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Organisation of genetic variation in multinucleate arbuscular mycorrhizal fungi

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Contents

Chapter 1

Introduction

1.1 The mycorrhizal symbiosis

The mycorrhizal symbiosis is widespread occurring in the roots of most terrestrial plants (Read, 1991). Three major types of mycorrhizal fungi that associate with plants have been described, ecto mycorrhizal fungi (basidiomycetous fungi), ericoid mycorrhizal fungi (mostly ascomycetous fungi) and arbuscular mycorrhizal fungi (zygomycetous fungi) (Smith and Read, 1997). Of these, ecto- and arbuscular mycorrhizal fungi (AMF) are the most abundant. They are estimated to associate with at least 80% of known terrestrial plant species (Smith and Read, 1997; Newman and Redell, 1987). Morphological structures similar to present day AMF have been found in fossils of the Early Devonian land plant *Aglaophyton major*, which is known to have existed at least 400 million years ago. The symbiosis is, therefore, considered ancient (Remy *et al.*, 1994). Phylogenetic analyses of ribosomal gene sequences of fossilized, *Glomus*-like spores suggest that the origin of glomalean fungi may be as early as 600 million years ago (Redecker, 2000).

The symbiosis is generally considered mutualistic because benefits are conferred to both partners. Plants benefit from the symbiosis with AMF because of improved phosphate acquisition (Marschner and Dell, 1994; Smith and Read, 1997), and because of improved uptake of micro nutrients such as zinc and copper (Smith and Read 1997; Harrison, 1999). Other important benefits are access to organic nitrogen sources (Hodge *et al.*, 2001), protection from root pathogens (Newsham *et al.*, 1994), improved drought resistance (Ebel *et al.*, 1996) and potentially improved heavy metal tolerance (Ricken *et al.*, 1996). In return, AMF receive photosynthates from the plant, and this can be up to 20 percent of total photosynthetically fixed carbon (Jakobsen and Rosendahl, 1990). Because of the age of the symbiosis and because of the conferred growth benefits by AMF it has been hypothesized that AMF may have played a crucial role for adaptation to terrestrial constraints during colonization of land by phototrophs (Simon *et al.*, 1993; Selosse and LeTacon, 1998; Redecker *et al.*, 2000). In addition to the ecological benefits derived by improved phosphate acquisition, over the past decade the ecological importance of AMF for terrestrial ecosystems has been further elaborated. Presence of AMF in soils has been shown to increase plant species diversity (Grime *et al.*, 1987) and co-occurring AMF species have been shown to have differential effects on growth of different plant species (Streitwolf-Engel *et al.*, 1997; van der Heijden, 1998a; Helgason *et al.*, 2002). This is one way

in which they contribute to plant community structure (van der Heijden, 1998b). Furthermore AMF species diversity influences plant species diversity and can increase ecosystem productivity (van der Heijden, 1998b; Klironomos *et al.*, 2000). Because AMF associate with most agricultural plants and because of their benefits to plants these fungi could also be useful in agriculture. This, along with their ecological importance has stimulated great interest in better understanding AMF biology and the functioning of the symbiosis.

1.2 Biology of AMF

AMF spores are produced on the termini of hyphae and are thought to be asexual structures. Spores germinate in the soil and after hyphal contact with the roots of a host plant. The fungus penetrates the surface via formation of an appressorium and colonizes the root cortex by intercellular hyphal growth. After entering living root cells the fungus then forms a highly branched dual membrane system known as an arbuscule. This is thought to be the site through which nutrient exchange occurs between the two partners. Following the colonization of roots, extraradical hyphae are produced that grow out into the soil. There, they take up nutrients and transport them back to the plant. Hyphae can also colonize other plants forming a hyphal network or can form new spores (further details are given in Harrison, 1997). Because AMF are obligate biotrophs, meaning they are completely dependent on green plants for their carbon energy supply (Ho and Trappe, 1973), the complete hyphal network can only be developed if an association with a host plant has been established.

The AMF hyphal network connects plants below ground (Wittingham and Read, 1982) and due to the lack of apparent host specificity this network also connects plants of different species (reviewed Sanders, 2002). AMF are coenocytic, meaning they possess a continuous cytoplasm and no cell separation occurs within hyphae. Therefore, within this hyphal network nutrients and nuclei have the potential to move in any direction. This is supported through the observation of bidirectional cytoplasmic streaming within single AMF hyphae (Giovannetti, 2000). Considerable transport of carbohydrates from one plant to another via the hyphal network that might be of ecological relevance was shown by Francis and Read (1984) and this is also true for other nutrients such as phosphate (Newman and Eason, 1993) and nitrogen (Frey and Schüepp, 1992). Differential allocation of nutrients from plant to plant via the hyphal network has been thought

Figure 1.1: Microscopic photographs of a) Spores of *Glomus* spp formed at the distal tips of growing hyphae b) intracellular highly branched structure of an arbuscule.

to mediate the effect of AMF on plant community structure and diversity, although at present there are no studies that provide direct evidence for this hypothesis (Grime *et al.*, 1987; Read, 1997).

Spores that are formed at the tips of AMF hyphae are fairly large (up to half a millimeter in diameter) compared to asexually produced spores of other fungi (several micrometers in diameter). Each spore contains many nuclei, ranging from several 100 nuclei in case of *Glomus* spp. up to several thousand nuclei in *Gigaspora* spp. (Burggraaf, 1989). During the whole life cycle spores are the only structures of a physically independent compartment that is formed in the hyphal network. Thus, it appears that there are no stages in the life cycle when the fungal material that can initiate a new generation is limited to only one nucleus. This seems to in contrast to almost all other eukaryotes. The organisation of nuclear movement in hyphae is poorly understood and, therefore, the effective origin of genetic material in spores is unknown. Whether only one nucleus moves into a spore and then replicates or whether several nuclei move into a spore has not been studied. However, germinating spores that originate from the same hyphal network have been shown to be able to anastomose (fuse). Thus it is possible that after germination a spore can establish cytoplasmic continuity within the network (Giovannetti *et al.*, 1999) and potentially exchange genetic information. The extent that this also happens in nature has not yet been studied.

Spores vary not only in number of nuclei but also in size and colour. Such morphological traits of spores have been taken as basis for a taxonomic AMF

classification. This resulted in the description of approximately 150 morphologically distinct species (Morton and Benny, 1990). Given this number of different species surprisingly little diversification has occurred during at least 450 million years of AMF evolution. Based on spore development and molecular phylogeny of rDNA AMF have been classified into five different families Paraglomaceae, Archeosporaceae, Glomaceae, Acaulosporaeceae and Gigasporaceae in the order Glomales (Morton and Benny, 1990, Morton and Redecker, 2001). Recently it has been proposed that AMF form a monophyletic group, probably having diverged from the same common ancestor as the Ascomycota and Basidiomycota (Schussler *et al.*, 2001). This has been suggested on the basis of rDNA sequences. As a result the current taxonomic classification of AMF families needs revising (Schwarzott *et al.*, 2001).

Despite several decades of intensive research on AMF many important features of the biology of AMF still remain unknown. This includes, knowledge about host preference or specificity, the extent of nutrient exchange and nuclear movement within hyphal networks or between hyphal networks (after anastomosis), the apparent discrepancies in taxonomic classification between AMF morphology and AMF phylogeny, the origin of genetic material in spores and basic knowledge about AMF genetics. With regards to the latter there is only little information about AMF genome size, about whether AMF are sexual or asexual and how genetic exchange might take place. There are no reports about the chromosome number, their arrangement or about the level of ploidy.

1.3 AMF Genetics

Only very few studies on AMF genetics have been performed. The reason why there is so little information about AMF genetics is mainly due to the technical difficulties in obtaining large quantities of pure AMF DNA and due to their obligate symbiotic character. AMF have never been grown successfully in pure culture (Ho and Trappe, 1973). Also, if growing symbiotically with a host plant, even fast growing *Glomus* species need a minimum reproductive time of 10-12 weeks to produce new spores resulting in long incubation periods for an experiment and limited amounts of fungal material. Successful cultivation of most AMF species is restricted to greenhouse cultures. Even when using sterilized soil and plant seedlings, this system presents a great risk for contamination with genetic material of non-AMF origin (as observed and discussed in Clapp *et al.*, 1999). In

addition, it has subsequently been shown that bacteria of the genus *Burkholderia* and viable fungi of the genera *Nectria* and *Leptospheria* live in the cytoplasm of some isolates of healthy surface sterilized AMF spores thereby indicating a serious problem for obtaining pure AMF DNA for genetic studies (Bianciotto *et al.*, 1996; Hijri *et al.*, 2002).

Some basic studies on the AMF genome have been performed. The GC content of AMF is low, with approximately 30%-35% in whole genome estimations and single gene sequences (Hosny, 1997; Requena, 2002). Based on flow cytometry, overall DNA content per nucleus was shown to range from 0.14pg DNA (*Scutellospora pellucida*) to 1.15pg DNA (*Scutellospora gregaria*) (Hosny *et al.*, 1998). The number of nuclei per spore ranged from a minimum of 1000 – 9000 for *Glomus caledonium* (Burggraff and Beringer 1989) to a maximum of 1000 – 35000 nuclei per spore for *Gigaspora decipiens* (Viera and Glenn, 1990). Unfortunately, because there is no clear knowledge about the ploidy level of AMF, the haploid genome size, based on DNA content per nucleus, remains unclear. Finally, because none of these studies accounted for the potential presence of fungi living in AMF spores, these estimations must be taken with great caution.

Using sensitive molecular methods (i.e. PCR-based) many studies have been performed that show variation in AMF genomes. Restriction fragment length polymorphisms (RFLP) on PCR amplified DNA fragments and cloning and sequencing of AMF DNA have been used to look at variation. This has mostly been performed with the aim of subsequent phylogenetic analyses and identification of AMF for field studies on AMF community structure. The first phylogenetic characterizations of AMF based on sequences of cloned AMF rDNA confirmed their classification within the true fungi and gave a first estimation of the age of AMF (Simon *et al.*, 1992; Simon *et al.*, 1993). However, despite the differences between species, unusually high intraspecific variation has been found within individuals and, after further investigation, was consistently found in all species of AMF that have been studied. Using PCR-RFLP on the two internal transcribed spacer regions (ITS) and DNA sequencing Sanders *et al.* (1995) showed that there are at least two variant ITS sequences in single spores of the AMF *G. mosseae*. Genetic variation was then shown within as well as between isolates of *G.mosseae* (Lloyd-McGilp *et al.*, 1996). Subsequently there were several more reports presenting high intraspecific ITS sequence variation in other AMF species (e.g. Lanfranco *et al.*, 1999; Antoniolli *et al.*, 2000). Even so, this variation has been largely ignored and was assumed to be unproblematic for

phylogenetic analysis based on the small ribosomal subunit (SSU) (Schuessler, 1999). This was mostly because at least part of this variation was thought to be due to contamination with foreign fungal DNA attached to spore samples (Clapp *et al.*, 1999; Redecker *et al.*, 2000) and due to less variation in SSU than in ITS regions (Schuessler, 1999). More recent studies concentrated on the variation in the large ribosomal subunit (LSU). In these studies high amounts of rDNA were PCR amplified from single spores and screened for presence of genetically different variants which were subsequently sequenced (Clapp *et al.*, 2001, Rodriguez *et al.*, 2001). Even with the most conservative estimates of this variation, where all potential contaminating sequences are removed the remaining intraspecific variation was considerable and, thus, could be problematic for phylogenetic classification. Again this variation has been assumed by phylogenetists to be due to contamination and was ignored. Therefore, although intraspecific genetic variation, even within single spores, is now widely accepted, there is obviously a general disagreement about its importance for AMF phylogeny and whether it presents a problem for studies using molecular techniques for analysis of AMF diversity in field communities.

Since the existence of this within isolate variation has been known for years, it seems surprising that none of these studies have attempted to analyze how and why this variation has been generated and maintained. Shortly after the first descriptions of intraspecific and even intraindividual AMF genetic variation, Sanders *et al.* (1996) have hypothesized that this could have arisen by divergence of nuclei in the absence of recombination. In this hypothesis, accumulation of non-purged mutations leads to divergent lineages of genetically different nuclei and, thus, to the evolution of a multigenomic state. The maintenance of such a state seems plausible given that it seems many nuclei are passed on to the next generation in one spore, but no test of whether there are multiple genomes has yet been performed.

At the beginning of this thesis only one study actually attempted to test whether AMF are recombinant. Amplified fragment length polymorhisms (AFLP) were used to generate a binary dataset based on the DNA of single AMF spores. Subsequent calculations of the index of association (a measurement of the degree of linkage disequilibrium) indicated that genetic variation among AMF spores deviated significantly from that expected by a recombinant population (Rosendahl and Taylor, 1997). Therefore, AMF spores are generally thought to be the result of little or no recombination. However, there are some serious shortcomings with this

study. First, there was no possibility to account for the reproducibility of the AFLP method as there was no replication of fingerprints originating from one spore, because of the very limited amounts of DNA that were obtained per spore. But this would have been important because with very small amounts of DNA there is a high likelihood for artefacts (e.g. incomplete genetic diversity due to increased probability for PCR amplification of frequent markers, differences in cutting efficiency of enzymes among samples). Second, the authors did not take into account that potential recombination might have occurred among genetically different nuclei within spores as they only compared polymorphisms among spores. Thus, the existence of recombination could have been masked at this level. And lastly, spores had been obtained directly from pot cultures, meaning that there was a high risk for contamination with foreign DNA. This is a serious problem with genome fingerprinting methods because there is no possibility to account for the origin of a polymorphic marker, i.e. whether it originates from the target organism or from a contaminant organism.

In addition to studies on rDNA sequences some information about other gene sequences from AMF has been collected using targeted and non-targeted approaches. The goal of most of these studies was to isolate important genes involved in metabolism and symbiosis-specific AMF genes (Harrier, 1998; Ferrol *et al.* 2000; Ulbalijoro *et al.*, 2001). Here, some genes involved in transport processes, such as phosphate transporters, genes that are differentially expressed during pre-symbiotic and symbiotic growth have been isolated (Bucher *et al.*, 2001; Harrsion *et al.*, 1998; Requena *et al*., 1999, 2000, 2002). However, in view of the considerable research efforts that have been undertaken during the last 10 years to understand the functional and molecular mechanisms of the AMF symbiosis, the number of genes clearly identified is limited. So far potential variation in these genes has been ignored. Besides the aforementioned difficulties during cultivation, one of the reasons for the limited success might be the unusually high genetic variation in AMF which gives problems for designing primers. Additionally, the difficulty in obtaining high quality DNA extractions, from which genes in low copy number can be successfully amplified is problematic as is getting DNA that is free of contaminants. Indeed, many of the current sequences available for AMF genes in the public databases are likely to be contaminations of non AMF-origin (N. Corradi, personal communication).

Unfortunately, until now genetic variation in AMF has only been studied in ITS and rDNA. Because these genes are multicopy it is difficult to identify the origin of this variation (i.e. within genome or between genomes). To date no single study has attempted to analyze whether and how much genetic variation exists in regions of the AMF genome other than rDNA, such as functional gene sequences.

A completely different experimental approach to analyze AMF genetics and its potential importance for symbiotic functioning has been based on quantitative genetic traits. Bentivenga *et al.* (1997) have shown that during cultivation in identical environments significant variation in spore morphological traits such as size and colour occurs within a single spore culture of *Glomus clarum*. This was considered as evidence for a type of nuclear genetic drift, indicating that genetic heterogeneity in the hyphal network had potentially been created based on differential distribution of nuclear genotypes. In a second experiment, Bever and Morton (1998) observed that selection of an ellipsoid spore morphotype of the AMF *Scutellospora pellucida* for initiating new cultures led to increased frequency of this morphotype after one period of growth. Thus, there is not only variation in quantitative traits but also that these traits are genetically based and heritable. Unfortunately, the first experiment had been performed on the basis of a culture that was started from one spore that was isolated from a natural field site. Therefore, there is no way of knowing what the orginal genetic diversity was in a field population and what the potential ecological relevance of this variation was. To date, no study exists that has analyzed the actual genetic and morphological diversity in a field population of an AMF. One reason for this is that to obtain a representative set of individuals for an experiment requires several years of intensive work in order to isolate and prepare the fungal starting material.

1.4 The importance of intraspecific genetic variation

At the beginning of this thesis a number of major gaps existed in our knowledge concerning the genetics of AMF. This includes: Whether nuclei are genetically different. How intraspecific variation is maintained in AMF. Whether AMF undergo recombination. In addition, the distribution of intraspecific variation in a natural population of AMF has never been studied. And lastly analysis of genetic variation in other regions than rDNA has never been performed.

Consequences of genetically different nuclei

The question whether nuclei are genetically different is of importance for research in evolutionary biology. Almost all organisms exist at one part of the lifecycle in a unicellular uninucleate stage. Thus, the effect is a strong genetic bottleneck that prevents within individual variation. This fact is reflected in the models that have been developed to understand the evolutionary forces which are acting and shaping genomes and how genetic diversity is created and maintained in these organisms. No such stages have been observed in AMF. Therefore, if nuclei are genetically different then new models would have to be developed to understand how, for example evolutionary forces could act on the genomes of AMF. Obviously, if nuclei are genetically different then one mechanism to maintain a high genetic diversity would be to pass many nuclei into newly formed spores. This way, the effects of evolutionary forces such as accumulation of mutations, genetic drift and selection could be different for AMF than in many other organisms.

Intraspecific genetic variation in ancient asexuals

AMF intraspecific and intraindividual variation has been interpreted as being the potential result of accumulated mutations during long term evolution without sexual reproduction (Sanders *et al.*, 1996). Only few groups of organisms are thought to have evolved without sex for millions of years (e.g. Bdelloid rotifers, darwinulid ostracods). These ancient asexuals have been considered scandalous because they directly contradict evolutionary theory (Judson and Normark, 1996; Maynard-Smith, 1986). Their existence challenges the idea of sexual reproduction as the major reproductive strategy among the great majority of living species. Understanding these mechanisms, firstly, why sexual prevalence has evolved, and secondly, how some organisms manage to be asexual for millions of years has fascinated evolutionary biologists. Several hypotheses have been put forward to explain the existence of ancient asexuals (Judson and Normark, 1996). Within sexually reproducing species it would be expected that recombination and segregation would allow random genetic drift to drive selectively neutral alleles towards fixation or extinction, thereby, limiting variation (divergence) between allelic sequences (Kimura and Crow, 1964). Conversely, for ancient asexuals it has been hypothesized that due to accumulation of mutations, these organisms should have evolved to contain highly divergent allelic sequences in their genome. In fact, evidence for this was found in Bdelloid rotifers (Welch and Meselson, 2000).

Ancient asexual *Darwinula stevensoni* have been shown to comprise particularly limited variation among allelic sequences and it is suggested that it is due to the evolution of highly efficient DNA repair systems (Schön and Martens, 2003). In AMF, the evolution of a multigenomic state (segregation of genetic material among nuclei) in AMF could provide a novel way of how AMF managed to evolve in a purely asexual way. Therefore, it would be important to test whether AMF are truly asexual.

Because recombination might occur among nuclei in one fungus it seems advisable to perform a test that could actually detect any recombination that had occurred among nuclei. Population genetic techniques could be used, but this would require isolation and analysis of single nuclei as individuals. Genotyping of single nuclei would then even increase the potential problems associated with small amounts of DNA (as described for AFLP studies on DNA from single spores). Alternatively, a different test could be performed by analyzing variation between allelic sequences. These can be obtained from a culture that had been propagated clonally from a single spore and had no possibility of exchanging genetic material with another fungus. Each variant sequence could be taken as an individual. Whether variation among the isolated sequences is the result of recombination could then be tested using various methods. However, in the absence of recombination, nuclei should have evolved to be genetically different. Two highly divergent sequences as differently labelled probes could be used in an experiment where nuclei are hybridized with both probes. If these sequences are segregated among nuclei this could be taken as evidence for genetically different nuclei.

Ecological relevance of intraspecific genetic variation in AMF

It is common habit to perform comparative ecological and molecular experiments by analyzing one representative AMF single spore culture for each species (e.g. Smith *et al.*, 2000; van der Heijden 1998a +b). These studies presume that within species variation is non-significant and would not change the outcome of these studies. However, given the amount of intraspecific variation that has been observed on rDNA within and between isolates there is a likelihood that there are considerable differences in populations. If differences exist between individuals then these would be the result of evolutionary forces such as genetic drift and or selection that have acted on the population. To my knowledge no population studies have yet been performed on AMF and thus there is neither knowledge

about the potential ecological relevance of intraspecific variation nor knowledge about evolutionary forces acting on AMF

For the identification of potential variation on the level of spores it would be best to sample a number of individuals from an AMF population from a field site and to characterize and compare them. This is important in order to know whether differences among individuals of one AMF species has potential biological (ecological) relevance and to identify what forces might have acted on the population to create or maintain the variation. Comparison of any observed genetic and phenotypic differences between species with the intraspecific differences would then be of use to identify how much of this variation exists within an AMF species. Intraspecific variation could account for the reported interspecific differences and, thus show that these experiments might have missed an ecological relevant level.

Intraspecific genetic variation in functional gene sequences

Based on the high variation that has been observed in rDNA and on the possibility that AMF contain multiple genomes there is a high likelihood that intraindividual and intraspecific variation also occurs among functional gene sequences. By functional genes I mean gene sequences which are translated into proteins and thus result in an amino acid sequence. This receives further support, because although rDNA is known to be arranged in multiple copies per genome these should be kept the same due to a process called concerted evolution. Variable sequences clearly indicate that homogenizing effects of mechanisms such as gene conversion or highly effective DNA damage repair systems (as discussed in Schön *et al.*, 1998) are not operating in such frequency or efficiency to cause this homogeneity in AMF. Therefore intraspecific or within isolate genetic variation might not only be restricted to rDNA.

To investigate variation in functional genes it would be best to analyze genes which are single copy (exist once per genome) and which are also translated into an amino acid sequence. First, if they were truly single copy in AMF then any observed variation could be interpreted as evidence for variation among nuclei. Second, if these genes were translated into amino acid sequences then further analysis at which sites variation occurs (e.g. non-synonymous : synonymous) would then help to elucidate how strongly selection might have acted or is acting on these parts of the genomes. Third, variation in a single copy gene could then

also result in a realistic estimate of total genetic diversity within an AMF. Fourth, based on whether there are functional variants that lead to different proteins and based on the number of copies per nucleus it might then be possible to know whether functional genetic information is partitioned among nuclei.

Importance of genetic diversity for commercial applications

In commercial production of AMF inoculum genetic diversity may be desirable to maintain functional diversity. The genetic diversity that is comprised within an AMF might be very high. Segregation of genetic information could occur among developing spores and result in only fractions of this diversity being present within each spore. However, depending on the amount of total genetic diversity the quality of inoculum could be increased by knowing how many spores should be sampled to initiate inoculum production to cover most of the naturally occurring diversity. Currently this amount of diversity is unknown and thus a study of genetic variation in an AMF population can also be directly useful to predict sampling strategies to develop genetically diverse inoculum.

1.5 Experimental outline

In this thesis I have used several different approaches to carry out a study on the genetic variation within AMF genomes. These approaches were aimed to fill the gaps identified in section 1.4 concerning whether AMF undergo recombination, the distribution of genetic variation in a population and presence of genetic variation in other regions than rDNA. The basic principle of my work is the dissection of the variation in AMF genomes at the level of an AMF population, within a single spore culture (one isolate) and within and among nuclei. In a first study, two fundamental problems have been approached, first whether nuclei are genetically different and second whether sequences of ITS regions, ribosomal genes and the binding protein gene (*BiP*) have evolved in the absence of recombination.

This first study was performed and published in *Nature* as presented in chapter 2 in collaboration with Mohamed Hijri. He performed a fluorescent *in situ* hybridisation experiment where the nuclei of single spores were hybridized with probes of 2 ITS variants labelled with different dyes to analyze whether nuclei are genetically different. Variation in the frequency of hybridization signals of one variant in relation to the other on individual nuclei was taken as evidence for

genetically different nuclei. Because the results of this experiment were published together with my studies on recombination they are presented in chapter 2 of this thesis.

A compatibility analysis was performed in order to identify recombination. This analysis is phylogenetically based, and tests whether polymorphic sites in sequences are likely to deviate from the predicted tree structure when recombination is assumed. Presence of significant deviation was taken as evidence for recombination. The test was performed on sequence data from ITS and a gene coding for a binding protein (*BiP*).

In a second study, spores from a population of *Glomus intraradices* at one field site were isolated and used to start single spore cultures. These were transferred into sterile laboratory cultures and were maintained for several months in identical conditions to remove maternal effects. These cultures were then analyzed with respect to differences in genetic markers using the AFLP fingerprinting technique. Analysis of molecular variance was performed to investigate patterns of genetic variation and for estimation of variance components at three different hierarchical levels in the population. This study was performed in close collaboration with Alexander M. Koch, who performed the complementary phenotypic measurements and subsequent analysis of variance on different fungal growth variables. This approach allowed the identification of mechanisms which might be acting on a natural population. The results of both studies are going to be submitted together to the Proceedings of the *National Academy of Sciences* they are presented in chapter 3 in this thesis.

Ribosomal gene sequences are not translated into amino acid sequences and all observed variation is non-synonymous. In order to identify evolutionary important mechanisms such as selection and drift, I further analyzed the variation among *BiP* sequences in the AMF *G. intraradices* by analyzing how much of the variation was likely to be selectively neutral or not. In order to avoid contaminating sequences, I used a culture of this fungus growing in sterile conditions on Ri T-DNA transformed *Daucus carota* roots (St-Arnaud *et al.*, 1996). This system can be used to obtain huge quantities of high quality and non-contaminated DNA.

In different fungal species the *BiP* homolog has already been shown to be single copy (Stedman and Buck, 1996) which made this gene a suitable candidate for analysis. Using the DNA of a single spore culture analysis of a representative population of these sequences, I aimed to estimate the total sequence diversity. The functionality of variants was then determined based on predicted amino acid

sequences allowing insight into the evolution of this gene in AMF and whether these variants are segregated among nuclei.

Using these approaches, variation at the level of single gene sequences, nuclei, and on single spores was analyzed and provided valuable insight into the organization and evolution of the AMF genome and its consequences for studies on AMF ecology, molecular biology and AMF commercial inoculum production.

Chapter 2

Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi

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Abstract and Introduction

Ancient asexuals directly contradict the evolutionary theories that explain why organisms should evolve a sexual life history (Maynard Smith, 1986; Welch and Meselson, 2000). The mutualistic, arbuscular mycorrhizal fungi are thought to have been asexual for approximately 400 million years (Judson and Normark, 1996; Rosendahl and Taylor, 1997). In the absence of sex, highly divergent descendants of formerly allelic nucleotide sequences are thought to evolve in a genome (Welch and Meselson, 2000). In mycorrhizal fungi, where individual offspring receive hundreds of nuclei from the parent, it has been hypothesized that a population of genetically different nuclei should evolve within one individual (Sanders *et al.*, 1996; Sanders, 1999). Here we use DNA–DNA fluorescent in situ hybridization to show that genetically different nuclei co-exist in individual arbuscular mycorrhizal fungi. We also show that the population genetics techniques (Rosendahl and Taylor, 1997) used in other organisms are unsuitable for detecting recombination because the assumptions and underlying processes do not fit the fungal genomic structure shown here. Instead we used a phylogenetic approach to show that the within-individual genetic variation that occurs in arbuscular mycorrhizal fungi probably evolved through accumulation of mutations in an essentially clonal genome, with some infrequent recombination events. We conclude that mycorrhizal fungi have evolved to be multi-genomic.

