogeny based on 18S ribosomal gene sequence regions (using the VANS1 primer), might not indicate the phylogeny of the AM fungi themselves but rather the phylogeny of those 18S gene sequences which were selected from spores because they contained the conserved VANS1 region itself. A high level of diversity in the 18S gene would then be missed. A similar problem has previously been addressed in relation to the phylogeny of bacterial lineages based on the 16S ribosomal gene (Woese, 1987, 1992); the question being whether the bacterial phylogenetic tree is really a phylogeny of the bacteria or of the 16S ribosomal gene. In the case of the bacteria, this question arises because genes can be transferred horizontally, i.e. bacteria contain an unknown number of promiscuous genes and, therefore, the molecular phylogeny based on some genes might have little relation to the true phylogeny of the organism. Establishing how much the ribosomal gene sequences can vary within single spores, between spores of identical morphology and between morphologically different spores will resolve the problem of relating ribosomal gene sequences to phylogeny in AM fungi and, therefore, whether ribosomal gene sequences can be used for constructing a phylogeny of AM fungi. In the case of the

ITS regions, only a small number of base differences were found between two sequences within a single *Glomus mosseae* spore and many more base differences in one ITS sequence obtained from *Glomus geosporum*, indicating that the differences between spores of different morphology are much greater than the differences within a single spore. It must be remembered, however, that the fungi came from single spore isolates and, therefore, replicate spores from the same isolate would be genetically similar. An experiment has been carried out at the University of York to compare the interspecific and intraspecific variation of ITS regions of several species of *Glomus* (Lloyd-MacGilp *et al.*, 1996). The intraspecific variation has been found to be as great as that between some species. In addition, intra-spore sequence variation has also been observed. This rRNA gene diversity supports the variation described by Sanders *et al.* (1995), Clapp *et al.* (1995) and Clapp *et al.* (unpublished).

However, the fact that phylogeny based on the 18S gene sequence containing the VANS1 primer site (Simon *et al.*, 1993) fits well with conventional studies of Glomales phylogeny, based on spore morphology, cannot be ignored.

Implications of distribution of AM fungi in the roots of natural plant communities

The knowledge that can be obtained regarding the distribution of AM fungi in the roots of natural plant communities is still limited by the lack of good species-specific molecular markers for AM fungi that can be used successfully in field investigations. One clear problem here is the lack of knowledge of genetics of AM fungi. If there is little homogeneity among gene sequences in a population then finding reliable markers is difficult. Despite these problems, the work of Clapp *et al.* (1995) with the family-specific markers clearly shows the coexistence of different AM fungi in the roots of an individual plant and, therefore, the potential for competitive interactions to occur among AM fungi. This has strong complications for mycorrhizal functioning since it is known that different isolates of AM fungi can have different effects on the nutrition and growth of plants (Jakobsen *et al.*, 1992). McGonigle (1988) found little evidence for a relationship between colonization by AM fungi and stimulation of growth under field conditions. It has been suggested by Fitter (1990) and Sanders & Fitter (1992) that coexisting AM fungi might be responsible for the lack of this relationship because the growth stimulation by one fungus might be masked by the presence, and different stimulation of growth, by another fungus. The development of further markers for AM fungi will allow the possibility of investigating this in the future.

Competitive interactions among coexisting AM fungi within the root have not been studied in the field. However, there is evidence from pot experiments that competition between AM fungi occurs in plant roots (Pearson, Abbott & Jasper, 1993) and that it is mediated by the host. The presence of several genera, and possibly species, within single roots raises questions regarding the mechanisms of coexistence of AM fungi and whether the fungi could be temporally and spatially separated in the root to avoid direct competition. Furthermore, the functional role of coexisting AM fungi needs to be examined. Such investigations may require the use of fluorescent oligonucleotide probes to identify the various AM fungi within the root.

FUTURE DIRECTIONS IN RESEARCH ON AM FUNGI

Understanding the genetics of AM fungi

We now have interesting data regarding the genetics of AM fungi but much more work is required to elucidate the mechanisms controlling the genetic diversity in these fungi. The task clearly is of high priority, not only for ecological studies of AM fungi but in order to understand the relationships between ecology, functionality and morphology in this group. If the genetic diversity in AM fungi is as great as the results from the York and Basle projects suggest, then it could also have important implications for investigations into the underlying molecular processes governing the development of the symbiosis. There is good reason to suggest that if the processes leading to the genetic diversity of AM fungi are not understood they could lead to inconsistent, irreproducible and uninterpretable results in these types of investigations. If we cannot find conservation in ribosomal gene sequences than what chances do we have of finding universal or conserved symbiosis-related genes from the fungal side of the arbuscular mycorrhizal symbiosis?

New molecular markers for field studies of AM fungi

The continuation of investigations into ecology of AM fungi in natural populations requires the development of reliable molecular markers. The use of ribosomal genes might turn out to be inappropriate for this purpose and the development of other likely candidates will probably have to await a better knowledge of the genetics of AM fungi. Until then we do not know whether other genes with a lower copy number can be conserved in the genomes of Glomales or whether these genes are suitable for use as markers in field studies. However, what we may find is that taxa of AM fungi have particular sets of sequences associated with them which allow a species to be identified by a sequence fingerprint. It

is also possible that a sequence might exist in this fingerprint that is unique and can therefore be targeted for the purposes of identification.

Descriptive vs. experimental investigations of ecology of AM fungi

There has been a trend in modern ecology away from descriptive studies of species or communities towards an experimental approach which identifies how organisms interact with the environment. For a wealth of plants and animals, in temperate communities at least, much of the descriptive work has been carried out already and forms a foundation for realistic experimental investigations into their ecology. In the case of AM fungi we neither have access to this information nor can we see what is happening below ground. For this reason, while the use of molecular techniques has, as yet, been for purely descriptive investigations of ecology of AM fungi they have a high value. In view of the fact that so little is known about the distribution of AM fungi in natural communities there is still much useful information to gain by using these techniques for descriptive purposes. Observational studies, however, will not be sufficient to elucidate the role of AM fungi in natural plant communities, and we stress that future studies that identify how AM fungi interact with the natural environment cannot be based solely on the use of molecular markers but, in addition, will have to include experimental investigations. The future success of this approach requires molecular markers for the identification not only of different AM fungi but also for important functional responses in the symbiosis.

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