Arbuscular mycorrhizal fungi (Class Zygomycetes; Order Glomales) are extremely successful fungi that form mutualistic symbioses with the roots of approximately 60% of all plant species (Smith and Read, 1997). They improve plant nutrition and promote plant diversity (van der Heijden *et al.*, 1998). These fungi have been assumed to be asexual (Smith and Read, 1997). This is supported by measurements of the degree of linkage disequilibrium, which indicated that genetic variation among the spores of arbuscular mycorrhizal fungi deviates significantly from that expected from a recombinant population (Rosendahl and Taylor, 1997). Genetic diversity in the ribosomal DNA occurs inside individual spores (Sanders *et al.*, 1995; Lloyd-MacGilp *et al.*, 1996; Redecker *et al.*, 1997; Lanfranco *et al.*, 1999), even though it is thought that several copies of rDNA are kept the same by concerted evolution (Hoelzel and Dover, 1991). It has been hypothesized that by accumulation of mutations, in the absence of recombination, individual arbuscular mycorrhizal fungi have evolved to comprise genetically divergent nuclei, or that one individual contains several genomes (Sanders *et al.*., 1996). Here we refer to an arbuscular mycorrhizal fungal spore as an individual.

Results and Discussion

We tested the hypothesis that individuals contain genetically different nuclei by performing specific fluorescent DNA–DNA *in situ* hybridization (FISH) on nuclei from spores of the arbuscular mycorrhizal fungus *Scutellospora castanea* (BEG 1). We used hybridization probes that specifically recognize two divergent sequences of the ITS2 region, known as T2 and T4, that were previously shown to co-occur within individual spores of this fungus (Hijri *et al.*, 1999). Probes were only used for variant ITS2 sequences that had previously been shown to be of glomalean origin (Redecker *et al.*, 1999). Single-target FISH showed that significantly more nuclei in *S. castanea* spores contained sequence T2 (40%) than T4 (17%) (Fig. 2.1a, b; Table 2.1). Double-target FISH using T2 and T4 probes showed that the divergent sequences T2 and T4 were indeed segregated in different frequencies among the nuclei (Fig. 2.1c, d; Table 2.1). Approximately 40% of nuclei contained only the T2 sequence and between 6 and 9% of nuclei contained only the T4 sequence. T2 and T4 co-occurred in between 8 and 9% of nuclei (Fig. 2.1c, d; Table 2.1). These results support the hypothesis that arbuscular mycorrhizal fungal spores contain a population of genetically different nuclei.

We need to know whether this genetic variation has been brought about by lack of recombination. Because several genomes exist within individuals, recombination could potentially occur among nuclei within individuals and this has not previously been considered. Using a theoretical approach we assessed whether the prediction from previous population genetic studies that mycorrhizal fungi are clonal (based on fingerprinting and calculations of index of association) is valid. We constructed artificial data sets representing presence or absence of alleles at 15 loci for a population of recombining nuclei within 30 spores. We then calculated the total number of nuclei containing an allele at each of the loci, for each spore. However, a fingerprinting technique performed on whole spores cannot take into account within-individual polymorphism (Fig. 2.2a). Therefore, whether the presence or absence of an allele at a given locus would be observed for each spore would depend on the number of nuclei in which that allele is present and the sensitivity of the fingerprinting technique (Fig. 2.2b). We therefore

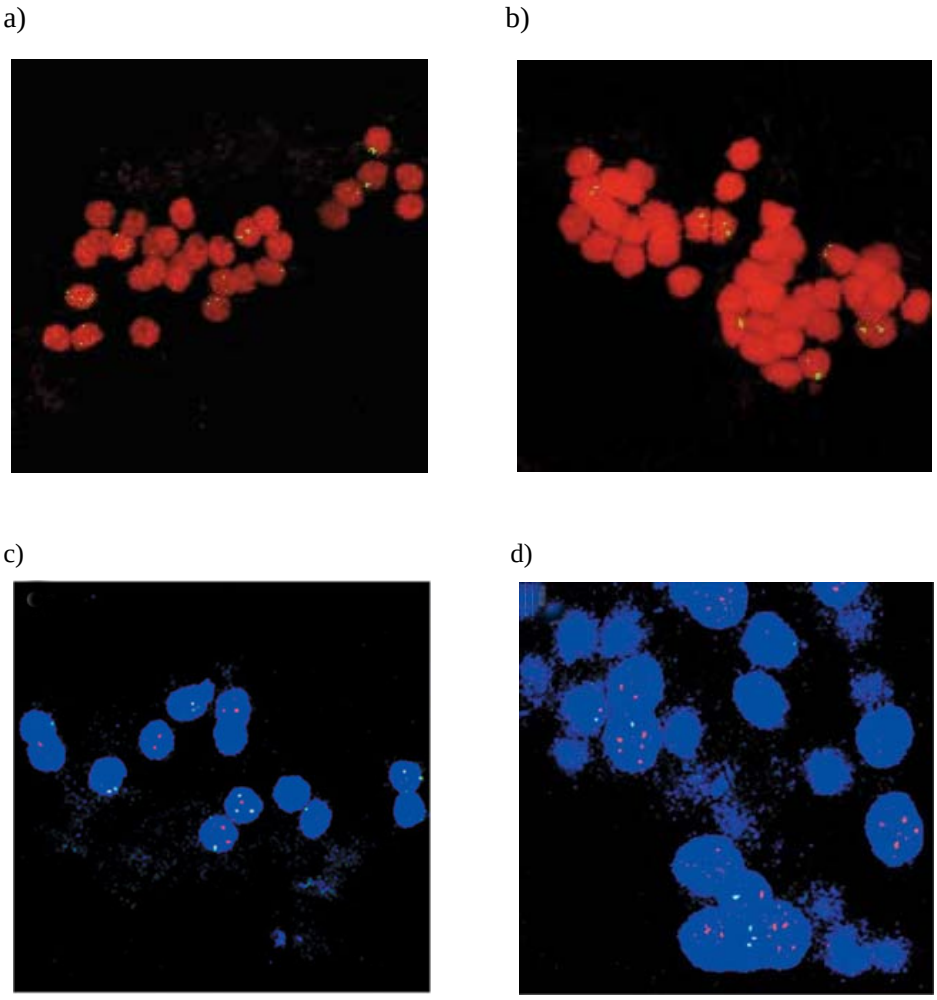

Figure 2.1: Nuclei of Scutellospora castanea taken with scanning laser confocal microscopy after single-target and double-target DNA–DNA FISH. a, Hybridization signals (green) of the probe T2-DIG to nuclei (red). b, Hybridization signals (green) of the probe T4-DIG to nuclei (red). c, Hybridization signals of the probes T2-DIG (light blue) and T4-biotin (red) to nuclei (purple). d, Hybridization signals of the probes T2-biotin (red) and T4-DIG (light blue) to nuclei (purple). The colours of images c and d have been adjusted to give better contrast between the colour of the two probes and the nuclei.

calculated the presence of alleles at each locus for each spore, simulating different levels of sensitivity of the fingerprinting technique. We then calculated the index of association (Maynard Smith *et al.*, 1993) for the population spores. All observed values of the index of association deviated significantly from zero (Fig. 2.3), allowing us to reject the null hypothesis that the population of hypothetical arbuscular mycorrhizal fungal spores were a recombining population. We concluded that any potential recombination was hidden within individuals and was

Table 2.1: Results of single and double DNA-DNA FISH on nuclei of the mycorrhizal fungus *Scutellospora castanea*.

Treatments	Number of nuclei observed	% nuclei hybridizing with T2	% nuclei hybridizing with T4	% nuclei hybridizing with T2 & T4	% nuclei unlabelled
Single FISH with T2 (digoxigenin)	1662	40.26 (3.04)	-	-	59.74 (3.04)
Single FISH with T4 (digoxigenin)	3405	-	17.03 (2.77)	-	82.97 (2.77)
Double FISH with T2 (biotin) & T4 (digoxigenin)	1120	40.16 (2.87)	6.52 (1.04)	8.20 (2.30)	45.12 (3.46)
Double FISH with T2 (digoxigenin) & T4 (biotin)	1364	41.02 (3.38)	9.64 (2.48)	9.63 (2.39)	39.71 (4.16)

Numbers in parentheses represent $\pm$ 1 s.e.m. Five spores were crushed on each slide and the number of slides observed were 34, 43, 28 and 23 for the treatments single-target FISH (T2-DIG), single-target FISH (T4-DIG), double-target FISH (T2-biotin and T4-DIG) and double-target FISH (T2-DIG andT4-biotin), respectively. Proportions of nuclei that were labelled with T2 or T4 did not differ from the presented results in experiments with a smaller sample size, where one spore was placed on each slide. Student's t-tests showed significant differences between the percentage of nuclei labeled with T2 and T4 in single-target FISH ($t_{1.75} = 30.31$, $P \leq 0.0001$) and between T2 and T4 in both double-target FISH experiments ($t_{1.44} = 121.76$, $P \leq 0.001$ and $t_{1.54} = 56.05$, $P \leq 0.0001$). The *t*-tests revealed no significant differences in the percentage of nuclei hybridizing to either T2 or T4 as a result of different labeling and also showed no significant differences in hybridization percentage with a given probe in single-target FISH experiments compared to double-target FISH experiments.

not detectable using this technique. Thus, the genomic structure of arbuscular mycorrhizal fungi gives rise to difficulties in detecting recombination with techniques that are based on linkage disequilibrium. To solve this problem, we used a phylogenetic technique known as character incompatibility analysis (Mes, 1998) to detect whether genetically divergent nuclei in these fungi are likely to have arisen by the accumulation of mutations in clonal nuclear lineages or by recombination events. We looked at sequence variation in ITS regions (including the 5.8S gene) within isolates of the arbuscular mycorrhizal fungi *Glomus geosporum*, *Glomus mosseae* and *Gigaspora margarita* and in the 28S gene in *G.*

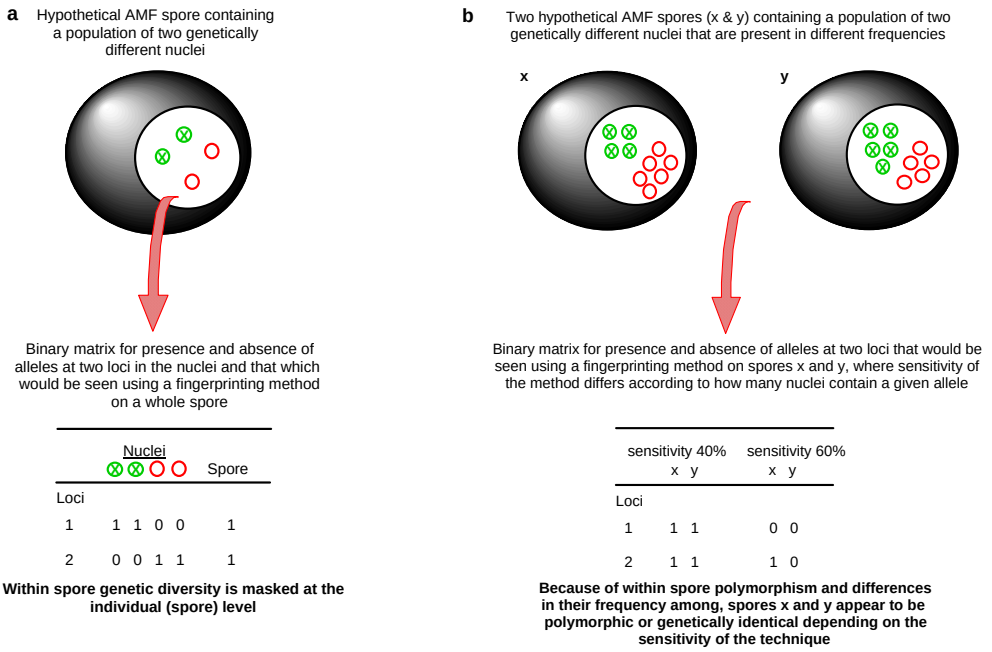

Figure 2.2: Within- and among-spore polymorphism. a, A spore of a hypothetical arbuscular mycorrhizal fungus contains a population of two genetically different nuclei (crossed and open circles). Within-individual polymorphism caused by differences among nuclei is masked in a fingerprinting method on the whole spores becaused the presence of both alleles is detected. B, Two hypothetical spores of an arbuscular mycorrhizal fungus, x and y, contain a population of two genetically different nuclei that are present in different frequencies. At a sensitivity of fingerprinting of 40%, where at least four nuclei carrying a given allele are required to show its presence in the spore, spores x and y appear to be genetically identical. At sensitivity 60%, where six nuclei are required to show the presence of an allele, spores x and y appear to be genetically different. Thus, spores can appear to be polymorphic or identical, depending on the fingerprinting sensitivity. Binary matrices show presence of alleles (1) or absence (0) during detection using a fingerprinting method at two different sensitivities.

geosporum, *Glomus coronatum*, *Glomus constrictum* and *G. mosseae* (Clapp *et al.*, 2001) (Table 2.2). Calculation of the Le Quesne probability (Mes, 1998) showed that for a large proportion of the variable characters the incompatibility count differed significantly from that which would be expected if it had arisen from recombination. A small proportion of the variation in rDNA sequences could be explained by recombination events, although this was higher in *Gi. margarita* than for the other fungi. Using a jacknife procedure we showed that only a small proportion of the sequences contributed nearly all the variation that is explained as

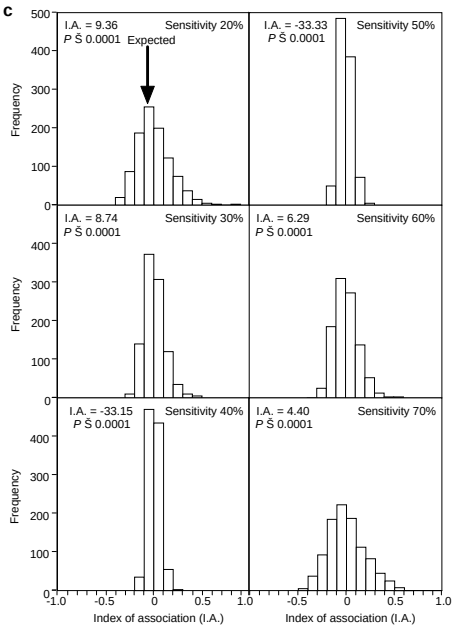

Figure 2.3: Frequency distributions for the index of association for a population of 30 hypothetical spores of an arbuscular mycorrhizal fungus. Each spore contains a population of recombining nuclei, calculated at 20% to 70% sensitivities of the fingerprinting. Data calculated with 80% sensitivity did not give a normal frequency distribution and is not shown. Each graph also shows the observed I.A. for the data set: in each case this differed significantly from the expected I.A.

being the result of recombination events (Table 2.2). This was also true for the variation seen in *Gi. margarita*. The results show, therefore, that most of the variant sequences are the result of accumulation of mutations in a clonal genome.

Until now, reports of within-individual sequence variation in arbuscular mycorrhizal fungi have been restricted to rDNA sequences. Our results demonstrate genetic differences only among nuclei for one region of rDNA. Therefore, we also provide evidence to support genetic differences among fungal nuclei, clonality and Muller's ratchet. We analysed the sequence of part of a gene encoding for a binding protein (*BiP* gene) that has high amino-acid sequence similarity (86% and 78%) to that of *Aspergillus niger* and *Saccharomyces cerevisiae*, respectively (van Gemeren *et al.*, 1997). This gene is highly conserved in eukaryotes and is a single-copy gene in other fungi. We sequenced 15 variant sequences of this gene from genomic DNA of one isolate of *Glomus intraradices*.

Table 2.2: Results of incompatibility analysis on the variation in rDNA sequences within different mycorrhizal fungi.

Analysis performed on variable sequences of 28S gene

AMF Species	No. variable sequences	No. clonal characters (% of variant characters)	No. recombinant characters (% of variant characters)	MIC 50¶	MIC 10¶
G. geosporum§	26	50 (83%)	10 (17%)	3%	7%
G. geosporum#	26	17 (85%)	3 (15%)	-	-
G. mosseae§	22	102 (68%)	47 (32%)	13%	54%
G. mosseae#	22	47 (70%)	20 (30%)	-	-
Gi. margarita§	18	53 (52%)	48 (48%)	11%	33%
Gi. margarita#	18	10 (31%)	22 (69%)	-	-

§ The analysis was performed with insertions or deletions in the dataset representing a fifth character. # The analysis was performed on sequences that only varied due to substitution. ¶ MIC 50 and MIC 10 are defined as the percentage of sequence variants that need to be removed in order to reduce the matrix incompatibility to less than 50% and less than 10% of the total matrix incompatibility in the dataset. This is performed by sequentially eliminating the sequences that contribute the most to the total matrix imcompatibility and then re-calculating the total incompatibility contained in the remaining dataset.

Along a length of 680 base pairs (bp), these variants ranged in similarity at the nucleotide level from 92% to 99% from one of the sequences that was randomly chosen as the comparison sequence (see the Supplementary Information). If this gene is also single-copy in arbuscular mycorrhizal fungi then the sequence variants that we observed must be segregated among nuclei. Incompatibility analysis indicated that no variation in these sequences was likely to be due to recombination. Furthermore, both synonymous and non-synonymous substitutions occurred in the sequences; that is, not all of the substitutions are selectively neutral. The mean number of substitutions compared to the comparison sequence was 26.6 (range 3 to 48) with a mean ratio of 1.02 (s.e.m. $\pm$0.10) synonymous to one non-synonymous substitution. We would expect a higher rate of synonymous substitutions to non-synonymous substitutions unless Muller's ratchet is in operation, because selection should act to conserve the sequence at non-synonymous sites. These data are consistent with evidence for Muller's ratchet in genomes of endosymbiotic prokaryotes (Moran and Wernegreen, 2000).

From our results, we predict that genetic variation is generated by accumulation of mutations in a predominantly clonal genome, leading to the creation of a population of genetically different nuclei. The evidence for (infrequent) recombination events is unsurprising in an organism that contains genetically different nuclei that co-exist coenocytically. However, our analyses indicate that these recombination events are rare and do not purge the majority of mutations in the genomes. Many other fungi have stages of heterokaryosis, where more than one nuclear genotype coexists, although genetic bottlenecks occur in the life cycle of each individual at each generation, limiting the number of nuclei that are transferred to the next generation to one per individual. No such stage in the life history of arbuscular mycorrhizal fungi is known and new spores receive many nuclei from the mother hyphae. Spores may also receive genetic material by fusion of hyphae (Giovannetti *et al.*, 1999; Giovannetti *et al.*, 2001), although the studies suggest that cross-incompatibility is most frequent. We suggest, therefore, that such a mechanism would not reduce the potential problems associated with Muller's ratchet as the nuclei received would also be accumulating mutations. Most theories of evolutionary and population genetics assume that one individual contains a single genome and so new models for evolution of multi-genomic organisms need to be developed. Our results may stimulate research on mycorrhizal symbiosis at the molecular and physiological levels, because several variants of the same gene exist in an individual and could lend to variation in expression. Our results provide essential information that is needed before any studies on genomics or proteomics of arbuscular mycorrhizal fungi are undertaken.

Methods

Slot-blot and DNA–DNA FISH

Hybridization probes were constructed for two different sequences of the ITS2 region, subsequently referred to as T2 and T4, that were known to be variable in *S. castanea* (GenBank accession numbers SCAJ2872 and SCAJ2874) (Hijri *et al.*, 1999; Redecker *et al.*, 1999). Probes were produced from cloned DNA that was amplified with the forward primer (5'-CACCTGCTTGAGGGTCAGT-3') and the reverse primer ITS4 (White *et al.*, 1990). The size of the probes were 274 bp and 258 bp for T2 and T4, respectively. Probes were labelled by polymerase chain reaction (PCR) with DIG-11-dUTP and Biotin-16-dUTP (Roche) and purified with QiaQuick PCR purification kit (Qiagen).

Spores of *S. castanea*, *G. geosporum* (BEG 18) and *Glomus* sp. (BEG 19) were collected from pot cultures and immediately fixed (in 4% formaldehyde, 100 mM Tris/HCl, pH 8, 100 mM NaCl, 2 mM $MgCl_2$ and 0.05% Triton X100, for 2 h at room temperature). Five spores or single spores were crushed on Frost Plus slides (Polylabo) and then dried overnight. For single-target FISH, 25 µl of the hybridization solution was loaded per slide. For double-target FISH, a mixture of digoxigenin and biotin probes in a 1:1 ratio was added to the hybridization solution. Hybridization was carried out at 37 °C overnight. The post-hybridization washes were made twice with 50% formamide in double-strength SSC (42 °C, 10 min), then twice with double-strength SSC, (37 °C, 5 min) and then rinsed again twice with half-strength SSC with 0.1% SDS (60 °C, 15 min). The post-hybridization washes were identical to those used for the slot-blot procedure. For full details regarding slot-blot conditions and slide preparation for FISH and all controls see the Supplementary Information.

For signal detection in single-target FISH, slides were incubated with anti-DIG-fluorescein conjugate antibody (Roche) and counterstained with propidium iodide. In double-target FISH experiments, signals were detected with a mixture of anti-DIG-fluorescein conjugate antibody (Roche) and Streptavidin Texas-Red conjugate. The slides were counterstained with TOTO-3 (molecular Probes). Slides were examined using a scanning confocal microscope (further details are given on signal detection and microscopy in the Supplementary Information).

Testing for detection of recombination

Thirty identical data sets were constructed, representing 30 spores of arbuscular mycorrhizal fungi, each containing a population of 50 genetically different nuclei that were variable at 15 loci. The nuclei within each spore were then recombined by the random rearrangement of alleles among nuclei, assuming that loci were independent of each other. The frequency of genetically different nuclei occurring in each spore was then altered by randomly selecting 10 nuclear genotypes that were then replicated a random number of times in the spore, with a maximum limit of 10 replicates of a given nuclear genotype per spore. The number of nuclei showing the presence of an allele at each locus was summed for each spore. The presence or absence of an allele at each locus was then calculated for each whole spore using different sensitivities of the fingerprinting method to give binary data sets. Sensitivity was defined by the percentage of nuclei containing a given allele that was required to give a positive signal with a fingerprinting technique. The sensitivities ranged from 100% (where the presence of an allele in 1 nucleus in a spore was sufficient to give a positive signal with a fingerprinting technique) through to 0% (where even if every nucleus contained the allele the technique would not be sensitive enough to detect presence of the allele). Polymorphism among the 30 spores at the 15 loci was calculated for data sets at 20%, 30%, 40%, 50%, 60%, 70% and 80% sensitivities. Sensitivity was calculated on a percentage basis rather than actual numbers of nuclei to account for differences in the numbers of nuclei among spores. Each of these seven data sets were then separately used to calculate the index of association.

Incompatibility analysis on rDNA and BiP gene sequences

Incompatibility analysis was performed on sequences of ITS and 28S rDNA from different arbuscular mycorrhizal fungi species. ITS sequences of *G. geosporum* (BEG 18) were obtained by extracting genomic DNA from spores (Qiagen DNeasy Plant Mini Kit) from a culture that originated from a single spore and amplified by PCR[9]. The 640-bp product containing ITS1, the 5.8S gene and ITS2 was purified (Qiagen QiaQuick purification kit), ligated into a pGEM-T vector and transformed into *Escherichia coli* JM109 (Promega). Clones were selected at random and both strands were sequenced using a dye-terminator cycle sequencing kit. Sequences were carefully edited by hand. ITS sequences from *G. geosporum* (BEG 18) and from *G. mosseae* (Antoniolli *et al.*, 2000) and *G. margarita* (Antoniolli *et al.*,

2000) were aligned. Sequences of the 28S gene for the species *G. geosporum* (BEG 11), *G. coronatum* (BEG 49), *G. constrictum* (BEG 130) and *G. mosseae* (BEG 25) (Clapp *et al.*, 2001) were also aligned and used for the analysis. All isolates originated from single spores except *G. coronatum* and *G. constrictum*. Further details on the compatibility analysis on rDNA and the gene encoding a binding protein are given in the Supplementary Information.

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Supporting information

Further information on probes, slot-blot hybridization, microscopy and FISH controls

Probes

We did not raise probes for the full reported ITS1-5.8S-ITS2 sequences because similarity of these sequences in the ITS1 and 5.8S regions is too high to satisfy the requirements for specific *in situ* hybridization.

Slot-blot hybridization conditions

Specificity of the probes was tested by slot-blot hybridization on nitrocellulose membranes. We used the same conditions for FISH that were found to give specific hybridization in the slot-blot tests. Conditions for specific hybridization for *in situ*

Figure 4. Slot-blot hybridization of the DIG-labeled probes T2 and T4 to DNA on nitrocellulose membranes. The top row on each membrane represents ITS DNA amplified

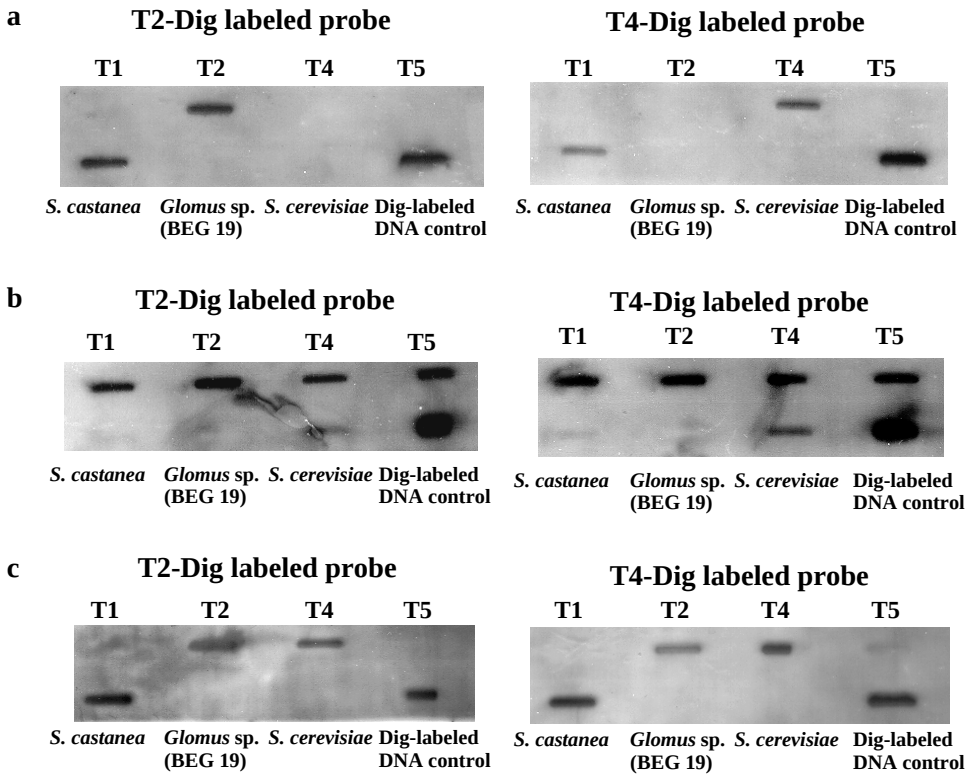

Figure 4. Slot-blot hybridization of the DIG-labeled probes T2 and T4 to DNA on nitrocellulose membranes. The top row on each membrane represents ITS DNA amplified from inserts of the clones T1/3, T2, T4 and T5. The bottom row represents genomic DNA of *S. castanea*, *Glomus* sp. (BEG19), *S. cerevisiae* and the DIG-labeled control supplied in the kit. (a) Results of specific hybridization with the conditions stated above and subsequently used for FISH. (b) Unspecific hybridization using the following post hybridization conditions; incubation in 50% formamide in 2 x SSC for 5 min at 37°C, 1 rinse in 2 x SSC for 5 min at 37°C, 1 rinse in 2 x SSC for 5 min at room temperature. (c) Unspecific hybridization using the following post hybridization conditions; incubation in 50% formamide in 2 x SSC for 5 min at 37°C, 1 rinse in 2 x SSC for 5 min at 37°C, 1 rinse in 2 x SSC for 5 min at room temperature, 2 rinses in 2 x SSC with 0.1% SDS for 5 min at room temperature and 2 rinses in 2 x SSC with 0.1% SDS for 15 min at 61°C.

were previously determined by slot-blot hybridization using the DIG-labeled probes only. DNA amplified from clones containing the insert T2 or T4, or genomic DNA of *S. castanea* (BEG 1), *Glomus* sp. (BEG 19) and *S. cerevisiae* were denatured (1 h in 0.4 M NaOH, 0.8 M NaCl, at room temperature) and were deposited on membranes using a hybridot apparatus (Gibco BRL). Purified DNA of two other ITS clones (T1/3 and T5) were also deposited on the membranes. These two sequences represent ITS sequences of ascomycete fungi that are known

DNA from herring sperm, 0.1% SDS, 5 ng/µl DIG-labeled probe T2 or T4). Hybridization was carried out at 37°C, overnight. The post-hybridization washes were made twice with 50% formamide in double-strength SSC (42°C, 10 min), then twice with double-strength SSC, (37°C, 5 min) and then rinsed again twice with half-strength SSC with 0.1 % SDS (60°C, 15 min). Hybridization signals were revealed using a DIG nucleic acid detection kit (Roche). The probes T2 and T4 hybridized to genomic DNA from *S. castanea* and not to control genomic DNA of another mycorrhizal fungus *Glomus* sp. (BEG 19) or to that of *Saccharomyces cerevisiae*. The probes hybridized specifically to the corresponding T2 and T4 DNA sequences that were amplified from cloned DNA of *S. castanea* containing the inserts T2 and T4 (Figure 2.4a). Two other post-hybridization treatments were previously assessed but did not give specific hybridization (Figure 2.4b-c).

Slide preparation for FISH experiments

Five spores or single spores were crushed on Frost Plus slides (Polylabo) and then dried overnight. Slides were incubated with 100 µl of DNase free RNase (100 µg ml^{-1}) in double-strength SSC (1 h, 37°C). Slides were rinsed in double-strength SSC (5 min, room temperature) and then incubated in a proteinase solution (1 µg/ml proteinase K, 20 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$, at 37°C for 10 min). Slides were rinsed in a 20 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$, 50 mM $MgCl_2$ at room temperature for 5 min, then dehydrated in ethanol and dried.

Signal detection and confocal microscopy

For signal detection in single-target FISH, slides were incubated with 20 µg ml^{-1} anti-DIG-fluorescein conjugate antibody (Roche), in PBS, 0.5% BSA pH 7.4 (37°C, 1 h). A final wash was made with PBS containing 0.1% Tween-20. Nuclei were counterstained with 1 µg ml^{-1} propidium iodide (molecular probes) and mounted in FluoroGuard Antifade Reagent (Biorad). In double-target FISH experiments, signals were detected with a mixture of 20 µg ml^{-1} anti-DIG-fluorescein conjugate antibody (Roche) and 20 µg ml^{-1} Streptavidin Texas-Red conjugate. The slides were counterstained with TOTO-3 (Molecular Probes).

Slides were examined using a confocal scanning microscope, equipped with Ar/Kr lasers (Leica TCS SP, Heidelberg, Germany). Sequential excitation was made at 494 nm, 536nm, 596 nm and 642 nm for fluorescein, propidium iodide, Texas-Red and TOTO-3, respectively. For each image, 16 to 32 optical sections with a single excitation were performed and stored as scanned files. The images were combined and processed using Imaris and Selima-processing software (Bitplane AG, Technopark Zürich, Switzerland). The number of nuclei showing the presence of clear hybridization signals in the different treatments was counted without the counter knowing the identity of the treatment.

In situ control for false hybridization signals

A control was also performed without using hybridization probes. In this case, no hybridization signals were observed on nuclei, showing that the antibodies were not hybridizing directly to molecules on the slide preparations and thus giving rise to false hybridization signals.

In situ control that results of double-target FISH were not biased by label type

In double-target FISH, the two probes were labeled differently, with biotin and digoxigenin (DIG). In order to control for the possibility that biotin and DIG-labeled probes exhibit differential hybridization efficiency, double-target FISH was carried out reciprocally with T2-biotin with T4-DIG and T2-DIG with T4-biotin labeling (Table 2.1). Both experiments gave similar results. T-tests indicated that there were no significant differences in the percentage of nuclei hybridizing to either T2 or T4 as a result of different labeling, indicating that the results of double-target FISH were not biased by the choice of label. Although slightly more nuclei hybridized with T2 in the double-target FISH experiments than in single-target FISH experiments, T-tests revealed no significant differences. There were also no significant differences in the numbers of nuclei hybridizing with T4 in the single and double-target FISH experiments.

In situ controls for specificity

FISH was performed simultaneously on nuclei of *G. geosporum* (BEG18) and *Glomus* sp. (BEG 19) as an extra control for specificity. The FISH using different

AMF were performed on separate slides but at the same time and in the same trays with the same solutions as the FISH experiments with *S. castanea*. No hybridization signals were seen on nuclei of the other two AMF with the probes T2 & T4, indicating that in the FISH conditions the probes are not unspecifically hybridizing to other ITS sequences or to non-target DNA such as centromeres or heterochromatic knobs. A positive control was made for the FISH performed with other AMF, where all fungi were placed together on single slides. A universal 18S probe of approximately 550bp in length was used for hybridization to confirm that sites of the other AMF nulcei were accessible to the probes. The universal 18S probe was amplified from genomic DNA of *S. castanea* using the universal primers NS1 and NS2 (White *et al.*, 1990). The universal 18S probe was used because it is more conserved among different AMF species than ITS regions and, therefore, a universal probe would be expected to hybridize to nuclei of all AMF species unspecifically. The universal 18S probe labeled approximately 98% of nulcei of all three AMF species.

In situ controls to test for efficient probe penetration

A universal ITS2 probe of approx. 270 bp was also raised by amplifying genomic DNA of *S. castanea* with the same primers that were used to amplify the probes T2 and T4. This universal probe represents amplifiable variants of the ITS2 sequence in *S. castanea*. This probe was used to ensure that ITS2 sites in FISH experiments were accessible to the probes and that probe penetration was successful in the same conditions.

Between 39 and 45% of nuclei were unlabelled in double-target FISH experiments with the probes T2 and T4, indicating that these nuclei probably contain other divergent rDNA sequences to which T2 and T4 probes do not hybridize. This interpretation is possible since we have characterized 7 more variant ITS2 sequences from cloned DNA from a genomic library of *S.castanea*, all of which phylogenetically fit to the glomales. The accession numbers for these sequences are AJ313169-AJ313175. This interpretation also is supported by our control hybridization with the universal ITS2 probe that contains a mixture of ITS variants that were amplified from *S. castanea*. Almost all nuclei (92.8%, S.E. $\pm$ 1.8) were labeled with this probe, showing that the lack of labeling with T2 and T4 on some nuclei is not due to inaccessibility of ITS sites or poor probe penetration.

In situ controls for hybridization with potentially contaminating ascomycete fungi

We have isolated an ascomycete fungus that grows inside some *S. castanea* spores which contains an ITS sequence, known as T1/T3[15]. We have made a study on the size of *S. castanea* nuclei and those of the ascomycete that lives inside this fungus. A document showing these results can be obtained from the authors on request. The nuclei that hybridize with probes T2 or T4 and with the universal ITS2 probe range in size between 3 and 5 µm in diameter. Nuclei of the ascomycete that contains the T1/3 sequence have a size of between 1-1.5µm. In our experiments we have only counted nuclei in the size range 3-5µm and no nuclei in the size range 1-1.5 µm have been observed on our slides. We do not find this result surprising since our electron microscope studies of the ascomycete fungus show that the fungus exists in the form of hyphae inside *S.castanea* spores. We doubt that these small hyphae are crushed when the AMF spores are crushed in preparation for *in situ* and therefore the nuclei of this fungus will not become bound to the surface of the slide. Subsequent washing to remove debris should, therefore, remove these hyphae containing ascomycete nuclei. As a control to test that nuclei that were not labeled with T2 or T4 were not of ascomycete origin, we performed *in situ* with a T1/T3 specific probe. This probe was shown to be specific in a slot-blot control with the same conditions (additional slot-blot figures can be supplied on request). This ascomycete probe never hybridized to nuclei of this size in our FISH experiments.

Additional comments on testing for detection of recombination in artificial populations of multi-genomic AMF

Because our empirical data show that nuclear genotypes exist in different frequencies inside AMF spores, we also altered the frequency of genetically different nuclei in each spore during the construction of the datasets. We defined sensitivity of the technique as being the proportion of nuclei possessing a given allele that is required to give a positive amplification using a given fingerprinting method. Each of the datasets except, with 0% or 100% sensitivities, revealed polymorphisms among spores.

Additional information on incompatibility analysis

Character incompatibility analysis can consider the evolutionary relationships among a number of variant sequences of the same gene that usually represent different taxa or individuals in a population. Here we consider the variant sequences from one AMF isolate as a population without knowing from which nuclei they originate. For the specific case of AMF this technique is particularly useful since population genetics techniques based on frequency and linkage of DNA markers would have to be applied to individual nuclei within a spore. This presents technical difficulties when performing PCR based methods on such small amounts of DNA since a negative result such as the failure to amplify an allele at a given locus does not necessarily mean that it is not present but may be an artifact caused by the sensitivity of the technique (see Fig. 2.2b).

In each case, the AMF had been isolated by taking single AMF spores so that all progeny had arisen from the same mother spore,meaning that the progeny would be expected to be clonal. Datasets representing sequence variation in ITS or 28S gene for each species were edited to remove all bases that showed no variation among the sequences. Calculations of the number of incompatibility counts were performed on the datasets using the program DNALQP from the package PICA 95 (available at http://www.bio.bris.ac.uk/research/markwilk/software.htm) for each species separately. Insertions and deletions were marked in the dataset as a fifth character; the other 4 characters being the nucleotides A, C, G and T. After removal of all insertions and deletions from the datasets, the incompatibility counts were repeated, considering only sequence variation that occurred by substitution. The observed and expected number of incompatibilities that were caused by each variable character in the sequences for each AMF species were calculated and Le Quesne probabilities (Mes, 1998) for each position were calculated after performing 1000 random permutations. The null hypothesis was that variation at a site was the result of recombination and this was rejected at $P \leq 0.05$. The contribution of each sequence variant to total matrix incompatibility counts was calculated for each dataset using the program JCPTP (PICA 95). The sequences that contributed the highest to the total matrix incompatibility were sequentially removed from the dataset and the total matrix incompatibility was recalculated after the removal of each sequence.

Additional information on the gene encoding for a binding protein (*BiP*) and analysis of sequence variation

Because rDNA genes are multicopy genes, investigations into whether genetic differences occur among nuclei using these gene sequences have to rely on techniques such as FISH, where large differences in the sequences are required over a long stretch of the DNA. For many gene sequences these differences will not be great enough to apply such techniques. Therefore, we took an additional approach to investigate genetic variation within AMF individuals by investigating sequence variation in another region of DNA that is single copy in other eukaryotes. This is also novel because until now all studies of sequence variation in AMF have looked exclusively at rDNA sequences.

Sequences of the BiP gene were obtained by PCR amplification from genomic DNA of *Glomus intraradices*. Genomic DNA was extracted from ground spores and mycelium of *G. intraradices* grown in sterile root cultures for 12 weeks using the DNeasy Plant Mini Kit (Qiagen). The primer pair BiP2.for (5-AAGACAAGCCACAAAAGATGCTGG-3) and BiP2.rev (5-AGTAGGGATTACAGTGTTACGAGG-3) was used for amplification. This primer pair amplifies an 805 bp region of the gene. Primers were designed according to a fragment of this gene found in a partial genomic library of *G. intraradices*. The PCR conditions were as follows: 40ng of genomic DNA were used in 20µl of PCR reaction mixture. The reaction mixture contained a final concentration of 1x PCR buffer without $MgCl_2$ (Invitrogen Life technologies), 250µM of each nucleotide, 2.5mM of $MgCl_2$, 1.25µM of each primer and 0.1U of taq polymerase (Invitrogen Life Technologies). PCR cycling conditions were 94°C for 3 min followed by 30 cycles of 94°C for 45s, 57°C for 45s and 72°C for 90s and a final extension step of 10 min at 72°C. Fragments were cloned and sequenced as described for the ITS sequences.

Clones that differed in sequence were found by screening with SSCP. 300ng of plasmid from positive clones were double digested using 10U of *Dra*1 and 10U of *Eco*R1 in 1x incubation buffer for restriction enzymes A (Roche) in a volume of 10µl and incubated at 37°C for 2h, followed by heat inactivation of the enzymes at 70°C for 10 min. Samples were mixed and then denatured at 95°C for 3 min. Submerged Gelelectrophoresis was carried out at 9°C and 6V/cm for 15hours on precast GMA™ Gels (Elchrom Scientific). Gels were stained with SYBR Gold for

40 min in the dark and rinsed for 20 min. Differences in the banding patterns indicated different clones. These clones were chosen for sequencing and analysis.

Fifteen different sequences were found (accession numbers AJ319763 - AJ319777). Every sequence was carefully edited. The incompatibility analysis was performed on a 680 bp region of the gene (shown in Figure 2.5), which was shown to be variable. 59 sites in this region were variable. Incompatibility analysis as described for rDNA sequences in the methods. Calculation of the Le Quesne probability (Mes, 1998) showed that for all 59 variable characters the incompatibility count differed significantly from that which would be expected if it had arisen from recombination. The sequences also differed considerably at the amino acid sequence level (variation shown in Figure 2.6). The number of substitutions that were synonymous (not altering the amino acid sequence) and non-synonymous (altering the amino acid sequence) were calculated. Comparisons of sequence differences among the sequences at the nucleotide and amino acid level were made by comparing all the sequences to one that was chosen at random (AJ319763).

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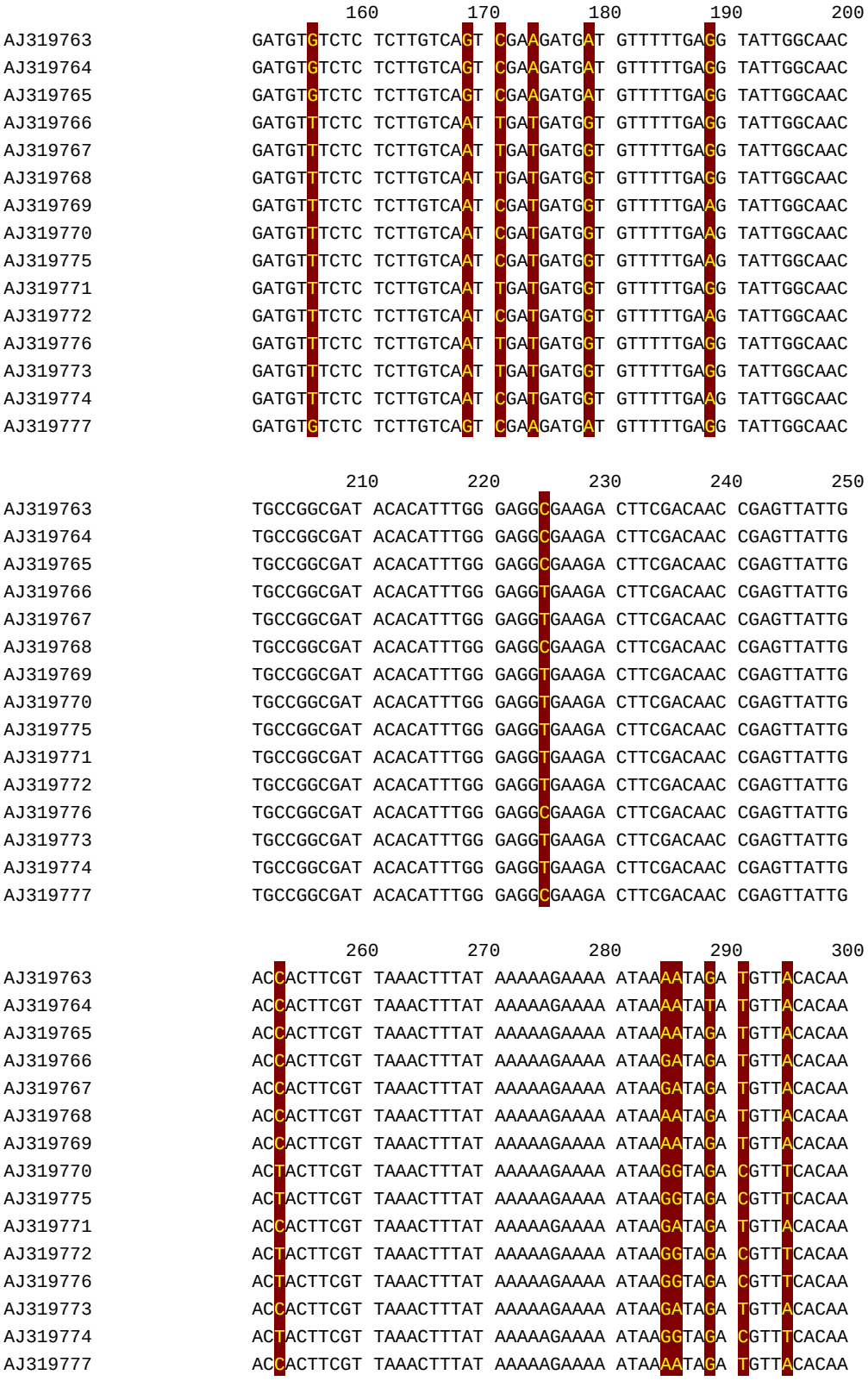
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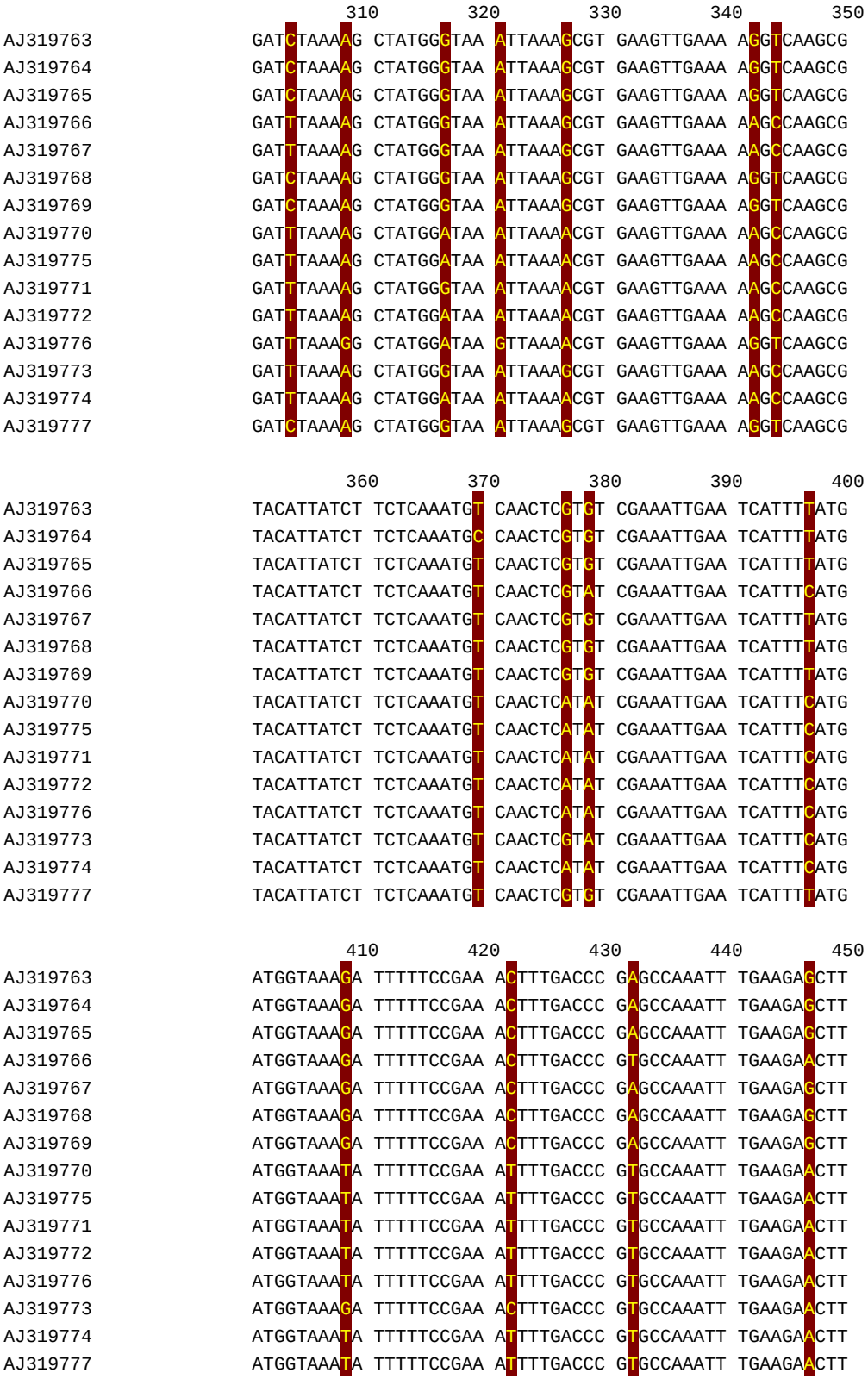
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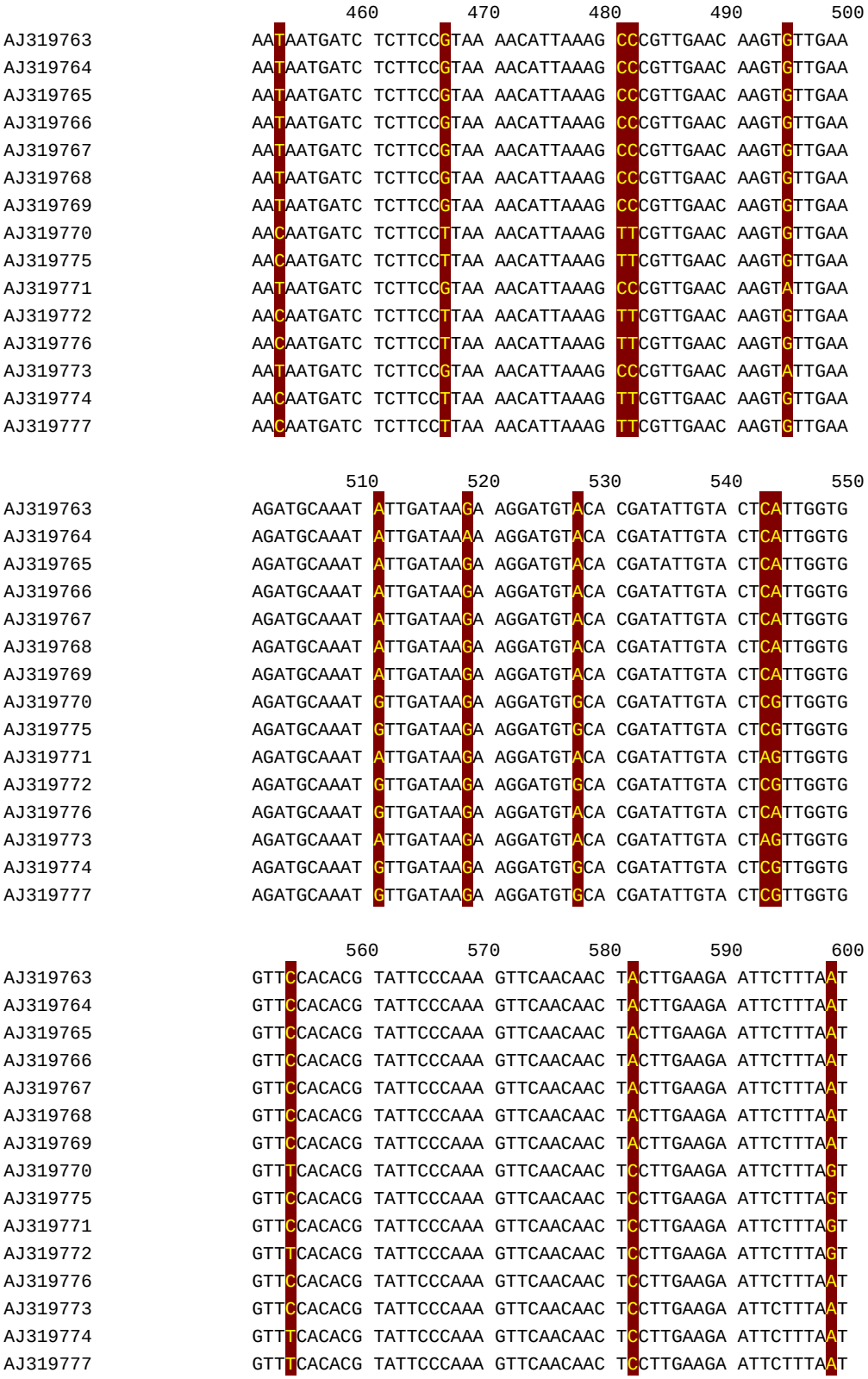
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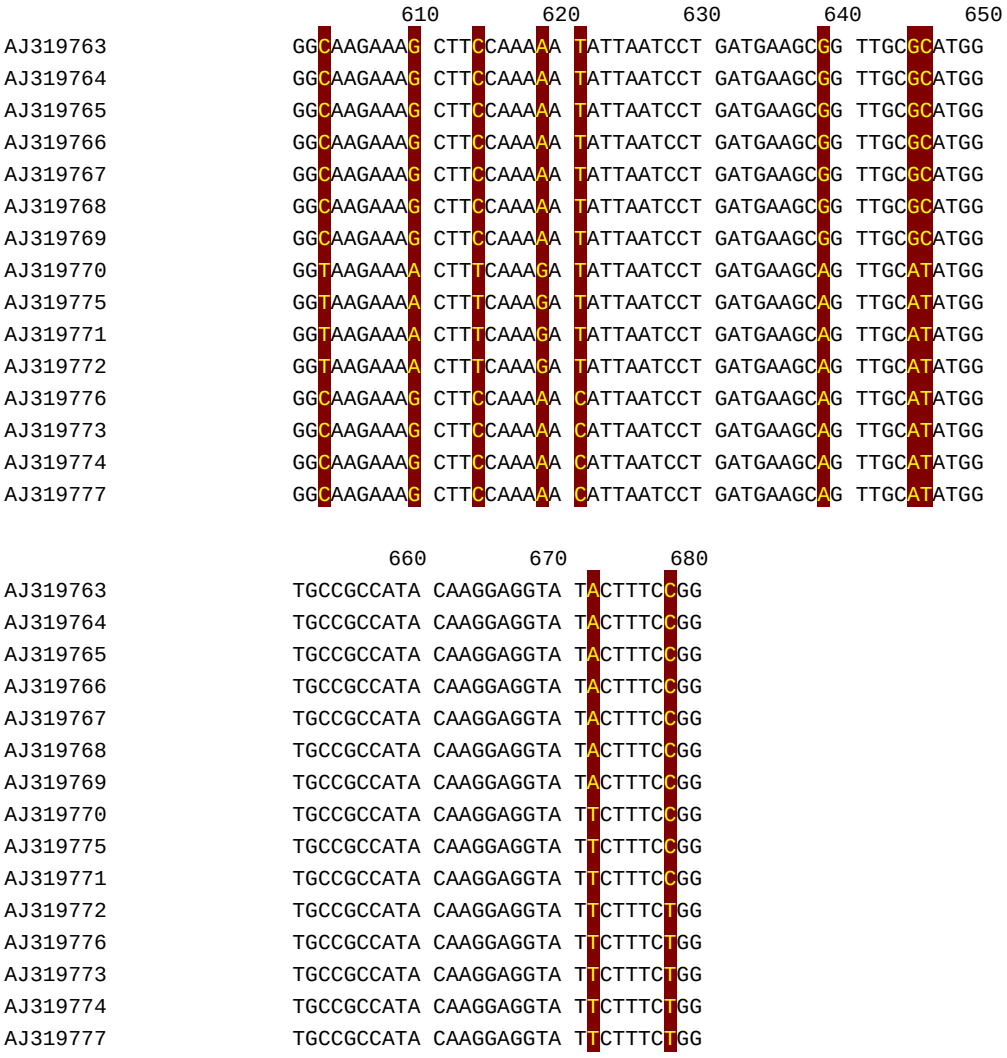
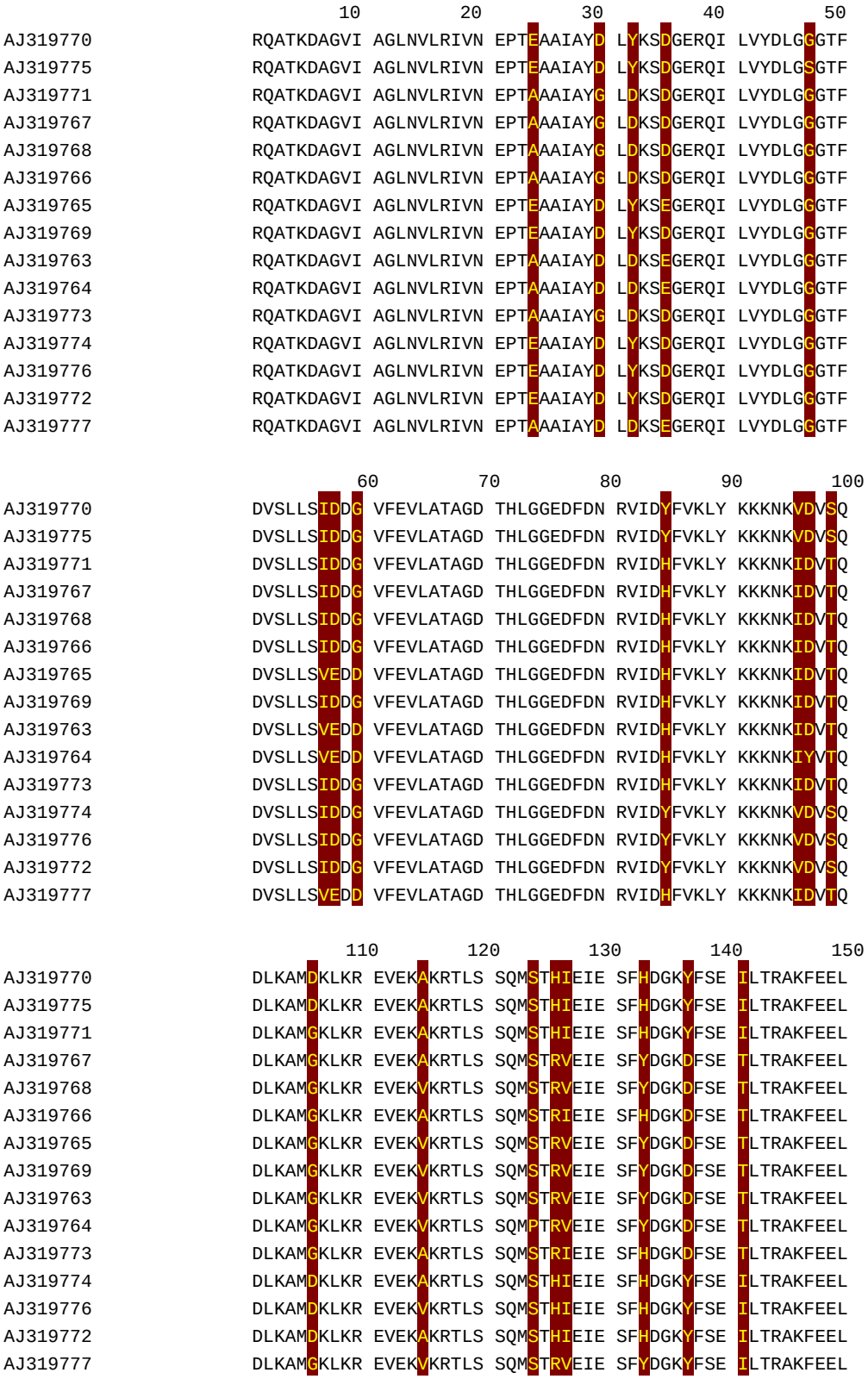


Figure 2.5: Alignment of 15 variant sequences of a 680 bp region of the BiP gene from *G. intraradices*. Each sequence is named with its respective accession number. 59 sites are marked in red and this indicates the sites that are variable and were used for the incompatibility analysis.


10 20 30 40 50
AJ319770 RQATKDAGVI AGLNVLRIVN EPTEAAIAYD LYKSDGERQI LVYDLGGGTF
AJ319775 RQATKDAGVI AGLNVLRIVN EPTEAAIAYD LYKSDGERQI LVYDLGSGTF
AJ319771 RQATKDAGVI AGLNVLRIVN EPTAAAIAYG LDKSDGERQI LVYDLGGGTF
AJ319767 RQATKDAGVI AGLNVLRIVN EPTAAAIAYG LDKSDGERQI LVYDLGGGTF
AJ319768 RQATKDAGVI AGLNVLRIVN EPTAAAIAYG LDKSDGERQI LVYDLGGGTF
AJ319766 RQATKDAGVI AGLNVLRIVN EPTAAAIAYG LDKSDGERQI LVYDLGGGTF
AJ319765 RQATKDAGVI AGLNVLRIVN EPTEAAIAYD LYKSEGERQI LVYDLGGGTF
AJ319769 RQATKDAGVI AGLNVLRIVN EPTEAAIAYD LYKSDGERQI LVYDLGGGTF
AJ319763 RQATKDAGVI AGLNVLRIVN EPTAAAIAYD LDKSEGERQI LVYDLGGGTF
AJ319764 RQATKDAGVI AGLNVLRIVN EPTAAAIAYD LDKSEGERQI LVYDLGGGTF
AJ319773 RQATKDAGVI AGLNVLRIVN EPTAAAIAYG LDKSDGERQI LVYDLGGGTF
AJ319774 RQATKDAGVI AGLNVLRIVN EPTEAAIAYD LYKSDGERQI LVYDLGGGTF
AJ319776 RQATKDAGVI AGLNVLRIVN EPTEAAIAYD LYKSDGERQI LVYDLGGGTF
AJ319772 RQATKDAGVI AGLNVLRIVN EPTEAAIAYD LYKSDGERQI LVYDLGGGTF
AJ319777 RQATKDAGVI AGLNVLRIVN EPTAAAIAYD LDKSEGERQI LVYDLGGGTF
60 70 80 90 100
AJ319770 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDYFVKLY KKKNKVDVSQ
AJ319775 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDYFVKLY KKKNKVDVSQ
AJ319771 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDHFVKLY KKKNKIDVTQ
AJ319767 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDHFVKLY KKKNKIDVTQ
AJ319768 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDHFVKLY KKKNKIDVTQ
AJ319766 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDHFVKLY KKKNKIDVTQ
AJ319765 DVSLLSVEDD VFEVLATAGD THLGGEDFDN RVIDHFVKLY KKKNKIDVTQ
AJ319769 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDHFVKLY KKKNKIDVTQ
AJ319763 DVSLLSVEDD VFEVLATAGD THLGGEDFDN RVIDHFVKLY KKKNKIDVTQ
AJ319764 DVSLLSVEDD VFEVLATAGD THLGGEDFDN RVIDHFVKLY KKKNKIYVTQ
AJ319773 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDHFVKLY KKKNKIDVTQ
AJ319774 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDYFVKLY KKKNKVDVSQ
AJ319776 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDYFVKLY KKKNKVDVSQ
AJ319772 DVSLLSIDDG VFEVLATAGD THLGGEDFDN RVIDYFVKLY KKKNKVDVSQ
AJ319777 DVSLLSVEDD VFEVLATAGD THLGGEDFDN RVIDHFVKLY KKKNKIDVTQ
110 120 130 140 150
AJ319770 DLKAMDKLKR EVEKAKRTLS SQMSTHIEIE SFHDGKYFSE ILTRAKFEEL
AJ319775 DLKAMDKLKR EVEKAKRTLS SQMSTHIEIE SFHDGKYFSE ILTRAKFEEL
AJ319771 DLKAMGKLKR EVEKAKRTLS SQMSTHIEIE SFHDGKYFSE ILTRAKFEEL
AJ319767 DLKAMGKLKR EVEKAKRTLS SQMSTRVEIE SFYDGKDFSE TLTRAKFEEL
AJ319768 DLKAMGKLKR EVEKVKRTLS SQMSTRVEIE SFYDGKDFSE TLTRAKFEEL
AJ319766 DLKAMGKLKR EVEKAKRTLS SQMSTRIEIE SFHDGKDFSE TLTRAKFEEL
AJ319765 DLKAMGKLKR EVEKVKRTLS SQMSTRVEIE SFYDGKDFSE TLTRAKFEEL
AJ319769 DLKAMGKLKR EVEKVKRTLS SQMSTRVEIE SFYDGKDFSE TLTRAKFEEL
AJ319763 DLKAMGKLKR EVEKVKRTLS SQMSTRVEIE SFYDGKDFSE TLTRAKFEEL
AJ319764 DLKAMGKLKR EVEKVKRTLS SQMPTRVEIE SFYDGKDFSE TLTRAKFEEL
AJ319773 DLKAMGKLKR EVEKAKRTLS SQMSTRIEIE SFHDGKDFSE TLTRAKFEEL
AJ319774 DLKAMDKLKR EVEKAKRTLS SQMSTHIEIE SFHDGKYFSE ILTRAKFEEL
AJ319776 DLKAMDKLKR EVEKVKRTLS SQMSTHIEIE SFHDGKYFSE ILTRAKFEEL
AJ319772 DLKAMDKLKR EVEKAKRTLS SQMSTHIEIE SFHDGKYFSE ILTRAKFEEL
AJ319777 DLKAMGKLKR EVEKVKRTLS SQMSTRVEIE SFYDGKYFSE ILTRAKFEEL

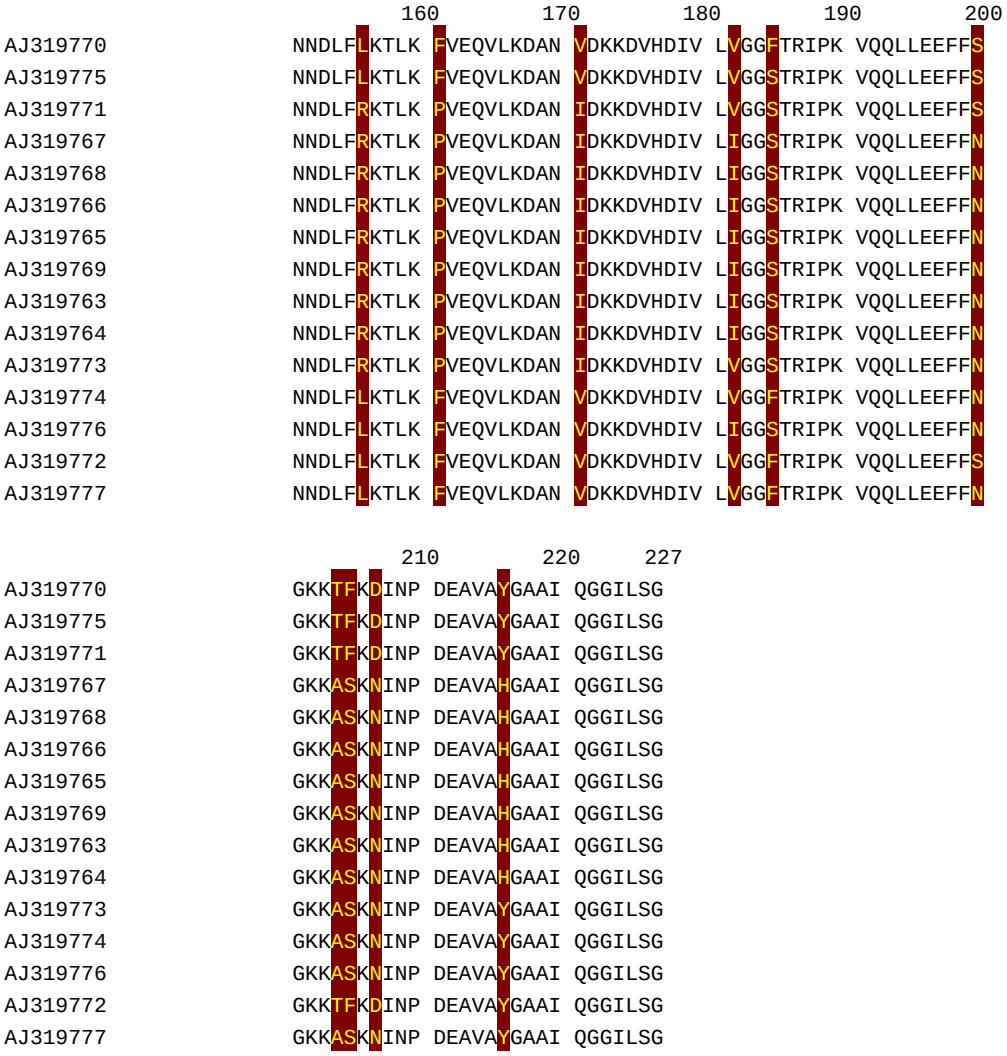


Figure 2.6. Variation in the amino acid sequences among 15 variants of the *BiP* gene from *G. intraradices*. The 30 variable sites are shown in red.

Table 2.3: Results of two analyses of substitution rates of 15 variable *BiP* gene sequences of *Glomus intraradices.*

	class 2		class 1		class 3	
	NG	mNG	NG	mNG	NG	mNG
S	183.167	227.048	183.722	226.028	181.250	225.875
N	603.833	561.952	603.278	559.972	607.750	563.125
s	5.900	5.900	9.267	9.267	3.500	3.500
n	5.429	5.429	13.067	13.067	6.667	6.670
ds	0.032	0.026	0.051	0.041	0.019	0.015
dn	0.009	0.010	0.022	0.023	0.011	0.012
ω	0.280	0.385	0.431	0.561	0.579	0.800

The analysis was performed on a total of 15 *BiP* gene variants. Variants were classified into 3 main classes (class 1-3). Two different models were used (Nei Gojobori and modified Nei Gojobori) for calculation. The total number of synonymous and non-synonymous positions per sequence are represented by S and N, respectively. The number of observed synonymous and non-synonymous substitutions are denoted s and n, while the proportion of observed substitutions compared to the total number are denoted ds and dn. The ratio of non-synonymous to synonymous substitutions is denoted ω.

Chapter 3

High genetic variability and low local diversity in an arbuscular mycorrhizal fungal population

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The study described in this chapter has been conducted in collaboration with Alexander M. Koch, Pierre Fontanillas, Luca Fumagalli, Jérôme Goudet and Ian R. Sanders. The manuscript is in preparation for submission to *Proceedings of the National Academy of Sciences.*

Abstract

Arbuscular mycorrhizal fungi (AMF) are ecologically important root symbionts of most terrestrial plants. Ecological studies of AMF have concentrated on differences between species; largely assuming little variability within AMF species. Even though AMF are clonal they have evolved to contain a surprisingly high within species genetic variability and genetically different nuclei can exist within individual spores. This could potentially lead to within-population genetic variation, causing differences in physiology and symbiotic function in AMF populations; a consequence that has been largely neglected. We found highly significant genetic and phenotypic variation among isolates of a population of *G. intraradices* but relatively low total observed genetic diversity. Because we maintained the isolated population in a constant environment, phenotypic variation can be considered as variation in quantitative genetic traits. In view of the large genetic differences among isolates by randomly sampling 2 individual spores, less than 50% of the total observed population genetic diversity is represented. Adding an isolate from a distant population did not increase total observed genetic diversity. Genetic variation exceeded variation in quantitative genetic traits indicating that selection acted on the population to retain similar traits. This might be because of the multigenomic nature of AMF, where considerable genetic redundancy could buffer effects of changes in the genetic content on phenotypic traits. These results have direct implications for ecological research, for studying AMF genes, for improving commercial AMF inoculum and understanding evolutionary mechanisms in multigenomic organisms.

Introduction

A Arbuscular mycorrhizal fungi (AMF) are widely distributed and extremely successful symbionts that colonise the roots of up to 80% of terrestrial plant species (Smith and Read, 1997). They are known to improve plant growth by increasing phosphate uptake (Harrison, 1997) and species diversity of AMF has been shown to increase plant species diversity and productivity (van der Heijden *et al*., 1998). Several studies describing the differential effects of AMF species, that were performed in an ecological context where all fungi originated from one plant community, have effectively compared a single spore of each AMF species because each isolate had been propagated clonally from one spore (van der Heijden

et al., 1998; Streitwolf *et al*., 2001; van der Heijden *et al*., 1998). Therefore, there was no replication of the species. So far, there have been no studies combining molecular genetic variation along with variation in quantitative genetic traits in an AMF field population. This is surprising given that a remarkably high amount of genetic variability exists in an AMF species and even within individual spores (Sanders *et al*., 1995; Schussler *et al*., 2001; Rodriguez *et al*., 2001; Lanfranco *et al*., 1999; Sanders *et al*., 1996). AMF are coenocytic, with many nuclei co-existing in a common cytoplasm and one species of AMF has recently been shown to be multigenomic; harbouring genetically different nuclei (Kuhn *et al*., 2001).

It has already been shown that single spore isolates of an AMF species of different geographical origin differentially affect plant growth and physiology (Stahl and Smith., 1984; Stahl and Christensen, 1991; Stahl *et al*., 1990). However, in two of those studies, the isolates were not kept for several generations under identical conditions, and therefore, observed differences could also be due to environmental differences. Furthermore, only 1 isolate was taken from each population so that within population variation was unknown. However, potential for considerable population variation exists. One AMF can form a hyphal network that connects the roots of many plants belowground. Considering the nature and the amount of genetic variation present in AMF (Sanders, 2002), there are several different possibilities regarding genetic and phenotypic variation in an AMF population. One possibility is that the fungi are clonal and that all parts of the hyphal network and all spores receive the same complement of genetically different nuclei, leading to little or no variation in the population. However, studies on variation in spore shape and colour indicate that heritable variation exists within AMF populations (Bever and Morton, 1999; Bentivenga *et al*., 1997). Even though AMF grow clonally, by either random or non-random processes, the nuclei could become unevenly distributed in the hyphal network or during spore formation. This would create both genetically and possibly phenotypic heterogeneity. It could also cause a spatial structure among different parts of the population. This has previously been described as a particular type of drift (Sanders, 2002). The third possibility, and not necessarily exclusive of the second, is that selection acts locally on nuclear genotypes due to environmental heterogeneity, thereby, also creating genetic variation (Sanders, 2002).

There have been several attempts using molecular techniques to demonstrate qualitative genetic differences between isolates within a population by picking out different sequences of rDNA from spores (Sanders *et al*., 1995; Pringle *et al*.,

2000). However, this approach is flawed because of high within-spore variation in rDNA sequences. Given that up to 23 different sequences of rDNA have been found in single spore isolates of *Glomus coronatum* (Clapp *et al.*, 2001), randomly picking out different rDNA sequences from a number of AMF spores is highly probable even if they all contain the same complement of sequence variants. Furthermore, no studies have successfully quantified genetic differences among AMF isolates using a large number of neutral markers although this is essential for knowing how much isolates actually differ from each other genetically.

The reason why the within population variation in AMF has not already been investigated in more detail is probably due to the time consuming methodology required to obtain a set of AMF individuals in the laboratory that are representative of an AMF population (Sanders, 2002). This requires the successful production of AMF spores from soil samples using trap cultures, setting up single spore cultures and the transfer of newly produced spores into sterile laboratory cultures, with subsequent propagation in laboratory conditions (Figure 3.1). Because of environmental heterogeneity in the field, each isolate has to be maintained for several generations in identical conditions to remove maternal effects. This is important for quantitative genetic studies because otherwise variation in traits may be due to environment rather than genotype. With subsequent replication of isolates, the preparation of material for an experiment would take approximately 3 years (Figure 3.1).

Whether or not genetic and phenotypic variation exists in AMF populations and how much is a fundamental question that directly contributes to many areas of mycorrhizal research. Variation among individuals of AMF could lead to strong differences in symbiotic efficiency with plants. Furthermore, knowledge of the extent of genetic variation in a population can help to predict how frequently individuals at one site could contain a variant of a given gene that is known to be polymorphic. Finally, knowledge about how many AMF should be sampled from a population to account for the majority of the genetic variation will clearly be of use for developing genetically diverse AMF inoculum.

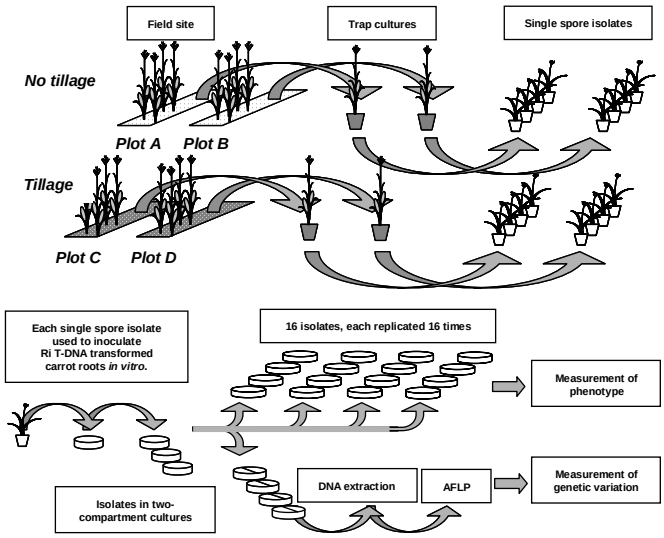

Figure 3. 1: Procedure to obtain laboratory cultures of isolates representing an AMF population and the subsequent design of an experiment to measure phenotypic variation and genetic variation of the isolates. Soil samples were taken from the field site and used to inoculate plants in pots in the greenhouse. Single spores from trap cultures that were identified as *G. intraradices* were used to inoculate individual plants (single spore isolates). Spores were then newly isolated from single spore isolates and used to inoculate sterile Ri-T DNA transformed carrot roots. The isolates were transferred 2 more times onto fresh medium in identical laboratory conditions. Sixteen replicate plates were inoculated with each culture. Another 16 plates of each isolate were inoculated on two-compartment plates. The phenotype was measured over a 15 week period to obtain an estimation of variation in quantitative genetic traits. The two-compartment plates were taken for harvest of fungal material with 2 independent extractions of DNA. AFLP fingerprint analysis of genomic DNA allowed measurement of genetic variation.

Our study aims to quantify and compare both neutral genetic variation and variation in quantitative genetic traits of an AMF population. We tested the null hypothesis that there are no phenotypic or genotypic differences among individuals in a field population of the AMF *Glomus intraradices*. An isolate is referred to here as a clonal culture of an AMF that was started from one spore (single spore culture) and, therefore, all its progeny is considered to represent its original genetic composition. Using a hierarchical design, we measured and analyzed the phenotypes of 16 individuals. Due to the lack of neutral genetic markers at either the spore or the nuclear level, AFLP fingerprinting was chosen for genetic characterisation. AFLP of DNA from single spores can suffer from artefacts due to low amounts of DNA and these artefacts cannot be quantified due to the lack of replication (Kuhn *et al*., 2001; Rosendahl and Taylor, 1997). Our study allowed the clonal growth of large amounts of hyphae and spores from each isolate, yielding

large amounts of high quality DNA from each individual that negates these problems (St-Arnaud *et al*., 1996). This allowed tests of AFLP reliability with the possibility of replication in order to quantify the potential AFLP artefacts. Our study of an AMF population is the first report to combine both quantitative genetic variation and quantitative molecular variation of a multigenomic organism.

Materials and Methods

Study site

A long-term experiment to study the impact of tillage on soil structure, soil organisms and crop development was started in 1987 (Anken *et al*., 1997). The field site of a size of 90m x 110m was located at Hausweid, Tänikon, Switzerland and comprised a nested design of two replicate plots of each of two tillage treatments (plots A and B for no-tillage; plots C and D for tillage) for this experiment within a larger design that comprised more plots and other soil management treatments (for spatial arrangement of plots see Figure 3.6 and Table 3.6 in supporting information). Each plot was 6m x 19m.

Isolation of a *G. intraradices* population

In 1999, thirty individual soil cores were randomly taken from the 4 plots. These were pooled and mixed by plot. Trap cultures were established on host plants of soybean (*Glycine max* L.), sunflower (*Helianthus annuus* L.) and leek (*Allium porrum* L.) (Jansa *et al*., 2002). After a growth period of five months in the greenhouse, fresh healthy spores of *G. intraradices* were sieved and single spores were used to start new cultures by placing them individually onto germinating roots of sterile *Plantago lanceolata* seedlings. These single spore isolates were then grown for a second period of three months in identical environmental conditions (Jansa *et al*., 2002) (Figure 3.1). One isolate was chosen from each plot (isolates A_4, B_3, C_2 and D_3) for sequencing analysis of the internal transcribed spacer region (ITS). A comparison with the sequences in the public database confirmed their belonging to the species *G. intraradices* (Accession numbers: AJ557006, AJ557009, AJ557007, AJ557008, respectively).

Cultivation of *G. intraradices* on Ri T-DNA transformed carrot roots

After cultivation on *P. lanceolata*, spores showing a creamy to white colour, indicating a healthy state, were collected and surface sterilized by shaking 4h with a mix of antibiotics (500mg l^{-1} Streptomycin, 500mg l^{-1} Rifampicin, 500mg l^{-1} Tetracyclin, 500mg l^{-1} Penicillin G, 500mg l^{-1} Neomycin and 200mg l^{-1} Polymyxin-B sulfate with a few drops of Tween 20). Spores were rinsed with sterile water and put onto a plate containing M medium with a piece of Ri T-DNA transformed carrots root that originated from one clone (Becard and Fortin, 1988). Cultures were incubated at 25°C. Each isolate was transferred for the first time after 15 weeks of growth by taking pieces of media (approximately $4cm^2$), containing roots and fungus, which were then placed onto a new plate containing M medium. In order to maintain isolates in identical conditions this was subsequently performed two more times with the same medium each time after 15 weeks of growth. Four isolates were taken from each of the different plots (A-D), resulting in a total of 16 single spore isolates of *G. intraradices* that were used in this study.

Experimental design

The aim of the experiment was to measure among isolate, among plot and between treatment (tillage and no tillage) genetic variation and phenotypic variation. Due to the cultivation under identical environmental conditions for a long time, differences in the observed phenotype are assumed to have a genetic basis and are, therefore, quantitative genetic traits. The isolates were cultured by the transfer of material from each of 4 replicate plates of each of the 16 isolates. Material from each replicate plate was then transferred onto 4 new plates. Therefore, there were 16 plates per isolate, resulting in a total of 256 plates (Figure 3.1). Contaminated plates or those with no root growth were excluded from analysis and this reduced the total number of plates to 229.

For measurement of genetic variation, 16 two-compartment cultures were established simultaneously for each of the 16 isolates and with the identical starting material as the cultures used for measurements of quantitative genetic traits (Figure 3.1). In addition, a *G. intraradices* isolate from Canada was used as an outgroup with a different geographic origin. A culture of this isolate (DAOM 181 602) was obtained from the group of Prof. G. Bécard (University Paul Sabatier, Toulouse, France) (St-Arnaud *et al*., 1996). Two-compartment plates allowed proliferation of

AMF hyphae and spores on one half of the plate (St-Arnaud *et al*., 1996). During the experiment, roots that directed their growth to the fungal side were cut to avoid contamination with plant material. After 15 weeks, the medium in the fungal compartment of all plates from each isolate was removed and pooled for extraction of hyphae and spores (Nagahashi *et al*., 1993). This compartment was then refilled with medium to allow the growth of more fungal material for a second extraction, after an additional growth period of 6 weeks (Douds, 2002).

Measurement of phenotypes

We decided to measure hyphal growth rate and spore production because they can be related to life history traits and tillage treatments (Pringle *et al*., 2002; Hart *et al*., 2001). Over a growth period of 15 weeks, the number of spores per cm^2 of medium and length of hyphae per cm^2 of medium were recorded every 3 weeks. The measurements made at several times allowed calculations of the maximum rate of spore production and the maximum rate of hyphal growth of each isolate. In addition, the ratio of spore number to hyphal length at the end of the experiment was also calculated. A description of the measurement procedure can be found in supporting information.

Measurement of genotypes

Freshly obtained hyphae and spores were immediately taken for extraction of DNA using the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. DNA extraction of 10 isolates gave sufficiently high yield for AFLP analysis with each of 10 different primer pairs. Further details of AFLP can be found in supporting information.

Analysis of phenotypic variation and population diversity

Variation in quantitative genetic traits among the 16 Swiss isolates was tested using the following growth variables: final hyphal length, final spore number, maximal rate of hyphal growth, maximum rate of spore production and the ratio of the final spore number to hyphal length.

A nested ANOVA (Analysis of Variance) was performed on these variables with the main factors treatment (2 levels, df 1), plot nested in treatment (4 levels, df 2), isolate nested in plot (16 levels, df 12) and plate nested in isolate (df 46). All variables were transformed before analysis to satisfy the assumptions of ANOVA (Sokal and Rohlf, 2000) (see Table 3.2 in supporting information).

Partitioning of variance of polygenic traits (Q_{st}) for all pairwise combinations of the four plots was calculated for all variables separately and as a combination of all five variables. For later comparison with genetic variation this was performed on the same 10 isolates as those that were used for the analysis of genetic variance. Isolates coming from the same plot were considered to be one sub-population. The following variance components were calculated for all pairs of plots: plot (V_p), isolate within plots (V_i) and the residual error (V_r). From these, the Q_{st} values were calculated according to the formula $Q_{st} = V_p/(V_p + V_i + 2V_r)$ modified from (Merila and Crnokrak, 2001) (see Table 3.3 in supporting information).

Variation in quantitative genetic traits was further analyzed to estimate the relationship between the number of isolates and the phenotypic diversity. Calculation of phenotypic diversity is described in detail in the supporting information. From the relationship between number of isolates and diversity we were able to estimate how many isolates comprised 90% of total observed phenotypic diversity.

Analysis of genetic variation

Ten isolates from the Swiss population were taken for analysis, using the binary data derived from AFLP with the 10 primer pairs. Analysis of molecular variance (AMOVA) was performed with the software ARLEQUIN (Excoffier *et al*., 1992) to investigate patterns of genetic variation and for estimation of variance components at three different hierarchical levels (isolate, plot and treatment). In a first AMOVA isolate was used as a factor with 10 levels, with 2 replicate DNA extractions of each isolate. Genetic variation among isolates (V_a) and between extractions of each isolate (V_b) was compared to investigate genetic differences among isolates in the AMF population. In a second AMOVA, the plot structure was used as a factor with 4 levels, where isolates originating from the same plot

were the replicates of the plots. The amount of genetic variation explained within (V_b) and among (V_a) the plots was used to examine whether a spatial genetic structure existed in the AMF population (for complete results of both AMOVAS see Table 4 in supporting information). The variance components for the plot structure (V_a,V_b) were used to calculate F_{st} values according to the formula $F_{st} = (V_a)/(V_a+V_b)$ (Merila and Crnokrak, 2001) (for F_{st} values see Table 3.5 in supporting information). Both AMOVAs were performed for each pair of primers separately and the means over all primer pairs were calculated in each analysis. Finally, isolates were grouped according to the treatment and taken as replicates in a third AMOVA with 2 levels to test whether there were genetic differences due to the tillage treatment.

Phylogenetic analysis was performed on the combined binary dataset of all 10 primer pairs to describe the relatedness among isolates, both with and without the isolate of Canadian origin. A maximum parsimony analysis was performed using PAUP 4.0 beta 10 with a heuristic search procedure using random stepwise addition and tree bisection-reconnection branch swapping options, repeated 10 times. The robustness of the branching pattern was evaluated with a bootstrap procedure (1000 replicates) (Swofford, 2002).

In order to know how much of the total population genetic variation is contained in a given number of isolates, a Monte Carlo simulation was performed allowing mean genetic diversity to be plotted against number of isolates. Details about the calculation of genetic diversity and the simulation can be found in supplementary information. The test was performed a second time including the isolate from Canada and with random sampling up to 11 isolates.

Relationship between phenotypic variation and genetic variation

The combined binary datasets of 10 primer pairs were used and genetic differentiation (F_{st}) between all pairs of plots was recalculated based on the plot structure of the second AMOVA. Plot F_{st} values were plotted against plot Q_{st} values for all 5 variables separately and for a Q_{st} where all 5 variables were combined. This pairwise comparison of the amount of phenotypic variation (Q_{st}) with the amount of genetic variation (F_{st}) allowed us to test the null hypothesis that

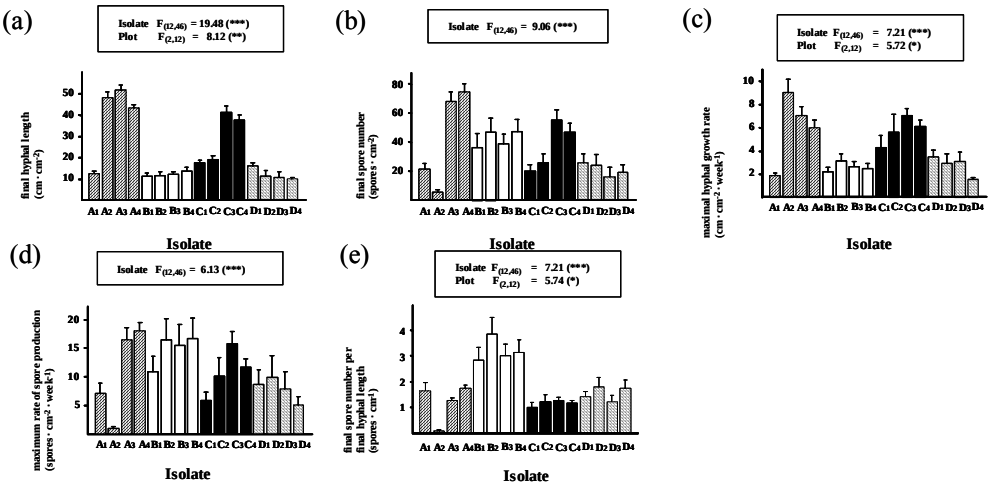

Figure 3.2: Mean final hyphal length (a), final spore number (b), maximal hyphal growth rate (c), maximum rate of spore production (d) and final spore number per hyphal length (e) of the 16 AMF isolates. Identical shading patterns indicate isolates originating from the same plot. Isolates A_1 – B_4 and C_1 – D_4 belong to no-tillage and tillage treatments, respectively. Only significant main effects from the ANOVA are shown in the graphs. Bars indicate standard error and significance levels are indicated with * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. Full ANOVA tables are given in supporting information (www.pnas.org).

a given trait evolved by genetic drift, in which case F_{st} will be equal to Q_{st} (McKay and Latta, 2002). Presence of selection on either the genetic or phenotypic level is expected to lead to deviation from this assumption.

Results

Analysis of variance

Isolates differed significantly in their final hyphal length and spore number (Figures 3.2a and b). The hyphal length ranged from 10 cm·cm^{-2} to 52 cm·cm^{-2} and spore number ranged from 5 spores·cm^{-2} to 75 spores·cm^{-2}. The isolates also differed strongly in their maximal hyphal growth rate, maximum rate of spore production and the ratio of spore number to hyphal length (Figures 3.2 c, d and e).

Table 3.1: Results of two analyses of molecular variance (AMOVA) showing the percentage of variation for (a) within isolate variation (between DNA extractions of the same isolate) and among isolate variation and (b) within plot variation and among plot variation. The analysis was performed on the binary dataset obtained from AFLP with 10 different primer pairs on DNA from 10 isolates of *G. intraradices.*

AFLP Primers		a) Isolates		b) Plots	
Eco	*Mse*	V_{among}	V_{within}	V_{among}	V_{within}
AGG	CA	93.1	6.9	64.5	35.5
AAG	CA	95.4	4.6	62.8	37.2
TC	CA	92.7	7.3	66.7	33.3
GA	CA	98.8	1.2	80.3	19.7
AG	CA	91.7	8.3	72.6	27.4
GT	CA	91.2	8.8	62.7	37.3
AGG	TT	94.1	5.9	70.8	29.2
AAG	TT	96.0	4.0	68.0	32.0
GT	TT	97.1	2.9	68.9	31.1
AA	CTG	92.1	7.9	65.4	34.6
mean		94.2	5.8	68.3	31.7

Final hyphal length, maximal growth rate of hyphae and the ratio of spore number to hyphal length also differed significantly among plots. There was no significant plot effect on final spore number and maximal rate of spore production. The tillage treatment had no significant effect on any of the 5 variables.

Genetic differences among isolates were large and this was true for all primer pairs (Table 3.1). A mean of 94.2% of the total population variation was explained by differences between the isolates. Only a small amount of the total variation (mean 5.8%) was due to differences among the replicate DNA extractions (Table 3.1). AMOVA with plot as factor showed that 68.3% of the total variation was explained by differences among the 4 plots, while 31.7% was explained by differences within the plots (Table 3.1). Only 1.3% of the genetic variation was explained between isolates of different treatments (data not shown).

Phylogenetic analysis

The maximum parsimony analysis of the 10 isolates used in the AFLP analysis revealed the presence of 3 monophyletic groups that were well supported with

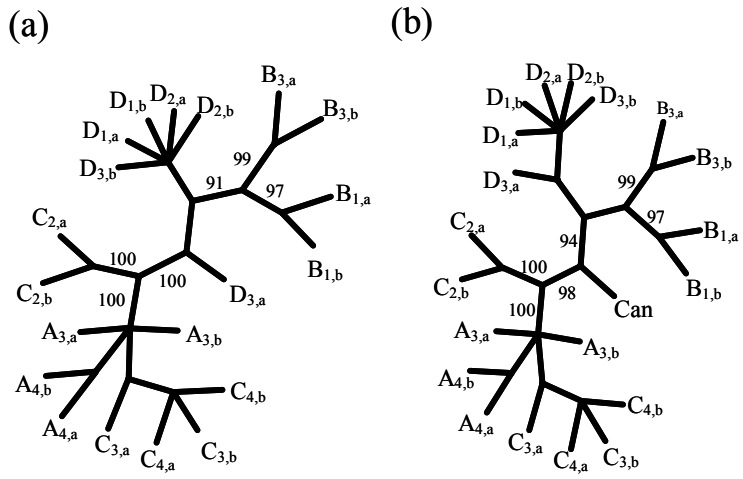

Figure 3.3: Phylogenetic analyses based on binary data generated using AFLP on 10 isolates of an AMF population from Switzerland. An unrooted consensus tree was obtained through a heuristic search procedure using stepwise addition and tree bisection-reconnection branch swapping options (with 10 additions). Support values are indicated at branches when found in at least 90% of the 1000 bootstrap trees. (a) The analysis was performed with AMF isolates from the Swiss population only. The Swiss isolate codes follow that described in the methods. The 2 independent DNA extractions are denoted with the lower case letters a and b after the isolate letter and number code. (b) The analysis was performed on 10 isolates of the Swiss population and an isolate of Canadian origin. The Canadian isolate is designated Can.

bootstrap values (100%, 100% and 100%) (Figure 3.3a). The analysis was repeated with the Canadian isolate and this did not greatly change the bootstrap values (100%, 100% and 98%) or greatly change the position of the other isolates in the tree (Figure 3.3b). One group was represented by isolate C_2. The second and the third group included isolates B_1, B_3, D_1, D_2, D_3 $^{+}$/- Can and A_3, A_4, C_3, C_4, respectively (Figure 3.3). The presence of 3 phylogenetic groups indicated that there is clearly a genetic structure within the population. Comparison of the group structure of the isolates with phenotypic traits of the isolates shows that isolates from the third group form a distinct class that also exhibited the highest values of final hyphal length and spore number.

Diversity analysis

The Monte Carlo simulations showed that genetic diversity increased with the number of randomly chosen isolates and the asymptotic curve indicated a limited

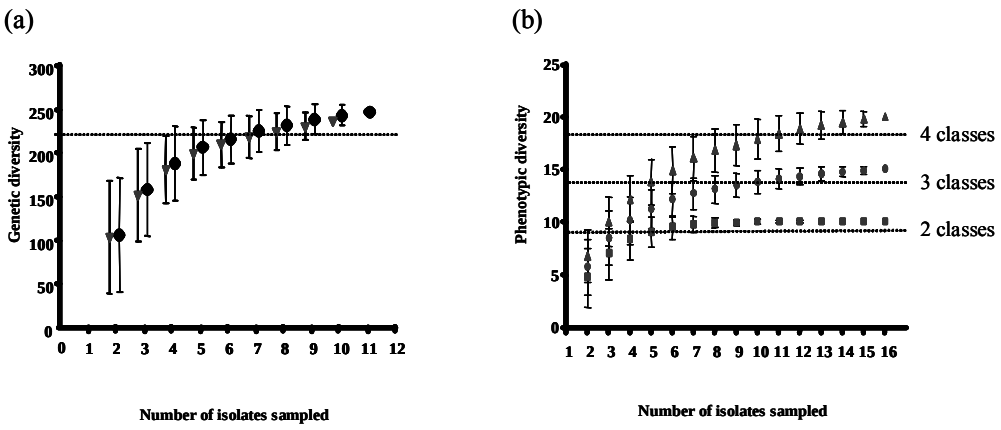

Figure 3.4: (a) Relationship between the amount of genetic diversity and the number of isolates sampled from an AMF population. The estimation of genetic diversity was performed including (♦) and excluding (▼) an isolate from Canada. Data points indicate the mean number of polymorphic bands for a given number of isolates. Error bars represent ±1 standard deviation. The dotted line represents the threshold for 90% of the observed genetic diversity. (b) Relationship between the amount of phenotypic diversity and the number of isolates sampled from an AMF population. The procedure for estimation of phenotypic diversity was performed on the basis of different numbers [2 (■), 3 (●) and 4 (▲)] of equally long intervals (classes) that were introduced to describe the variation in a trait. For each of the three different classes the threshold for 90% of the observed diversity is indicated with a dotted line.

amount of diversity in the population (Figure 3.4a). Two isolates, chosen at random, accounted for less than 50% of the total observed genetic variation. After selecting 7 or more isolates at random the curve levels off and accounted on average for more than 90% of the total observed variation. Thus, adding more isolates after this point did not greatly increase total observed variation. A subsequent reanalysis including the isolate from Canada in the dataset did not change the shape of the curve. Any two isolates, chosen at random, accounted for less than 50% of the total observed phenotypic diversity and this was true for all models of phenotypic diversity. Using the most conservative assumptions of the model (2 classes) the asymptotic curve started levelling off after selecting 6 or more isolates at random and accounted on average for more than 90 % of the observed total phenotypic diversity (Figure 3.4b). Again, adding more isolates did not greatly increase total observed variation. Increasing the number of classes in the model (3 or 4) shifted the point where the curve started levelling off to 10 or more isolates.

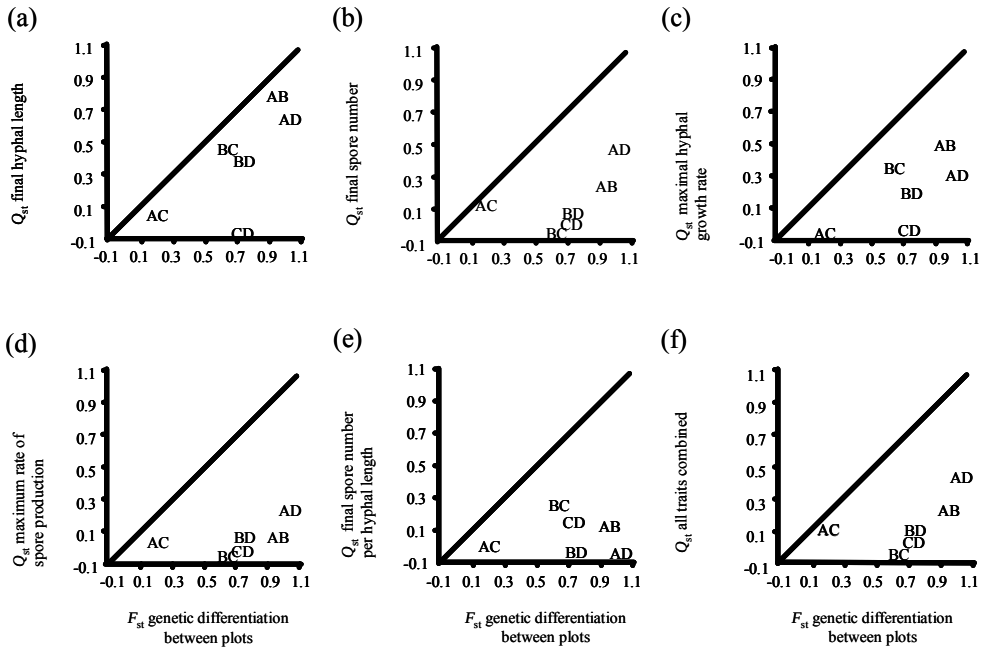

Figure 3.5: Relationship between phenotypic differentiation (Q_{st}) and genetic differentiation (F_{st}) for all 6 possible combinations of pairs of plots. Q_{st} values are shown for the phenotypic traits (a) final hyphal length, (b) final spore number, (c) maximal hyphal growth rate, (d) maximum rate of spore production, (e) final spore number per hyphal length and (f) a combination of all five variables. Data points are denoted with 2 letters describing which plots have been compared. All variance components of phenotypic traits used in the calculations are shown in supporting information.

Comparison of phenotypic differentiation (Q_{st}) to genetic differentiation (F_{st})

Phenotypic differentiation (Q_{st}) of each pair of plots was compared to the corresponding genetic differentiation (F_{st}) giving 6 data points representing each possible combination. Genetic differentiation was generally bigger than phenotypic differentiation for all 5 variables (Figures 3.5a – e). This was also true for the relationship between the combined values of phenotypic differentiation and genetic differentiation (Figure 3.5f).

Discussion

Differences between AMF individuals

In this study, we have shown for the first time that large genetic differences exist between individuals in an AMF population in an area of 90m x 110m. Therefore, we reject the null hypothesis that there are no phenotypic or genetic differences among individuals in an AMF population. Five-fold differences in hyphal length between isolates, as observed among isolates in this population, have previously only been described between AMF species and have been shown to explain differences in plant phosphate uptake (Jakobsen *et al*., 1992). Thus, the variation observed in phenotypes of this AMF population has, indeed, the potential to alter plant nutrition and growth, indicating that variation in AMF populations may be ecologically important. The level of variation is surprising given that AMF have only evolved about 150 morphologically distinct taxa in 400 Million years of terrestrial evolution (Remy *et al*., 1994; Redecker *et al*., 2000).

The variation that we show could either be created by drift, as described by (Sanders, 2002), or by selection due to different environments in the field or by a combination of both. From our comparison of genetic and phenotypic differentiation, it is likely that the variation is not the result of drift alone. However, using this test it is not possible to quantify the strength of selection and how much it contributes (Merila and Crnokrak, 2001). In our study, individuals from different plots showed higher genetic differentiation than differences in phenotypic traits. Similar comparisons for other organisms showed that phenotypic differentiation typically exceeded that of neutral genetic markers indicating divergent selection (Merila and Crnokrak, 2001; McKay and Latta, 2002). Our data are in contrast to these studies and, therefore, suggest that there could be selection on similar quantitative genetic traits in the presence of great genetic diversity. Since the AMF population originates from a highly managed agricultural system, perhaps certain management practices that are common to all plots of the field have caused this selection. A possible explanation is that functional redundancy in gene sequences among multiple genomes could lead to a similar phenotype. For example, minor changes in a population of alleles such as replacement of rare alleles or slight changes in allele frequencies might not affect the resulting phenotype if it represents an average of all alleles. It could, however, lead to considerable changes in the genetic content. The observed variation in quantitative genetic traits is sufficiently large to detect selection but our results show no

indication that one practice, that of tillage, had any significant effect. However, it is possible that other agricultural management practices or environmental differences could act selectively.

Population structure

The data indicate there could be a spatial genetic structure within the population. This means that AMF from different parts of a 90m x 110m field are likely to be genetically different and that genetic differences are not randomly distributed. Due to small sample size per plot caution needs to be taken with the interpretation of this result. A genetically structured AMF population is also supported by the phylogenetic analysis that revealed 3 major groups within the population. Evidently, a link between the pattern of variation in quantitative genetic traits and the phlylogenetic structure in the population could exist as one monophyletic group was found to comprise the isolates that exhibited the highest hyphal growth. Indeed, a Mantel test comparing genetic relatedness between pairs of isolates (the number of shared polymorphic bands) with differences in mean hyphal density (after 15 weeks of growth) showed a highly significant correlation ($R^2 = 0.6812$; $p = 0.0003$). Differentiation among isolates of one population might, therefore, not only occur on a genetic but also on a functional level and this has not previously been considered.

Anastomosis, the fusion of hyphae, has previously been shown in AMF (Giovannetti *et al*., 2001), allowing possible exchange of nuclei within the hyphal network. This should reduce the effects of drift and selection by allowing re-mixing of nuclear genotypes after their distribution has been rendered heterogeneous. However, to date experimental studies have only revealed anastomoses between hyphae of spores coming from the same isolate (Giovannetti *et al*., 2001; Giovannetti *et al*., 1999) and not between isolates of different geographical origin (Giovannetti *et al*., 2003). At the scale of our study, the spatial genetic structure of the population shows that exchange of nuclei could not have been frequent enough to cause genetic homogeneity in the population.

Limited diversity in an AMF population

Our analyses of genetic and phenotypic diversity show that six to seven isolates are sufficient to cover the great majority of the total observed population diversity. Both diversity analyses and the phylogenetic analysis suggest that only a limited

number of very different main types exist and, thus, that total genetic diversity might be relatively low. However, by choosing only two isolates at random we would be sampling less than 50% of the diversity in the population. Despite the large genetic differences among isolates found within 1 field the Canadian isolate was not genetically distant and phylogenetically it fitted into one of the main branches of the population. This suggests either potential gene flow between the Canadian isolate and some of the Swiss population or that the total diversity comprised within a small scale may already account for most diversity on a much larger scale. This, however, is in contrast to the findings that geographically distant isolates do not anastomose (Giovannetti *et al*., 2003).

These results have strong implications for commercial inoculum development and for ecological studies of AMF that rely on the use of molecular methods. The functional and genetic diversity of a commercial inoculum could be altered according to the number of single spore isolates used to initiate an inoculum. Using several isolates would increase the likelihood of genetic exchange with a local AMF population. Similarly, molecular studies aimed at characterizing AMF communities in field-sampled roots should consider the high within population genetic variation, because primers developed from single spore isolates may not necessarily allow detection of all individuals of the same AMF species in the field. Additionally, given the large genetically based phenotypic differences that we have shown in an AMF population from a small field, studies concentrating on rDNA sequence diversity among AMF species is unlikely to be targeted at the ecologically relevant level.

We conclude that ecological and molecular studies of AMF should take this surprisingly high variation into account. Because of the potential that this genetic diversity is translated into functional differences, within-population variation should be considered when designing ecological and molecular experiments. Furthermore, because of sequence variation in AMF, including regions of functional genes (Kuhn *et al*., 2001), genetic differences in a population might lead to segregation of alleles of genes among individual spores which could lead to complete presence and absence of certain alleles depending on the isolate studied. Molecular biologists studying functionally relevant genes and their molecular regulation in a multigenomic organism should consider this potential segregation of alleles and possibly ascertain their findings through analysis of several isolates, because otherwise important information could be missed.

Acknowledgements

This work was supported by grants 3100-050481.97 and 631-058108.99 from the Swiss National Science Foundation (SNSF). I.R.S. was in receipt of a professorial fellowship from the SNSF, which is gratefully acknowledged. Gerrit Kuhn was supported by a grant from the Roche Research Foundation. We thank Jan Jansa and Hannes Gamper for isolating *G. intraradices* from the field site and help with the transfer of these onto Ri T-DNA transformed carrot roots. We thank Nicolas Corradi for sequencing the ITS regions of 4 *G. intraradices* isolates and 3 anonymous reviewers for their comments that greatly helped to improve the manuscript.

Supplementary information

Procedure for the measurement of phenotypes

Hyphal length cm^{-2} medium was measured by counting the number of hyphal intersects on an L-shaped line at 4 randomly chosen positions on the plate. The mean number of intersects was then multiplied by the conversion factor 1.057 to give values of hyphal length in $cm.cm^{-2}$. Spore density was measured by counting the number of spores in 4 randomly chosen squares. The 4 measurements per plate were pooled to give 1 value per plate. Measurement of these variables at regular intervals also allowed the calculation of hyphal growth rates and rates of spore production.

Additional details about AFLP

DNA extraction was performed on the 16 isolates and the Canada isolate. Two independent extractions were made for each of the 16 Swiss isolates and 0.25µg of DNA was used for AFLP (AFLP Kit for Microorganisms, Invitrogen). Isolates were not analyzed if there was less than 0.25µg of genomic DNA per extraction available. The Canada isolate (Can) and ten of the 16 isolates were retained for AFLP analysis. The 10 Swiss isolates (A_3, A_4, B_1, B_3, C_2, C_3, C_4, D_1, D_2, D_3) were distributed over the 4 plots, where 2 isolates originated from each of plots A and B and 3 isolates originated from each of plots C and D. Ten different radioactively (P^{33}) labeled primer pairs (*Eco*R1 + AGG, AAG, TC, GA, AG, GT with *Mse*1 + CA; *Eco*R1 + GT, AGG, AAG with *Mse*1 + TT and *Eco*R1 + AA with *Mse*1 + CTG) were used for AFLP. Amplified DNA fragments were separated on 6% denaturing acryl amide gels for 3,5h at 70W, dried for 2h at 70°C and exposed on a film. The polymorphic fragments were scored in two ways. First, all fragments that were clearly polymorphic were scored. Second, three fragments were scored that were the most obviously polymorphic (as judged by fragment intensity) among all fragments in each of 4 equally sized partitions of the gel.

Calculation of the relationship between number of isolates and phenotypic diversity

Phenotypic diversity was calculated with the following procedure. The means of all 5 phenotypic traits of each isolate were used as the raw data in this analysis. For each variable, the range of the 16 means was divided several times into equally long intervals, ranging from 2 up to 14 classes. We defined phenotypic diversity for each trait as the total number of classes in which at least one of the 16 means lay. When all means were in the same class the diversity was considered to be zero. The total diversity was then calculated by adding up the phenotypic diversity of each of the five traits for each number of classes. For each number of classes the following sampling procedure was performed. X isolates (X = 2, 3, …, 16) were randomly sampled 1000 times and the average total diversity was recorded for each X (in the case of X = 16 this was by definition the maximum diversity).

Calculation of the relationship between number of isolates and genetic diversity

The total number of observed polymorphic bands in the dataset was considered as the total population diversity. The number of polymorphic fragments was counted after randomly sampling 2 isolates. This was repeated successively with random sampling without replacement from 3 to 10 isolates. The mean genetic diversity and the standard deviation were obtained by performing a bootstrap over 1000 repetitions of the procedure. Values of mean diversity obtained were plotted against the number of randomly sampled isolates.

Table 3.2: Results of nested ANOVA for five quantitative traits

a) final hyphal density

level	df	*SS*	*F*	Prob>F
treatment	1	0.660	0.090	0.792
plot	2	14.635	8.118	0.006
isolate	12	10.816	19.481	<< 0.001
plate	46	2.128	1.183	0.221
error	167	6.529		

b) final spore density

level	df	*SS*	*F*	Prob>F
treatment	1	65.521	1.797	0.312
plot	2	72.913	0.774	0.483
isolate	12	565.518	9.063	<< 0.001
plate	46	239.185	1.219	0.185
error	167	712.490		

c) maximal hyphal growth

level	df	*SS*	*F*	Prob>F
treatment	1	0.006	0.005	0.951
plot	2	2.723	5.718	0.018
isolate	12	2.858	7.207	<< 0.001
plate	46	1.820	1.100	0.326
error	167	5.017		

d) maximum spore production

level	df	*SS*	*F*	Prob>F
treatment	1	3.515	1.012	0.420
plot	2	6.944	1.056	0.377
isolate	12	39.443	6.129	<< 0.001
plate	46	24.670	1.316	0.108
error	167	68.045		

e) ratio of final spore density per hyphal density

level	df	*SS*	*F*	Prob>F
treatment	1	1.013	0.332	0.623
plot	2	6.099	5.741	0.019
isolate	12	6.373	7.213	<< 0.001
plate	46	3.387	1.236	0.169
error	167	9.949		

Table 3.2: Results of nested ANOVA on 5 dependent variables of fungal growth (a-e). The F-values were calculated by using MS of the nested level as denominator, and the MS of the residual error term was only used as denominator for calculating the F-valuesfor the plate effect. The five variables were transformed using different root transformations: $^{2}\sqrt{}$(final hyphal density), $^{2}\sqrt{}$(final spore density), $^{4}\sqrt{}$(maximum hyphal growth), $^{3}\sqrt{}$(maximum spore production), $^{3}\sqrt{}$(ratio of final spore density per hyphal density).

Table 3.3: Phenotypic variance components for all pairs of plots for 5 variables of AMF growth and a combination of the variables

a) Final hyphal density

plots		V_p	V_i	V_r	Q_{st}
A	B	0.3051	-0.0029	0.0209	0.8868
A	C	0.0161	0.0391	0.0281	0.1444
A	D	0.3025	-0.0127	0.0664	0.7160
B	C	0.1196	0.0379	0.0303	0.5480
B	D	-0.0057	0.0095	0.0541	-0.0505
C	D	0.1199	0.0330	0.0597	0.4404

b) Final spore density

plots		V_p	V_i	V_r	Q_{st}
A	B	3.5482	-0.1757	4.3200	0.2954
A	C	1.7683	1.1394	3.3132	0.1855
A	D	8.4803	-0.3700	4.0341	0.5242
B	C	-0.3847	0.9403	4.8935	-0.0372
B	D	0.9930	-0.3474	5.7543	0.0817
C	D	1.4669	0.9154	4.4964	0.1290

c) Maximal hyphal growth rate

plots		V_p	V_i	V_r	Q_{st}
A	B	0.0518	-0.0010	0.0213	0.5544
A	C	-0.0013	0.0026	0.0245	-0.0252
A	D	0.0353	-0.0031	0.0339	0.3530
B	C	0.0402	0.0025	0.0291	0.3982
B	D	0.0008	-0.0005	0.0373	0.0100
C	D	0.0257	0.0008	0.0382	0.2498

d) Maximum rate of spore production

plots		V_p	V_i	V_r	Q_{st}
A	B	0.0810	0.0004	0.3446	0.1051
A	C	0.0479	0.0508	0.2579	0.0779
A	D	0.3120	-0.0337	0.4105	0.2838
B	C	-0.0293	0.0447	0.4364	-0.0330
B	D	0.0582	-0.0291	0.6065	0.0469
C	D	0.0578	0.0264	0.4757	0.0558

e) Final spore density per final hyphal density

plots		V_p	V_i	V_r	Q_{st}
A	B	0.0251	-0.0027	0.0607	0.1748
A	C	0.0035	0.0013	0.0272	0.0587
A	D	-0.0006	0.0012	0.0390	-0.0080
B	C	0.0526	-0.0030	0.0606	0.3079
B	D	0.0373	-0.0039	0.0775	0.1979
C	D	0.0006	-0.0014	0.0449	0.0068

f) Q_{ST} all traits combined

plots		ΣV_p	ΣV_i	ΣV_r	Q_{st}
A	B	4.0112	-0.1819	4.7675	0.3001
A	C	1.8345	1.2332	3.6509	0.1769
A	D	9.1295	-0.4183	4.5839	0.5106
B	C	-0.2016	1.0224	5.4499	-0.0172
B	D	1.0836	-0.3714	6.5297	0.0787
C	D	1.6709	0.9742	5.1149	0.1298

Table 3.3: Variance components (V) obtained from a nested ANOVA with the two levels for plot ($_p$) and isolates ($_i$) nested within plot for all pairs of plots. V_r indicates the variance components of the residual error. These values were used to calculate Q_{st} values for all five traits separately (a – e) and combined (f).

Table 3.4a: Variance components from an AMOVA for all pairs of isolates

Source of variation	d.f.	Sum of squares	Variance components		Percentage of variation
Among populations	9	941.5	50.70556	V_a	94.06
Within populations	10	32	3.2	V_b	5.94
Total	19	973.5	53.90556		

Table 3.4b: Variance components from an AMOVA for all pairs of plots

Source of variation	d.f.	Sum of squares	Variance components		Percentage of variation
Among populations	3	667.5	41.22466	V_a	68.31
Within populations	16	306	19.125	V_b	31.69
Total	19	973.5	60.34966		

Variance components (V_a and V_b) obtained from an AMOVA with isolate as a factor with 10 levels (4a) and with plot as a factor with 4 levels (4b). These values were calculated on the basis of the combined binary datasets obtained with 10 different AFLP primer pairs.

Table 3.5: Population pairwise F_{st} between each pair of plots

plots		Population pairwise F_{st}
A	B	0.87529
A	C	0.11952
A	D	0.94934
B	C	0.56392
B	D	0.65576
C	D	0.66707

Percentage of genetic variation between pairs of plots obtained from an AMOVA with 2 levels (between plot populations and within plot populations). These values were calculated on the basis of the combined datasets obtained with 10 different AFLP primer pairs.

Table 3.6: Geographic distance between each pair of plots

plots		geographic distance [m]
A	B	29.2
A	C	58.4
A	D	46
B	C	85.2
B	D	71.2
C	D	58.4

The geographic distances between plots were determined as the distances from the center of a plot to the center of another.

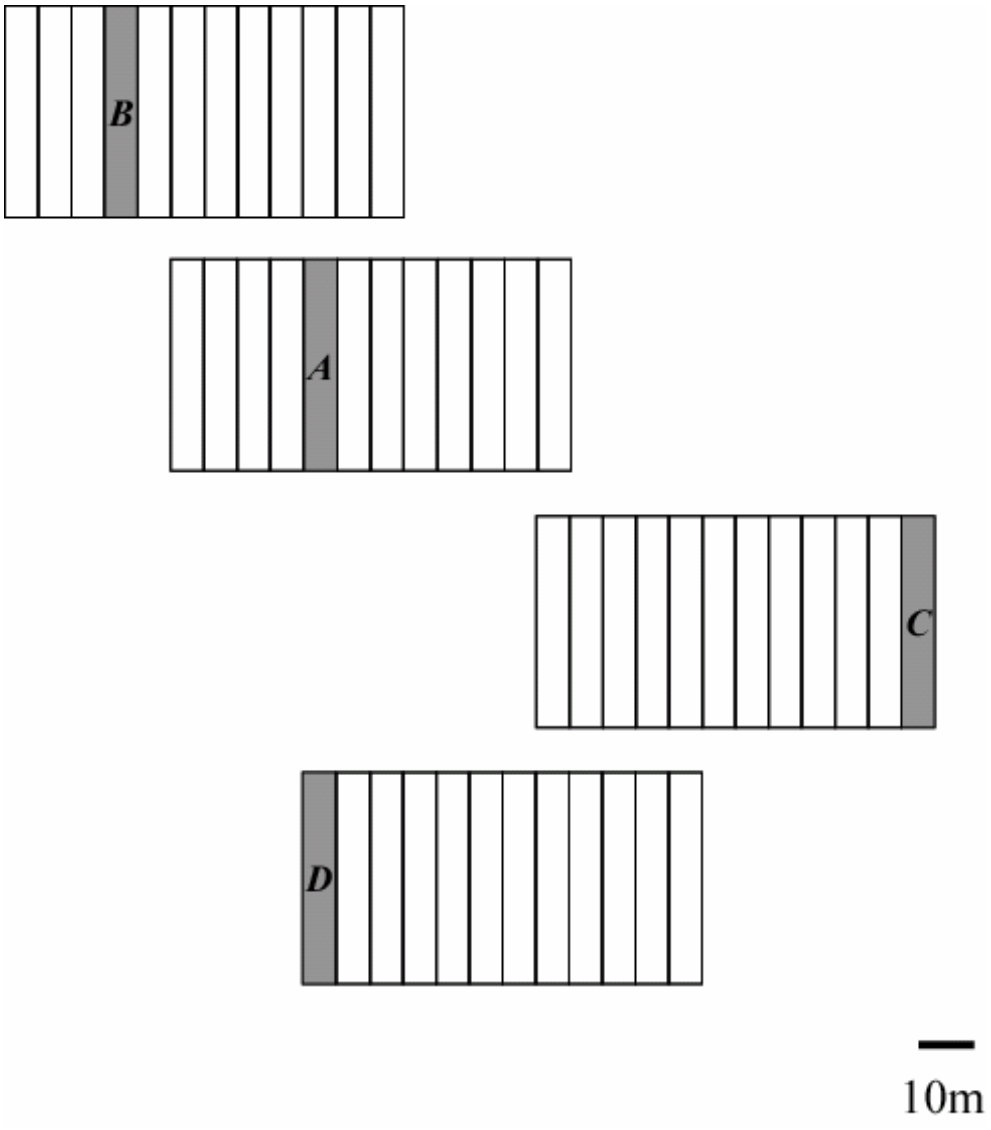

Figure 3.6. The spatial arrangement of the 4 plots (A, B, C, D) at the field site within a larger design that comprised additional plots that were not used in this study. The dimensions of each plot were 6m x 19m. A 4m distance separated the 4 main blocks. Each block contained 12 plots.

Chapter 4

Within individual genetic variation in *BiP* gene sequences of *Glomus intraradices* and its limits to diversity and functionality

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The study described in this chapter has been conducted in collaboration with Mohamed Hijri and Ian R. Sanders and is currently in preparation for submission.

Abstract

Arbuscular mycorrhizal fungi comprise an unusually high within individual genetic diversity. The reports of this diversity are almost exclusively restricted to rDNA. There is evidence that part of this diversity is shared between nuclei, because of a multigenomic structure and that this diversity might also exist among sequences for functional genes. Because rDNA is known to exist in multiple copies per genome it is difficult to identify sources of genetic variation and it is also difficult to assign a functional relevance of the observed variation. Consequently, we have analyzed genetic variation in DNA sequences of a binding protein (*BiP*). This gene is known to be single copy in other fungi and was thus deemed a useful tool to study potential segregation among nuclei and to study the potential effects of selection on the functional genetic information in the AMF genome.

We identified a highly variable region among *BiP* DNA sequences within a single spore isolate of *G. intraradices*. We then PCR amplified and cloned DNA fragments of this variable region. Sequencing of 166 clones chosen at random resulted in identification of 31 variants within one AMF individual. More than half of as many as 55 substitutions were non-synonymous indicating that there was no apparent selection to retain only one functional sequence. Approximately one third of all variants were predicted to be non-functional because of early termination codons and these are considered as pseudogenes. Using the complete *BiP* DNA sequence as a probe in a single FISH experiment nuclei of one spore displayed an average of 1 to 2 hybridization signals.

AMF contain a high within individual genetic variation also in functional gene sequences. A considerable amount of the variation in *BiP* is clearly non-neutral indicating absence of purifying selection. We predict also the presence of a high number of *BiP* pseudogenes in AMF individuals. Comparing the small number of hybridization signals per nucleus with the number or potential pseudogenes we hypothesize that not all nuclei might contain a functional variant of *BiP* and thus that complete functional genomic information might be partitioned among nuclei within spores.

Introduction

Arbuscular mycorrhizal fungi form mutualistic symbioses with the roots of approximately 80 percent of all known terrestrial plant species (Smith and Read, 1997). Plants benefit from the symbiosis due to increased phosphorus acquisition, improved water uptake and protection from root pathogens (Newsham and Fitter, 1995; Selosse and Le Tacon, 1998; Harrison, 1998). These benefits have, therefore, provoked a great interest in finding fungal genes that are involved in symbiotic functioning and establishment of the symbiosis (Harrison, 1995; Rausch *et al*., 2001).

However, recent studies show that organisation of the genome in AMF comprises some unusual properties. There is very high within and among species genetic diversity and this is even true for individual spores (Sanders *et al*., 1995; Antoniolli *et al*., 2000; Clapp *et al*., 1999; Lanfranco *et al*., 1999). AMF are coenocytic and are assumed to pass hundreds (sometimes thousands) of nuclei to individual spores during formation. The nuclei within one spore have been shown to be genetically different (Kuhn *et al*., 2001) although it is not known to what extent the total genetic diversity is arranged within a nucleus or segregated among nuclei.

Until now, studies showing high genetic diversity in AMF within single spores and within single spore isolates have been conducted almost exclusively on rDNA sequences. Given the high amount of genetic variation and given the presence of multiple genomes there is also potential for variation among gene sequences that code for the same protein. Since high within-spore variation in rDNA has been known for several years now it is surprising that the variation in other AMF genes has not already been addressed.

Because rDNA exists in many copies per nucleus it is extremly difficult to attribute the genetic variation to either among-nuclei or within nucleus variation. Moreover, because these genes are not translated into proteins it is difficult to predict whether variation in these sequences is neutral or may result in functional changes on which selection could act. It is, therefore, desirable to know whether nuclear genes that are coding for an amino acid sequence vary in AMF spores. This would allow us to estimate how much of the variation could lead to functional changes, how much may be neutral and whether some variants may be pseudogenes.

In order to know whether any variation among sequences of a functional gene is segregated among nuclei it is important to look at the variation in a single copy gene. Furthermore, the simplest way to know whether these differences occur in one spore and not among spores is to work with a single spore isolate, where all fungal material originated from one spore that has not had the possibility to exchange genetic information with another isolate (clonal propagation). Knowledge about the total diversity of the variants of a single copy gene and how many of these variants are potentially functional would then provide information about how functional and non-functional genetic information is segregated among nuclei.

There are already some indications that functional genes are variable in AMF isolates (Kuhn *et al.*, 2001; Ubalijoro *et al.*, 2001; Ferrol *et al.*, 2000), although it was not the goal of these studies to study within isolate diversity and the results have not been interpreted in this way. The existence of chitin synthase and ATPase genes in the AMF genome has been demonstrated using PCR with highly degenerated primers and Southern hybridisation. Sequences of cloned DNA were variable for a given gene within an isolate of a species and several bands were detected after Southern hybridisation. There are several explanations for the presence of multiple bands and due to the design of the experiments it is unclear which one is true. Firstly, both genes are known to be organized in gene families in other organisms and, therefore, are present in multiple copies even in a single genome (Palmgreen 2001, Roncero 2002). Secondly, the ploidy level of AMF is not known and given that AMF contain genetically different nuclei the observed results could be due to both polyploidy and / or multiple genomes. In the case of AMF, presence of multiple genomes could result in several bands in present Southern hybridisation even if the gene is only present in one copy per nucleus.

For our studies, we have used the AMF *Glomus intraradices* growing in a sterile culture with Ri T-DNA transformed carrot roots. The culture was started using one spore and had no possibility to exchange genetic material with any other *G. intraradices* isolate. No visible contaminations of other fungi were observed during cultivation. This single spore culture provided us with large amounts of pure fungal material that could be used for extraction of DNA, all of which originating from 1 spore. We have based our study on variation in DNA sequences of a gene that is present in *G. intraradices*. Homologues of this gene occur in many other eukaryotes including fungi (ascomycetes) and are known to code for a binding protein BiP. It belongs to the HSP70 family of proteins which are among the most

highly conserved proteins known (Gupta and Golding, 1993). *BiP* is known to be single copy in ascomycetes (Stedman and Buck, 1996) and has previously been found to be variable in AMF (Kuhn *et al*., 2001). After sequencing the whole *BiP* gene we conducted single fluorescent in situ hybridization (FISH) using this sequence to estimate the number of hybridization signals that are present per nucleus. We amplified, cloned and sequenced 166 fragments of *BiP*. The resulting dataset was then used in a resampling procedure where sequence diversity was plotted against sampled sequences. The resulting curve allowed a prediction of the total number of different sequences that might be present in this isolate. A functional analysis of the sequence variants based on the deduced amino acid sequences was carried out in order to predict how many were likely to be pseudogenes and how much of the variation is neutral or not. In addition, we analyzed the phylogenetic relationship among the different sequences in order to identify potential main sequence groups. We then looked at where the predicted pseudogene sequences were located in the phylogenetic tree and whether there was observable pattern of distribution. A final experiment was aimed to see whether RNA transcripts were variable and to see whether pseudogene transcripts were present. Knowledge about the total number of variant sequences in a single spore isolate, the proportion that may be pseudogenes and the number of copies of the gene per nucleus would allow us to predict whether each nucleus is likely to contain a functional copy of *BiP*. If they do not, then this could be indication of evolution of cooperation among genetically different nuclei or possible “cheating” by some nuclei.

Material and Methods

Cultivation of *Glomus intraradices* and preparation of genomic DNA

Glomus intraradices (isolate DAOM 181 602) growing on Ri T-DNA transformed *Daucus carota* roots was obtained from the group of Prof. G. Bécard (University Paul Sabatier, Toulouse, France). The fungus was maintained on split plates which allowed proliferation of large amounts of hyphae and spores in a compartment that is free from roots (Stedman and Buck, 1996). Roots that directed their growth to the fungal compartment were removed to avoid contamination with plant DNA. The cultures were grown for an average period of three months at 25°C before DNA extraction. Spores and hyphae were harvested by dissolving the medium in

Table 1. Summary of the primers that have been used in different experiments to isolate and characterize the *bip* gene in the AMF *G. intraradices*.

Primer	Sequence 5' – 3'	Application / Experiment	Fragment (bp)
GiBiPinv1.forward	TACTCACTATAGCGGGACTATTAG	Inverse PCR	2300
GiBiPinv1.reverse	AGAAGTCACAAAATCTTCTCTACC		
GiBiPinv2.forward	CAGGAAAATTATTAAAGATTCA	Inverse PCR	900
GiBiPinv2.reverse	CCTCGTAACACTGTAATCCCTACT		
GiBiP.forward	ATACCCATCCTTTTCTGTACAGGG	PCR of complete *bip*	3812
GiBiP.reverse	AAATCCTTAGTAATTATCTCGAAC	gene	
GiBiP2.forward	AAGACAAGCCACAAAAGATGCTGG	PCR of a fragment in 2nd	792
GiBiP2.reverse	AGTAGGGATTACAGTGTTACGAGG	exon	
GiBiPvar.forward	CGTTGAACAAGTGTTGAAAGATGC	PCR of highly variable	220
GiBiPvar.reverse	ACCAGTTGTTTCAATACCGAGTGT	region	
GiBiP2.forward	AAGACAAGCCACAAAAGATGCTGG	RT-PCR	890
GiBiP3.reverse	CCTGTTAATTCGAACTTGCCGAGC		

citric acid (Nagahashi *et al*., 1993). The fungal material was collected on a 22µm sieve and taken for DNA extraction. Freshly harvested mycelium was placed into sterile 2ml microcentrifuge tubes and disrupted using a magnetic stirrer for 10min (1200rpm). DNA was then extracted from the resulting suspensions using the DNeasy plant mini kit (Quiagen). DNA concentration was estimated using a fluorimeter (Hoefer DyNA Quant 200) and DNA quality was checked using 150ng on a 0.6% TAE agarose gel after electrophoretic separation for 30min at 8V per cm.

Characterization of the complete *BiP* gene

Inverse PCR. A partial genomic library was used to obtain a fragment of the *BiP* gene (Kuhn *et al*., 2001). Based on this fragment, the inverse primers GiBiPinv1.forward and reverse plus GiBiPinv2.forward and reverse were designed to amplify the flanking regions of DNA (Table 4.1)(Sambrook and Russel, 2001). Inverse PCR (iPCR) was performed using 1µg of *Bcl*I, *Eco*RI, *Hind*III, *Sal*I and *Mse*I digested and re-ligated genomic DNA as a template with the following reaction components: 1 times reaction buffer containing $MgCl_2$ (Roche), 0.25mM dNTP´s, 0.1µM of 2 inverse primers and 1U High Expand Polymerase (Roche).

Conditions for amplification were: denaturation at 94°C 3min, then 34 cycles of amplification, denaturation at 94°C for 1min, annealing at 54°C for 30s, extension at 68°C for 4min and final extension for 10min at 68°C. The iPCR amplification products were then cloned and sequenced. Because iPCR can result in artefacts (Kaplinski *et al*., 2002), as a control, a fraction of the iPCR amplification product was then taken as template for a nested PCR with primers that specifically amplified a known part of the *BiP* gene. Amplification of this fragment was taken as positive control for successful amplification of DNA with iPCR containing an unknown flanking part of the *BiP* gene. Amplification of the complete *BiP* fragment with specific primers from genomic DNA, cloning and subsequent sequencing confirmed that artefacts had not been incorporated during iPCR.

Cloning and sequencing. PCR products were purified using the PCR Quick Kit (Quiagen) according to the manufacturers instructions. The cleaned PCR product was cloned into pGEM T easy vector (Promega) and then transformed into ultracompetent JM 107 *Escherichia coli* (Invitrogen). Bacterial clones carrying the plasmid with insert were taken for amplification and plasmid rescue (Sambrook and Russel, 2001). Sequencing reactions were performed using BigDye vs 3.0 (Applied Biosystems) and products were separated on a 377 ABI Prism automated gel sequencer (Applied Biosystems). Each sequence was manually corrected for reading errors. Alignments were made using the program ContigExpress and AlignX (software package Vector NTI).

DNA-DNA fluorescent *in situ* hybridization (FISH).

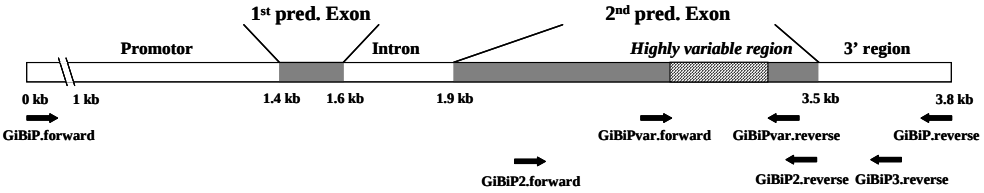
The complete *BiP* gene including the predicted promoter region was used as a probe for FISH in order to look for its presence and distribution in *G. intraradices* nuclei. The size of the probe was approximately 3.8kb. It was synthesized by PCR amplification with the primers GiBiP.forward and GiBiP.reverse (Table 4.1). The probe was labeled by PCR using DIG-11-dUTP (Roche) according to the manufacturers recommendations. The labeling of the probe was checked using agarose gel electrophoresis and by observing the size shift between the labeled and unlabeled probe. The probe was then purified using Qiaquick PCR purification kit (Qiagen) according to the manufacturers instructions.

Spores and hyphae of *G. intraradices* were extracted from the cultures and immediately fixed with 4% formaldehyde in nuclear isolation buffer (NIB) containing 100mM Tris/HCl, pH 8, 100mM NaCl, 2mM $MgCl_2$ and 0.05% Triton X100, for 2h at room temperature. Spores and hyphae were rinsed in fresh NIB

then crushed on Frost Plus slides (Menzel-Gläser, Germany) then air dried overnight. Slides were incubated with 100µl of DNase free RNase (100µg ml^{-1}) in double-strength SSC (1h, 37°C). Slides were rinsed in double-strength SSC (5min at room temperature) and then incubated in a proteinase solution (1µg ml^{-1} proteinase K, 20mM Tris-HCl, pH 7.5, 2mM $CaCl_2$, at 37°C for 5min). Slides were rinsed in 20mM Tris-HCl, pH 7.5, 2mM $CaCl_2$, 50mM $MgCl_2$ at room temperature for 5min, then dehydrated in ethanol (70%, 90% and 100%) and dried. Twenty five µl of the hybridization solution containing 50% formamide, 1mM EDTA, 10% dextran sulfate, 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.1% Ficoll, 0.5µg $µl^{-1}$ DNA from herring sperm, 0.1% SDS, 5ng $µl^{-1}$ purified DIG-labeled probe, were loaded per slide. Slides were covered with plastic cover slips and denatured for 3min at 80°C. Hybridization was carried out at 37°C, overnight in a moist chamber. The post-hybridization washes were performed with 50% formamide in double-strength SSC (37°C, 10min), followed with double-strength SSC (37°C, 10min) and then rinsed again with double strength SSC at room temperature for 15min.

The signal was amplified for detection by a three-step immunoreaction (Pinkel *et al.*, 1986). All reactions were carried out in PBS buffer (160mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8mM Na_2HPO_4, pH 7.4) containing in addition 0.5% BSA and 0.1% Tween-20 at 37°C. Slides were incubated with 0.5 µg ml^{-1} monoclonal mouse anti-digoxigenin antibody for 1h. Following 2 washes in buffer without antibody (2 x 5min each), slides were incubated with 10µg ml^{-1} of anti-mouse IgG digoxigenin conjugate antibody for 1h. This was followed by an incubation in the dark with 2µg ml^{-1} sheep anti digoxigenin fluorescin isothiocyanate (FITC) conjugate for 1h in a humidified atmosphere. A final wash was made with PBS containing 0.1% Tween-20. Nuclei were counterstained with 1 µg ml^{-1} propidium iodide (Molecular Probes) and mounted in FluoroGuard Antifade Reagent (Biorad). Slides were examined using a TCS-SP laser confocal scanning microscope (Leica).

Figure 4.1: Scheme of BiP gene organization



Variation in the BiP gene

Primer design, PCR conditions and optimisation. We aimed to identify the total sequence diversity present in one region of the *BiP* gene. Conserved regions are important for the design of primers that do not selectively amplify a subset of variants and to be able to amplify a rather accurate representation of the sequence diversity of a region. Therefore, we firstly aimed to identify the distribution of variable and conserved regions within the original fragment of *BiP* that was previously sequenced by Kuhn et al. (2001). This fragment is located in an open reading frame (ORF) of the *BiP* gene. Using the primers GiBiP2.forward and GiBiP2.reverse (Table 4.1) we amplified a 792 bp fragment of *BiP*. This was repeated several times and the product was cloned (as described above). In this study, a total of 60 sequences of the 792 bp fragment were aligned and analyzed for variation. Based on the alignment showing conserved sites we designed the primers GiBiPvar.forward and GiBiPvar.reverse (Table 4.1) that were flanking a highly variable region of the *BiP* gene. Their annealing temperature was optimised with a temperature gradient cycler (Biolabo) using a temperature range from 50-65°C. Each of the PCR products amplified with these primers was separated on TAE agarose and TMA sephadex gels (SSCP gels) and checked for the presence of unspecific amplification products (data not shown). The highest temperature (56°C) that still resulted in an amplification product was used to look at variation. Each PCR reaction was carried out in 50µl volumes and was composed of the following components: 20ng of *G. intraradices* DNA as template, 1 times reaction buffer (Invitrogen), 1.5mM $MgCl_2$, 0.1mM dNTP´s, 0.2µM of both primers and 0.2U of Taq Polymerase (Invitrogen). Conditions for PCR amplification were: 3min at 94°C for a first denaturation, followed by 30 cycles of 15s at 94°C for denaturation, 15s at 56°C for annealing, 1min at 72°C for extension and a final 7min at 72°C for extension. The resulting PCR amplification products were cloned and sequenced. In order to obtain a representation of the population we sequenced 166 clones chosen at random. This allowed us to determine the frequency of

variants and later on to predict the total sequence diversity using a Monte Carlo Simulation.

Functional and phylogenetic analysis. The similarity of each sequence to existing *BiP* sequences of other fungal species in the NCBI database was confirmed using the Blast program (Altschul *et al*., 1990). The closest match was with the *BiP* gene from the Ascomycete *Aspergillus awamorii* (similarity on the amino acid level of 76%; accession number: gi|11277119|pir|T43723). The number and distribution of substitutions and indels per sequence was then estimated by comparing all sequences to the sequence matching closest to the *BiP* sequence in GenBank. The nucleotide sequences were analyzed with regards to the numbers of synonymous and non-synonymous substitutions. Sequences were translated into amino acid sequences using the vector NTI software package. Indels in the ORF that resulted in a change of an amino acid into a termination codon were counted as well as those which were in frame.

The alignment of sequences that differed in at least 1 substitution was then analysed using a maximum parsimony analysis both with and then without indels. This showed relatedness among sequences and the distribution of non-functional variants. A maximum parsimony analysis was performed using PAUP 4.0 beta 10 (Swofford, 2002) with a heuristic search procedure using random stepwise addition and tree bisection-reconnection branch swapping options, with 10 repetitions. The robustness of the branching pattern was evaluated with a bootstrap procedure (100 replicates). An unrooted tree was chosen for graphical display.

Monte Carlo simulation for predicting maximum sequence diversity. The goal of this analysis was to test whether the number of different sequences of the *BiP* gene was limited within an AMF spore and, if possible, to estimate a maximum number. The dataset of 166 sequenced clones served as the basis for this analysis. The number of variant sequences was plotted against the total number of sequences. By means of random sampling starting with 2 sequences, successive sequences were chosen without replacement and the cumulative number of sequences that were different was scored until all sequences had been sampled. The mean values and standard deviation were obtained by performing a bootstrap over 1000 repetitions of the procedure. The test was performed twice, first with the assumption that a difference of a single substitution was regarded as a different sequence (real estimate) and second with the assumption that only sequences containing at least 2 substitutions in comparison to all other sequences were scored as truly different sequences (conservative estimate). Although the chosen PCR

conditions limited the mistake rate to less than 1 false incorporated nucleotide along a stretch of 1000bp, we did the second calculation to obtain a more conservative estimate. An asymptotic curve function then used to fit the curve, in order to test whether it fitted the data better than a linear function. This curve was used to predict the maximum number of different sequences that existed in one isolate.

RNA extraction and reverse transcription. Spores and hyphae of *G. intraradices* were harvested (as described above) and put into RNAse free tubes containing ceramic beads (Q-Biogen) at 4°C. The material was disrupted with a bead beater (Q-Biogene) by shaking twice at maximum intensity for 15s. The resulting suspension was transferred into new tubes and taken for extraction using the RNeasy plant mini kit (Quiagen) following the manufacturer's instructions. RNA extracts were treated with DNAse and purified using the messenger clean kit (Genhunter). To check for absence of contaminating DNA, two independent PCR reactions were performed as controls. In these reactions, 200ng of total RNA were used as template containing the primers GiBiP2.forward and GiBiP.reverse. These primers can only anneal with genomic DNA because GiBiP.reverse anneals in the non-transcribed region of the 3' end. The other reaction used the primers GiBiPvar.forward and GiBiPvar.reverse that can only anneal with genomic DNA and cDNA. If no amplification was visible in both cases then RNA was deemed DNA-free and used for reverse transcription.

Reverse transcription was performed using 1µg of total RNA, denatured at 70°C for 10min, in a total reaction volume of 25µl containing the following components in a final concentration: 1 times reaction buffer (Invitrogen), 0.5µM oligo dT_{15} Primer, 1mM dNTPs, 1.5mM $MgCl_2$, 0.01M DTT and 5U Superscript Reverse Transcriptase (Invitrogen) and incubation at 42°C for 1h. After heat inactivation a PCR to amplify sequences that were then screened for genetic variation was performed using the primers GiBiP2.forward and GiBiP3.reverse (Table 4.1). The PCR conditions were: 3min at 94°C for denaturation followed by 32 cycles of 15s at 94°C for denaturation, 15s at 56°C for annealing,

a) b)

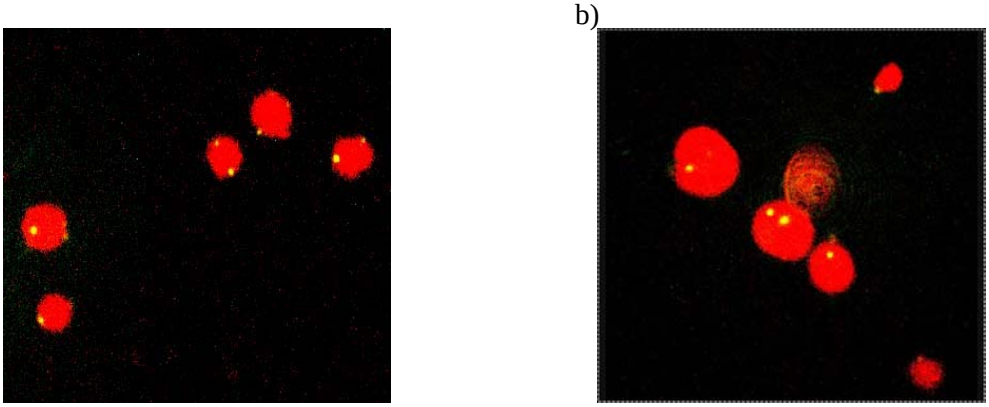


Figure 4.2: Nuclei of *Glomus intraradices* taken with laser confocal microscopy after single target DNA-DNA FISH. Hybridization signals (yellow) of the 3.8kb BiP gene probe labeled with DIG to nuclei (red). a) and b) nuclei showing 1-2 hybridization signals.

2min at 72°C for extension and finally 10min at 72°C for extension. The resulting PCR product was cloned and sequenced.

Results

Size of *BiP* and presence in nuclei. Using iPCR we successfully amplified, cloned and sequenced the whole *BiP* gene including the promoter region and non coding regions. The size of this fragment was 3.8kb (Figure 4.1). A genetic map of the *BiP* gene organization, based on the comparison of the predicted amino acid sequence with that of the ascomycete *A. awamorii* indicated a 1.4kb promoter region and 2 exons of approximately 186 bp and 1.6kb, which are separated by an 340bp intron (Figure 4.1).

Using the *BiP* gene as a probe for FISH revealed an overall range of 0 - 4 hybridization signals per nucleus. Eighty nine percent of the nuclei showed between 1 and 2 hybridization signals (n = 117)(Figure 4.2). Six percent of the nuclei were unlabeled and 5% exhibited 3 or 4 signals.

Variation in the *BiP* gene. Aligned sequences of the region amplified by GiBiP2.forward and GiBiP2.reverse showed the presence of a highly variable region of approximately 230 bp located in the second exon. By randomly

Table 4.2: Analysis of 166 sequences of a variable region in the *bip* gene based on predicted aligned amino acid sequences

Variant	Class	size of insertion	size of deletion	presence of termination codons	frequency n = 166	Information	no. synonymous substitutions	no. non-synonymous substitutions	proportion ns:s substitutions
GintBiP1	1	0bp	0bp	no	2.4%	pi	0	1	1.00
GintBiP2	1	0bp	0bp	no	1.2%	pi	0	2	1.00
GintBIP3	1	0bp	0bp	no	0.6%	pi	0	1	1.00
GintBiP4	1	0bp	0bp	no	0.6%	pi	0	1	1.00
GintBiP5	1	0bp	0bp	no	0.6%	s	0	1	1.00
GintBiP6	1	0bp	0bp	no	0.6%	s	1	0	0.00
GintBiP7	1	0bp	0bp	no	22.3%	pi	0	0	0.00
GintBiP8	2	0bp	1bp / 6bp	yes	0.6%	s	9	8	0.89
GintBiP9	2	0bp	1bp / 6bp	yes	0.6%	s	10	7	0.41
GintBiP10	2	0bp	6bp	no	0.6%	pi	11	7	0.39
GintBiP11	2	0bp	6bp	no	0.6%	s	12	8	0.40
GintBiP12	2	0bp	6bp	no	0.6%	s	12	8	0.40
GintBiP13	2	0bp	6bp	no	0.6%	s	12	8	0.40
GintBiP14	2	0bp	6bp	no	0.6%	s	13	7	0.35
GintBiP15	2	0bp	6bp	no	1.2%	pi	13	7	0.35
GintBiP16	2	0bp	6bp	no	0.6%	s	12	8	0.40
GintBiP17	2	0bp	6bp	no	1.2%	pi	12	8	0.40
GintBiP18	2	0bp	6bp	no	0.6%	pi	10	6	0.38
GintBiP19	2	0bp	6bp	no	1.2%	pi	10	6	0.38
GintBiP20	2	0bp	6bp	no	0.6%	pi	11	11	0.50
GintBiP21	2	0bp	6bp	no	30.2%	pi	12	7	0.37
GintBiP22	3	5bp	0bp	yes	0.6%	pi	1	0	0.00
GintBiP23	3	5bp	0bp	yes	1.2%	pi	5	7	0.58
GintBiP24	3	5bp	0bp	yes	0.6%	pi	6	7	0.54
GintBiP25	3	5bp	0bp	yes	0.6%	s	5	7	0.58
GintBiP26	3	5bp	0bp	yes	0.6%	s	5	7	0.58
GintBiP27	3	5bp	0bp	yes	0.6%	pi	5	6	0.55
GintBiP28	3	5bp	0bp	yes	0.6%	s	6	6	0.50
GintBiP29	3	5bp	0bp	yes	0.6%	s	5	7	0.58
GintBiP30	3	5bp	0bp	yes	0.6%	pi	6	6	0.50
GintBiP31	3	5bp	0bp	yes	25.9%	pi	9	6	0.40

pi = parsimoniously informative s = singleton

sequencing 166 cloned DNA fragments that had been amplified with the primers GiBiPvar.forward and GiBiPvar.forward we found 31 different variants of the variable region that were assigned with numbers from 1 to 31. If variants that only carried one singleton substation and were only found once were removed a more conservative estimate of at least 18 variants was obtained. Among all variants, three main categories of sequences appeared. The classification was based on an indel mutation that occurred at position 200. Compared to a first category (variants 1-7) that matched most closely to the *BiP* gene in *A. awamorii*, a second category (variants 8-21) was characterized through a deletion of 6bp and a third category (variants 22-31) through an insertion of 5bp (Table 4.2). The frequency at which sequences of each class occurred in the 166 clones was 28%, 40% and 32%, respectively. Remarkably, in each of the three classes there was one variant that was present in very high frequency (*GintBiP7*, class 1, 22.3%; *GintBiP21*, class 2, 30.2%; *GintBiP31*, class 3, 25.9%) and the sum of all these was 78.4%.

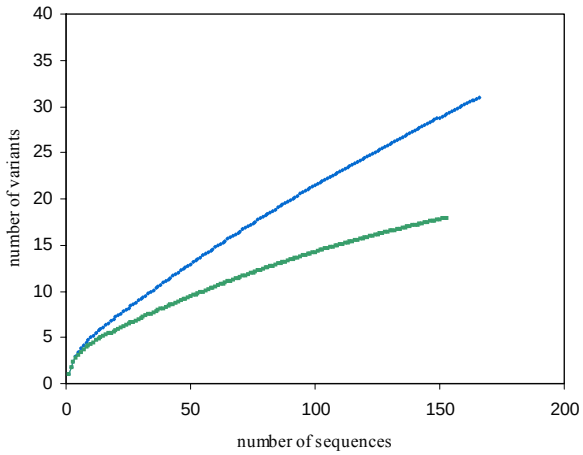


Figure 4.3: Two Monte Carlo simulations based on a population of 166 sampled sequences that have been found in the DNA from a single spore culture of the AMF *G. Intraradices.* Details of the sampling procedure are given in the text. The simulation was performed under the assumption that all sequences that carried (a) a single substitution and (b) more than one substitution were considered as variants.

Fifty-five sites out of 274bp in the variable region were found to be polymorphic. Fourteen sites only differed from the other sequences by 1 base and we called these singletons. Variants that differed from all other sequences because of only one singleton were considered to be potentially due to polymerase errors and were removed for the conservative estimate of total genetic diversity. Forty one sites were "truly" polymorphic, in that they occurred in 2 variants or more and were informative for parsimony analysis. Eight variants were found only once (frequency = 0.6%) but differed in more than 1 site from all other variants. Nine variants were found at least twice among 166 sequences (frequency ≥ 1.2%) (Table 4.2).

Analysis of the predicted *BiP* gene functionality. The amino acid sequence of the *BiP* gene was predicted for all variants. Variants belonging to class 1 (with no indel) resulted in a "functional" ORF. This was also true for class 2 variants (6bp deletion) although 2 variants (8 and 9) had an additional single deletion which led to a shift in the ORF. In both cases, the amino acid sequence was then shortened because of the appearance of a termination codon and was, therefore, considered non-functional. Variants of category 3 carried a 5bp insertion which led to a shift in the ORF and resulted in all cases in a shortened amino acid sequence because of the appearance of a termination codon.

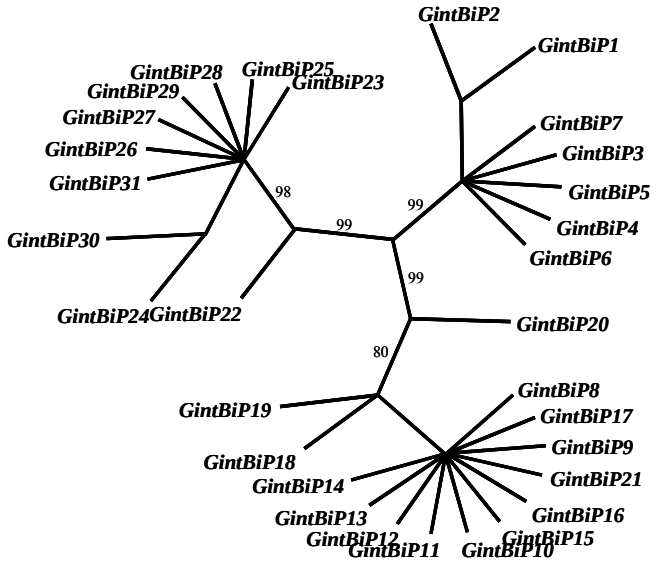


Figure 4.4: Phylogenetic analysis of 31 *BiP* gene variants that have been found in the DNA from a single spore culture of the AMF *G. Intraradices*. A Parsimony analysis was performed with a heuristic search procedure using 10 stepwise random additions and tree bisection-reconnection branch swapping options. Bootstrap values for a procedure with 1000 repetitions ≥ 80 are indicated at branches.

An average of 51% of all substitutions were non-synonymous when the number of substitutions based on an amino acid alignment was compared with the number of substitutions based on a nucleic acid sequence alignment. However the ratio of non-synonymous substitutions to synonymous substitutions (ω) based on the Nei-Gojobori (modified Nei Gojobori) method is 0.53 (0.69) (Table 4.3). However, if the class of sequences containing potential pseudogene sequences is excluded from the dataset ω is reduced to 0.28 (0.36), indicating stronger evidence for selection (Table 4.3).

Maximum sequence diversity of *BiP* gene sequences. Two Monte Carlo simulations performed assuming that either 31 *BiP* variants or 18 *BiP* variants existed in the population of 166 sequences showed that the relationship between the number of variants and the number of randomly sampled clones deviated significantly from a linear relationship and fitted an asymptotic curve with the function $n_{hap} = a_{max} * (n_i/(n_i + b))$(Figure 4.3). Here, n_{hap} represents the number of variants, n_i is the number sampled sequences and a_{max} is the maximum number of variants. From this curve we predicted that the maximum number of variants (a_{max})

was 69.7 if each sequence that carried a single substitution was interpreted as a variant (Figure 4.3 a). The second more conservative estimate that accounted for potential polymerase errors gave a very similar curve function and a maximum number of 27.9 different sequences was predicted.

Phylogeny. Using the complete set of variants a heuristic analysis also showed three well supported groups (bootstrap values 99%, 99% and 99%) (Figure 4.4). These corresponded to the three classes of sequences that were found on the basis of indels. This finding even held true, when indel mutations were removed from the dataset with resulting bootstrap values of 83%, 100% and 80% (data not shown). An entire group contained a 5bp insertion and, therefore, consisted only of non-functional variants and this corresponds to the class 3 variants shown in table 4.2.

Transcription of the *BiP* gene. The negative control showed that no contaminating DNA was present in the extracted RNA. An 890bp fragment of the *BiP* gene was amplified from cDNA with the primers GiBiP2.forward and GiBiP3.reverse. The PCR product was cloned. Thirty clones were taken at random for sequencing and an alignment of sequences showed that all sequences were of category 2. Of these, only 7 single substitutions (singletons) were found. This sequence matched exactly to that of variant 7, that was amplified from genomic DNA.

Discussion

High variation among *BiP* gene sequences. In a population of 274bp sequence fragments of the *BiP* gene that were isolated from a single spore culture of the AMF *G. intraradices*, a surprisingly high number of variable sites was found (total 55 variable sites). This variation was comprised within a total of 31 variants. While the variation remained high (with 43 variable sites) even when sequences that could be due to methodological errors were removed the total number the number of variants that comprised it was only 18. Thus, most of the diversity is comprised in about half of all variants. Among all 31 variants 51% of all substitutions have been shown to be non-synonymous and led to changes in the predicted amino acid sequence. However, if the ratio of non-synonymous substitutions to the number of synonymous substitutions is calculated weak evidence for purifying selection is found. Because one of the classes comprised only variants with non functional ORF's that are thought to evolve differently from functional gene sequences these

were excluded for a second analysis. The second analysis then showed stronger evidence for selection. This is congruent with the data of functional gene sequences from other organisms where synonymous substitution rates generally exceeded the rate of non-synonymous substitutions (Hartl and Clark, 1997). This has been interpreted as evidence for selection to retain functional traits in the protein of a gene. Equal or even reverse ratios of non-synonymous substitutions to synonymous substitutions have previously been reported for selectively neutral regions of a genome such as pseudogenes or for parts within an ORF that code for a region of little importance for the protein functioning and which has been under relaxed selection. But also adaptive or diversifying selection has been described to lead to similar substitution patterns in gene sequences as for example in immunoglobulin genes in mammals (Hartl and Clark, 1997). The presence of pseudogene sequences is supported by our findings because the strength of selection has been reduced drastically when the pseudogene sequences were included in the dataset.

Phylogenetic analysis showed that termination codons do not appear randomly in each of the mains groups but with 2 exceptions exclusively in one major group through an insertion event. We also show, through recalculation of the tree without indels, that this structure has not been confined through these insertions. We conclude that mutations do not occur randomly even in the presence of a surprisingly high number non-synonymous substitutions and that selective forces are in operation. Moreover, this pattern of mutations was observed within the progeny of one single AMF spore and this presents several possibilities for how gene expression might occur. First it is possible that variants are differentially and specifically expressed, by means of temporal or spatial variation. Second it could be that several variants are simultaneously expressed. Whether several variants of the *BiP* gene are expressed simultaneously has been tested and revealed that under the given experimental conditions only one variant was transcribed. The expressed variant is identical with that variant that showed closest similarity to the *BiP* gene homolog in *A. awamorii* based on the amino acid sequence. However, future studies with varying experimental conditions are needed in order to know whether the other variants are also transcribed and expressed.

Copy number. In view of the high variation and low copy number of the *BiP* gene in other eukaryotes, getting an estimate of the total diversity, the diversity that might be functional and the copy number can allow us to predict whether every nucleus could carry a functional copy of the *BiP* gene.

Our single FISH experiment on nuclei showed that a great majority of nuclei displayed between 1 (37%) and 2 (52%) hybridisation signals, suggesting that only a small number of *BiP* sequences are located on one nucleus. Unfortunately, due to the methodology of FISH, it is not possible to know whether a hybridization signal corresponds to one or to several *BiP* sequences arranged in a cluster. Given that the *BiP* gene is among the most highly conserved proteins and is single copy in other fungal species (Stedman and Buck 1996) the likelihood that *BiP* is organized in clusters seems small. Given that one nucleus, depending on ploidy, resembles a distinct number of genomes the prediction of only very few *BiP* sequences per nucleus receives further support from the fact that the estimated genome size of AMF compared to other eukaryotes is very small (Mohamed Hijri, personal communication).

Analysis of total diversity showed that when the population of sampled sequences was analyzed the relationship between the number of variants and the number of sampled sequences resulted in an asymptotic curve. From this we could predict that a minimum estimate of 28 variants and a maximum estimate of 70 variants coexist in this isolate of *G. intraradices*. This is the first report in AMF to show that there are limits to the genetic variation. In future experiments analyses of different isolates and isolates that have been started with several spores would help to identify a more accurate estimate of maximum genetic diversity within AMF. Clearly, the amount of variation among gene sequences could differ according to which gene is studied as these might be under different selective constraints (Hartl and Clark, 1997). Therefore, to get a more general estimate of the average amount of genetic variation that can be found in functional genes sequences in AMF also studies on other genes are needed.

The functional genome. Among the 31 isolated variants 12 have been predicted to be pseudogenes and the sum of the frequencies of these variants within the population of 166 sequences was 34%. Our FISH results show one or two copies per nucleus. This means that the chance that some nuclei do not possess a functional *BiP* gene sequence is considerable. Consequently, we hypothesize that nuclei carrying one or two non-functional copies of the *BiP* gene might exist within an AMF spore and, thus, that these nuclei co-exist with other nuclei that contain a functional copy of *BiP*. These could be considered as cheating nuclei. However functional genetic information might generally be partitioned among nuclei, resulting in nuclei that do not all contain the same functional copies of genes and indicate the evolution co-operation among nuclei to comprise a complete functional

genome. Unfortunately based on our findings we cannot say whether cheating or co-operation or a mixture of both has evolved in AMF.

Overall, in multigenomic AMF, the unit of a functional genome might therefore no longer be seen as one single nucleus but rather the sum of the diversity of all different nuclei and all their functional variants. AMF pass on hundreds and sometimes thousands of nuclei into newly formed spores. The reason for this has never been clear. Bearing in mind the chances that not all functional variants might be located on each nucleus our study implies the need of AMF to pass a relatively high number of nuclei in order to maintain their genetic diversity and not loose any functional genetic information due to accumulation of deleterious mutations.

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Table 4.3: Results of two analyses of substitution rates of *BiP* gene sequences of *Glomus intraradices*, comprising all 31 variable sequences and only functional sequences.

	all variants		only functional variants	
	NG	mNG	NG	mNG
S	60.22	72.79	60.79	73.28
N	194.78	182.21	194.21	181.72
ds	0.1	0.083	0.089	0.073
dn	0.053	0.057	0.025	0.026
	0.53	0.69	0.28	0.36

The analyses were performed on all 31 *BiP* gene variants and on functional variants only. Two different models were used (Nei Gojobori and modified Nei Gojobori) for calculation. The total number of synonymous and non-synonymous positions per sequence are represented by S and N, respectively. The number of observed synonymous and non-synonymous substitutions per synonymous and non-synonymous position are denoted ds and dn. The ratio of non-synonymous to synonymous substitutions is denoted .

Chapter 5

Discussion and Conclusion

The results presented in this thesis demonstrate that the AMF genome is highly unusual. Analysis of the organization of within species genetic variation showed that significant within individual genetic differences exist among sequences of rDNA and a putative single copy gene as well as among nuclei. In addition significant genetic differences have been found between individuals of an AMF population. These results have implications for evolutionary biology, for the ecological importance of AMF and for analysis of AMF gene function. In this discussion, I bring together the most important aspects of the results for all three experimental chapters.

5.1. Evolutionary aspects

5.1.1 Evidence for recombination

Using a compatibility analysis, I found evidence for some recombination between sequence variants of rDNA and no evidence among sequences of *BiP*. Although it was only a few sequences that showed recombination, it has been shown in all rDNA datasets of 4 different species and, therefore, seems consistently present. This is a highly important finding because it puts into question whether AMF are truly ancient asexuals as it has long been thought due to the complete absence of any sexual structures and due to a previous experimental test (Rosendahl and Taylor, 1997). One of the reasons for my contrasting finding might be the that my test has been applied to sequences and, thereby, allows detection of recombination among nuclei while the previous study relied on genetic markers being present or absent in different spores and was, therefore, unable to detect recombination among nuclei.

The compatibility test presented in chapter 2 has only revealed recombination in rDNA. These genes are multicopy and known to be organized in tandem repeats. Due to concerted evolution they are likely to be homogenous within individuals (Hoelzel and Dover, 1991). But in recent studies doubt has arisen about whether these genes really co-evolve because variation has also been observed within individuals (Harris and Crandall, 2000) and this might be especially true for AMF.

Included in chapter 2 is the work of Mohamed Hijri who has demonstrated in a double FISH experiment that there was segregation of two divergent ITS sequences among nuclei and, thus, that nuclei are genetically different. Therefore

in AMF individuals, the presence of divergent rDNA sequences between loci of the same nucleus and between nuclei is possible. While copies of ribosomal sequences on one nucleus are considered to have originated by gene duplication and are therefore considered sequence paralogues, divergent copies of the same rDNA gene locus between nuclei can be considered as true orthologues. Both types are known to undergo different evolutionary fates and thus doing a phlygenetic analysis only orthologs of a sequence should be compared (Graur and Li, 2000). In the present study there is no way of knowing whether the analyzed sequences satisfy the assumptions. Although this has not been reported to be problematic for other organisms (sequence variation within individuals has been low or absent) genetic variation in AMF is unusually high and might therefore lead to a false positive indication of recombination.

Although various recombination tests have been developed and used for sequence data, it was after the experiments in chapter 2 that studies have been performed to compare and analyze the performance of these tests (Wiuf *et al*., 2001; Posada and Crandall, 2002). Comparison of the outcomes of different recombination tests on artificially created datasets showed that many tests rarely indicate false positive recombination, but are unlikely to detect very infrequent recombination (Posada and Crandall, 2001). Unfortunately, the test that was used here was not included in these analyses. Gandolfi *et al*. (2003) have recently demonstrated by using several tests on the same datasets as in chapter 2 that all tests indicated recombination for ITS sequences and 4 of 5 different tests also indicated high likelihood for recombination among *BiP* sequences. Therefore, based on the applied tests it seems rather likely that at least some recombination has occurred.

5.1.2. Evolutionary strategies of ancient asexuals and AMF

Genetic diversity of putative ancient asexuals Darwinula and Bdelloid rotifers has been previously examined. In the ostracod, *Darwinula stevensoni*, extremely little genetic variation was found and it was suggested that they possess extremely efficient DNA repair mechanisms preventing accumulation of mutations (Schön and Martens, 2003). Limited genetic diversity should still confer a fitness disadvantage in changing environmental conditions, but a population study of *Darwinula stevensoni* showed that this is not necessarily true because of the evolution of so called “general purpose genotypes”. In this study, a certain

genotype seemed to be extremely tolerant to various changing environmental conditions and did not seem to suffer any disadvantage (Van Doninck, 2002). In contrast, very high genetic variation has been found in individuals of ancient asexual *Bdelloid rotifers* where the number of synonymous substitutions consistently outnumbered the number of non-synonymous substitutions (Meselson and Welch, 2000). This has been predicted due to the Meselson effect where accumulation of neutral mutations over long periods of time should lead to the evolution of highly divergent lineages of formerly allelic sequences within individuals. Analysis of the variation as observed among analyzed *BiP* sequence variants of a *G. intraradices* single spore culture showed weak evidence for selection in the case of 2 classes that are predicted to comprise functional ORFs (class 1, $\omega = 0.385$; class 2, $\omega = 0.561$) indicating an elevated number of synonymous substitutions compared to non-synonymous, as predicted by this effect. However the variants that are predicted to be pseudogene variants of *BiP* show even less evidence for selection (class 3, $\omega = 0.8$), thereby indicating the pseudogene state of these variants. Comparison of the variants that were most frequent in each class showed strong evidence for purifying selection in the case of a variant from class 1 and 2, while comparison with a variant from class 3 clearly shows random accumulation of mutations (N. Corradi personal communication). Thus, in AMF there seems to be selection for the maintenance of functional variants in the genome and this is true even for the accumulation of mutations among the variants within functional groups. However, purifying selection seems to be absent in the case of the pseudogene variants and thus supports the finding that these variants are non-functional.

5.1.3. What has been predicted for being asexual over long periods of time?

Muller states that deleterious mutations accumulate in limited asexual populations and lead to successive loss of the fittest genotype which ultimately leads to extinction (Muller 1964). This accumulation of mutations does not seem to be disadvantageous for AMF. For other organisms it has been shown that asexuals are often polyploid and it has been proposed that increased levels of ploidy could slow down the ratchet (Mogie and Ford, 1988). However recent studies suggest that *G. intraradices* is likely to be haploid (Mohamed Hijri, personal communication). One way to overcome the problems of Mullers Ratchet in AMF might have been to be evolve a multinucleate state, thereby, increasing the effective population size,

but also to pass many genomes from one generation to the next thereby avoiding the effects of severe genetic bottlenecks.

Even if Mullers Ratchet is overcome then asexuals should accumulate slightly deleterious mutations irrespective of their population size (Crow, 1994), resulting in the necessity to evolve other mechanisms to reduce the mutational load. It is difficult to imagine ways to overcome this problem although one has been proposed from Gabriel et al (1993), where populations of haploid organisms that do not repair mutations should have an advantage over others that do. This strategy should lead to quick loss of less fit genotypes due direct selection and would lead to reduced accumulation of mutations in populations in the long term.

In chapter 4 it is clearly shown that about one third of the isolated variants of *BiP* in AMF have accumulated mutations of which the predicted amino acid sequence seemed non-functional and doubt might arise about whether AMF effectively repair mutations. Accumulation of deleterious mutations is predicted in the absence of sexual reproduction from the "Mullers Ratchet" hypothesis and the "mutational load reduction" hypothesis. Even if recombination has occurred in AMF there is doubt whether it was frequent enough to purge all deleterious mutations. Apparently AMF do not seem to be negatively affected by their presence. They, therefore, seem to have taken a different strategy to highly efficient repair systems or frequent recombination and this might be not only in the form of being multinucleate but also to be multigenomic.

5.1.4. Evolution of a multigenomic state

Accumulation of mutations in multinucleate AMF has been proposed to lead to the evolution of a multigenomic state in AMF (Sanders *et al*., 1996). Assuming nuclei would randomly accumulate mutations, this could also lead to accumulation of mutations in functional genes leading to loss of genome information on individual nuclei. Individual spores might, therefore, develop a state where the complete genetic information for a functional genome is partitioned among nuclei. One of the reasons how these genetically different nuclei could co-exist in one cytoplasm could be the interdependence between nuclei. Such a state would not be expected to evolve in the presence of frequent recombination because then the complete genome information might be maintained on each nucleus and no interdependence would evolve. Thus, the proposed accumulation of mutations as due to asexual

reproduction might even be a prerequisite to evolve a stable equilibrium between nuclear types.

Depending on the genetic differences between nuclei, the maintenance of such a state could probably not be achieved if only one nucleus would lead to the development of a new spore. However, no uni-nucleate state has yet been observed in AMF at any stage of its life cycle. One way to overcome the risk of lacking genetic information in AMF spores might be that many nuclei enter a newly forming spore. However, whether the formation of a spore still resembles a genetic bottleneck in the life cycle of AMF cannot be concluded, because firstly the divergence between nuclei is not known and secondly because also the actual number of nuclei that enter a spore during formation is unknown.

5.1.5. Advantages and disadvantages of a multigenomic state

Because there are potentially many "backups" of each gene within an AMF individual accumulation of deleterious mutations might be less problematic than for other organisms. Depending on the number of genomes (effective genome size) AMF might be able to cope with accumulated mutations even over long periods of time although this needs to be modelled. Due to these mutations divergent functional copies of a gene could evolve that stably co-exist on interdependent nuclei in one individual. This way AMF could evolve to strongly promote their functional genetic diversity. Based on this variability AMF could be able to adapt to a variety of environmental conditions, thereby, increasing their ecological diversity. It could be speculated that the lack of host specificity might be due to the maintenance of such this in functional copies of those genes that are important during interaction with a plant host and allow the fungus to infect different hosts simultaneously.

Disadvantages to being multigenomic are also conceivable. The possibility exists that there could be nuclei that have lost functionality in many of their genes and only retained their capacity to replicate. These could be called "cheating" nuclei because they do no longer contribute a functional benefit to the fungus and could clearly be considered as a mutational load bringing a disadvantage to the multigenomic state. They could potentially increase in frequency and, thereby, pose a problem for AMF fitness.

Evidence whether functional genetic information is partitioned among nuclei has been sought in chapter 4. I showed that due to the low number of hybridization signals per nucleus it is highly unlikely that all 31 variants of *BiP* are harboured on each nucleus. One third of the variants are predicted non-functional. If each hybridization signal is taken as evidence for the presence of one copy of *BiP*, those nuclei that displayed no signal and those that displayed one signal contained either no or, with a considerable probability, only a non-functional copy. Unfortunately it cannot be concluded whether some nuclei have not been labelled because of bad probe penetration or real absence of a *BiP* copy because no other positive control was performed in the experiment. Also it remains unknown whether nuclei with single signals are actually containing a non-functional copy, because divergence between sequences is too little to be able to perform a specific double FISH experiment, where functional and non-functional variants can be labelled differently. But as the experimental conditions were deemed optimal and specific (conditions similar to Double FISH in chapter 2) and because of the great number of non-functional sequences there is a likelihood that some nuclei have lost a functional *BiP* copy indicating the possibility of co-operation and or cheating between nuclei. However, that I isolated far more variants of *BiP* that differ in their amino acid sequence (30) than hybridization signals per nucleus (mostly one or two per nucleus) still suggests that there is segregation of functional genetic information and that not all *BiP* variants are likely to co-exist on one nucleus. It is clear that for future studies the isolated variants of *BiP* are a useful tool to test such hypothesis as whether functionally different copies are partitioned within or among nuclei.

5.1.6. Genetic differences among spores of a field population.

In Chapter 3 I have shown that great genetic differences exist between AMF individuals (single spore isolates) of a field population of *G. intraradices* by analyzing binary datasets that were obtained with AFLP fingerprinting of each single spore isolate. If the DNA of the organisms only carries a single genome then polymorphisms observed by AFLP can be interpreted as real presence and absence of restriction sites in the genome (Vos *et al*, 1995). Such a genomic structure is not given in the case of AMF. However, because AMF may have multiple genomes it is impossible to know whether the observed changes in banding patterns of AMF are real presence-absence polymorphisms or rather changes in the presence of the

most frequent markers. However, irrespective of the origin of genetic differences the variation observed is large and as shown in chapter 3 leads to changes in the phenotype.

5.1.7. Selection and anastomosis on multiple genomes.

It is important to understand how selection acts on multiple genomes in AMF. Selective forces normally act on the phenotype of individuals which are produced by the given genomes in individuals (genotypes). Therefore, a genetic change which affects one genome as e.g. accumulation of a deleterious mutation might only confer a small effect on the individual fitness. Thus, selection could only have a less direct effect on nuclei. However, the functional traits of the resulting phenotype might strongly depend on the genetically different nuclei that are present in spores. In chapter 4 I have shown that not all functional genetic information might be comprised within each nucleus. Thus the composition of nuclei within each spore might be of great importance for the functioning of the fungal phenotype and thus for its fitness. AMF, therefore, comprise two potential levels at which selection could act, first on the level the whole fungus and second on the level of the genetically different nuclei.

However, it is equally important to understand the effects of genetic drift in AMF. Assuming that genetically different nuclei are coexisting in the hyphal network, these could become unevenly distributed by means of differential allocation or replication of nuclei thereby creating genetic heterogeneity. Spores that are formed at different parts of the hyphal network could thus contain representations of these different nuclear populations. Depending on the effective differences in functional genetic information among nuclei and depending on the differences in the composition of nuclei within spores there is a possibility for functional differences among spores. There is evidence for such a mechanism in a study of *Scutellospora pellucida*, where spores of a spherical or ellipsoid shape were used to initiate new cultures. Bever and Morton (1999) could show that this shape was heritable and the pattern of the frequencies of these shapes indicated the presence of more than one nuclear genotype. These could have been differentially allocated to the previously formed spores. In this thesis through comparison of plot populations with respect to genetic and phenotypic differentiation I could show that genetic differentiation was higher than phenotypic, indicating that differences are most likely not the result of pure drift and that selection is in operation.

Anastomosis could be an important mechanism to counteract the effects of selection and drift among nuclei. Via anastomosis within the hyphal network genetic homogeneity could be maintained (Giovannetti *et al*., 2001). If the isolates that have been studied in chapter 3 would have been interconnected by a hyhpal network then there cannot have been complete genetic homogeneity. One of the reasons could be that anastomoses have not been very frequent. In contrast to maintenance of genetic homogeneity anastomosis could also be thought of as a mechanism that is able to create genetic diversity in an AMF by fusion of isolates that can exchange genetically different nuclei. Recently Giovannetti *et al* (2003) performed an experiment to investigate anastomoses between geographically distant isolates but no fusion of hyphae could be observed. However, there are several explanations possible for this observation. For example it is unclear whether these isolates were cultivated in the correct environmental conditions to be able to fuse.

At this point it can be summarized that still only very little knowledge exists about how evolutionary forces could have acted and shaped the genome structure in AMF. The results obtained in this thesis suggest that considerable genetic differences exist on the level of gene sequences, nuclei and spores. But it is obvious from this study that new evolutionary models need to be developed in order to study the effects of mutation, selection and genetic drift in populations of AMF.

5.2. Ecological aspects

5.2.1. The ecological relevance of variation between AMF isolates

As mentioned in section 5.1. variants of *BiP* were shown to comprise differences in the amino acid sequence. The differences are therefore potentially of functional relevance. Additionally the variation could also be segregated among nuclei.

The genetic differences that have been observed between individuals in an AMF population suggest the possibility that the complete genetic information and diversity is partitioned among spores. AFLP is known to likely show polymorphisms of only neutral regions of DNA. But since I could show in chapter 4 that in AMF DNA substitutions are neither mainly restricted to non-coding DNA nor to synonymous sites it must be taken into account that observed differences

could also be the result of polymorphisms in functional gene sequences. Therefore it is possible that the observed differences partly include differences in functional genetic information between spores of the same species. In addition, because in chapter 3 maternal effects were eliminated when phenotypic traits were measured, the observed phenotypic differences between isolates, indeed, have a genetic basis. The amount of differences has previously only been described between AMF species and has been shown to explain significant differences in plant phosphate uptake (Jakobsen et al, 1992). Therefore the measured phenotypic differences in a population of *G. intraradices* indicate an ecologically relevant variation.

In addition it would then be highly important for research in AMF ecology to find out in future experiments by comparing intra- and interspecific differences what is the ecologically relevant level. The importance of these intraspecific differences has previously not been considered because comparative ecological experiments only rely on one AMF isolate per species. Because one isolate is usually started with one spore these studies have effectively compared one spore per species and there has been no replication of species. In this thesis I could also clearly demonstrate that one isolate showed less than half of the total amount of genetic variation present in a population. Given that there are also large phenotypic differences that have a genetic basis ecological studies aimed at understanding the functional significance of AMF should not ignore within species variation.

5.2.2 Studies of diversity in AMF field communities and populations

Analysis of AMF field communities relies on the development and availability of molecular markers that distinguish AMF at the ecologically relevant level (Clapp et al, 1999). Development of these markers has been attempted based on rDNA but has only had limited success because of high intraspecific genetic diversity. Due to this diversity it has been impossible to design primers that would amplify the great majority of rDNA sequences that are known for a given AMF species without amplifying sequences that belong to a different species. However, specific amplification of all variants present in one species is a prerequisite for analysis. In order to be able to do develop specific molecular markers it is necessary to know the total genetic diversity in an AMF species. Analysis of total genetic diversity has been performed in chapter 3 on the level of spores and in chapter 4 on the level of *BiP* sequences.

Genetic diversity as number of polymorphic bands has been assessed in relation to the number of sampled AMF spore isolates. Using this resampling procedure it has estimated that only an average of 7 spores of this field population is likely to contain at least 90% of the total genetic diversity. Doing a similar resampling procedure on the phenotypic level using the most conservative assumptions resulted in an average of at least 5 spores to cover 90% of the total observed phenotypic variation. Thus, in order to find molecular markers of an AMF species that have a high probability of being amplified, then the variation in a population comprising at least 7 isolates of this species originating from this field should be studied.

Variation in other genes was also high. The total number of isolated *BiP* variants was 31. However, because I sampled a representative population of sequences that is present within a single spore culture I was also able to resample this dataset with respect to the number of different sequences (variants) that could be obtained in relation to the total number of sequences sampled. The resulting curve indicated that there was limited number of *BiP* variants and based on an asymptotic curve function this could be deduced as being approximately 70. In contrast to the unknown limits of variation in rDNA the *BiP* gene seems to be a more promising candidate to serve as a marker for which specific primers could be developed. In order to achieve this goal it would now be important to repeat the analysis of diversity firstly with more isolates of *G. intraradices* but then also to analyze this gene in other AMF species to be able to identify the effective differences between species.

5.3. Consequences for studies of AMF gene function using molecular methods

Clearly the studied AMF *G. intraradices* comprises a remarkably high intraspecific genetic diversity of *BiP* variants. It is known from a comparative study of *BiP* in two divergent strains of the *ascomycete* fungus *Pneumocystis carinii* that infect two different hosts that sequence divergence of this gene is higher than compared to other gene sequences, such as *b-tubulin* and 18S nuclear rDNA (Stedman and Buck, 1996). The variation of *BiP* in *G. intraradices* has been observed within one individual and this has never been reported before. This seems even more surprising because *BiP* belongs to the *HSP* 70 family of genes which is known to

have diverged considerably among species (Gupta and Golding, 1993) but which is thought to belong to the most highly conserved proteins in nature (Hughes, 1993). Thus, in contrast to the current opinion in AMF research, genetic variation does not seem to be restricted to multicopy rDNA and this has many consequences for future studies on AMF genes.

AMF genes have been studied with the assumption that only copy is present in a given AMF species. If variation in gene sequences was thought to be present it would have been expected to be negligible and certainly not of functional relevance. That the variation in gene sequences might be functionally relevant is shown in chapter 3 and 4. Firstly, in chapter 3 Alexander M. Koch has shown clear proof for differential growth patterns among isolates of the AMF *G. intraradices*. This must have a functional relevance, and because environmental effects were removed, also a genetic basis. In chapter 4 I then clearly show that 50% of all substitutions in *BiP* are non-neutral leading to changes in the amino acid sequence. Thus, although I could not show any mechanistic link between the observed variation in growth patterns and variation in functional gene sequences I could show that both exist within *G. intraradices* and thus that the general assumption is wrong and that variation in gene sequences must be taken into account for research on AMF genes.

If translated into amino acid sequences two thirds of all *BiP* variants result in a functional but different protein sequence. Thus, within one individual there is the possibility of simultaneous expression of more than one variant. However, these could also be differentially expressed in space and time. So far the great majority of studies analyzing a gene function in AMF rely on methods that have been applied are based on only one isolated sequences per species. Depending on the method this might lead to some major shortcomings. For example hybridization techniques only have a limited specificity and if a probe is used then it is likely to also recognise sequences that diverge in the range of 5% from this probe. Thus, it is possible with a Northern blot experiment that during subsequent hybridization one probe binds to a population of variants. Whether observed changes of expression of a gene in such an experiment are the result of differential expression of one or several variants of this gene cannot be known. Also, if no changes were observed then it could simply have been that differential expression of a certain variant had been masked because overall expression of variants has not changed. This has never been accounted for. In chapter 4, I attempted with a similar approach as based on DNA to analyze potential genetic variation present on the

level of transcripts. But the chosen experimental conditions showed the presence of only one transcript. Thus it remains unknown whether AMF simultaneously express more than one variant at a time. This is clearly an area that needs further attention and more study.

Another example of how experiments could be affected by the presence of many sequence variants of a gene are in the use of Southern blotting and subsequent hybridization. Because of the multiple genomes in AMF presence of several bands would be expected even for single copy genes in an experiment. Therefore, it would not be possible to identify the number of copies in an AMF per genome because there are many genomes analyzed simultaneously. Thus, there is a high probability that those studies that have used these methods will have to be interpreted in another way. Multiple bands in Southern experiment have been interpreted as evidence for a gene family but it could also indicate the presence of multiple gene copies of the same family (e.g. Ulbalijoro *et al*., 2001).

Lastly, there are genetic differences not only within but also between isolates of one AMF species. Thus, there is a likelihood that variants of a gene are not homogeneously allocated among spores. This is not only a certain risk for ecological experiments but also for molecular experiments. In this case, comparison of only one isolate per species with regards to presence of absence of a certain gene might present a certain risk that only partial genomic information of each species is actually compared, thereby, leading to incomplete results. Thus, also for research in molecular biology, sampling strategies for AMF species should be reconsidered in the sense that either more than one isolate should be analyzed per species or more spores should be used per isolate from a natural population to initiate an inoculum.

5.4. Conclusions and future perspectives

This thesis shows the existence of high intraspecific genetic variation in AMF in the *BiP* gene and within and among isolates of a population of *G. intraradices*. These previously unknown sources of genetic variation increase the level of complexity of how evolutionary mechanisms are potentially acting on AMF populations and thereby drive AMF evolution. As no other organism has been shown to contain multiple genomes AMF present a completely different and obviously successful evolutionary strategy to survive. However, this also means

that completely new models must be developed to derive testable hypotheses and this should be of great interest for evolutionary biologists.

The molecular approaches (AFLP, analysis of gene diversity) that have been successfully established and taken to characterize the AMF *G. intraradices* are useful tools to extend these studies to other AMF species but also to reanalyze previously reported differences between species. This should be done in a twofold sense, with respect to consequences in ongoing research on AMF ecology and with respect to gene function in AMF. Based on the results of this thesis, changes in sampling practice are likely to improve AMF research in these areas.

The genetic markers that have been isolated in this thesis should be highly useful in future experiments to test such hypotheses as whether AMF individuals might only be different with regards to frequency of genetic markers and should thus also be useful to detect the effects of drift and selection that must have acted to shape the AMF genome.

Abstract / Résumé

Abstract

Arbuscular mycorrhizal fungi (AMF) are root symbionts with about 80% of all known species of vascular land plants. AMF are ecologically important because of the benefits that they confer to plants. Molecular studies on AMF showed that rDNA sequences were highly variable between species and within species. Because AMF are coenocytic and multinucleate there are several possibilities how this intraspecific genetic variation could be organized. Therefore, the organization and evolution of this variation in AMF were investigated in the present work.

Based on fossil records the AMF symbiosis has existed for 450 Million years and is therefore considered ancient. First molecular data indicated no evident sexual reproduction and gave rise to the hypothesis that AMF might be ancient asexuals. The first part of this thesis (Chapter 2) shows evidence for recombination in different AMF but also indicates that it has not been frequent enough to purge accumulated mutations. Given asexual reproduction, it has been predicted that the many nuclei in AMF should diverge leading to genetically different nuclei. This hypothesis has been confirmed by an experiment of M. Hijri and is also included in chapter 2 as the results were published together.

In chapter 3 I then investigated whether intraspecific genetic variation also exists in a field population of the AMF *Glomus intraradices*. Comparing genetic fingerprints of individuals derived from single spores I could clearly show that large genetic differences exist. A similar result, based on quantitative genetic traits, was found for the same population by A. Koch. The two studies taken together show that the genetic variation observed in the population is high enough to be of ecological relevance.

Lastly, in chapter 4, I investigated within individual genetic variation among *BiP* gene sequences. It is the first study that has analyzed genetic diversity in the AMF genome in a region of DNA other than rDNA. I found 31 sequence variants of the *BiP* gene in one *G. intraradices* isolate that originated from one spore. Genetic variation was not only restricted to selectively neutral parts of *BiP*. A high number of predicted non-functional variants compared to a likely low number of copies per nucleus indicated that functional genetic information might even be partitioned among nuclei.

The results of this work contribute to our understanding of potential evolutionary strategies of ancient asexuals, they also suggest that genetic differences in a population might be ecologically relevant and they show that this variation even occurs in functional regions of the AMF genome.

Résumé

Les Champignons Endomycorhiziens Arbusculaires (CEA) forment une symbiose racinaire avec environ 80% des espèces connues de plantes vasculaires. Ils occupent une position écologique très importante liée aux bénéfices qu'ils confèrent aux plantes. Des études moléculaires effectuées sur des gènes ribosomaux ont révélé un très grand polymorphisme, tant à l'intérieur des espèces qu'entre celles-ci. Ces champignons étant coenocytiques et multinucléés, l'organisation de cette variabilité génétique intraspécifique pourrait avoir différentes origines. Ce travail se propose d'examiner l'organisation et l'évolution de cette variabilité.

Sur la base de fossiles, l'existence des CEA remonte à au moins 450 millions d'années. Cette symbiose peut donc être considérée comme ancienne. Les premières données moléculaires n'indiquant pas de reproduction sexuée, une hypothèse fut élaborée stipulant que les CEA seraient des asexués ancestraux. La première partie de cette thèse (chapitre 2) met en évidence l'existence de recombinaison dans différents CEA mais montre également que celle-ci est insuffisante pour purger les mutations accumulées. La reproduction étant essentiellement asexuée, on peut prédire que les nombreux noyaux ont probablement divergé génétiquement. En collaboration avec M. Hijri nous avons pu vérifier cette hypothèse (chapitre 2).

Dans le chapitre 3 j'ai cherché à comprendre si le polymorphisme était également présent dans une population naturelle du CEA *Glomus intraradices* au niveau intraspécifique, ce qui n'avait encore jamais été examiné. En comparant les empreintes génétiques d'individus obtenus chacun à partir d'une spore mise en culture, j'ai clairement démontré que d'importantes différences génétiques existent entre ceux-ci. Un résultat similaire, portant sur des traits quantitatifs d'individus de la même population, a été trouvé par A. Koch. Les deux études en ensemble montre que le polymorphisme génétique dans cette population est suffisamment grand pour être important au niveau écologique.

Dans le chapitre 4, j'ai cherché a examiner le polymorphisme des séquences du gène *BiP* au sein d'un individu. C'est la première étude qui examine la diversité génétique du génome de CEA avec un autre marqueur que l'ADN ribosomique. J'ai trouvé 31 types de séquences différentes du gène *BiP* issu d'un isolat de *G. intraradices* mis en culture à partir d'une seule spore. Cette variation n'était pas restreinte à des zones sélectivement neutres du *BiP*. Mes résultats montrent qu'il y a un grand nombre de variants non-fonctionnels, proportionnellement au faible nombre de copies attendues par noyau. Ceci va dans le sens d'une partition de l'information génétique entre les noyaux.

Les résultats de ce travail contribuent à la compréhension de stratégies évolutives potentielles des asexués ancestraux. Ils suggèrent également que le polymorphisme génétique au sein d'une population est probablement écologiquement importante et que celui-ci se situe également dans des régions non codantes du génome des CEA.

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Curriculum Vitae

Personal Background

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Educational Background

School Education

1980 - 1984	Grundschule Fuldatal Simmershausen
1984 - 1993	Friedrichsgymnasium Kassel
”Matura”	Biology, Latin, Sports, Social studies

University Education

1993 - 1995	Georg-August-Universität Göttingen, biological studies
Vordiplom subjects	Genetics, Botany, Chemistry, Physics
1995 - 1998	Philipps-Universität Marburg, biological studies

Diploma, second level of university studies

Diplom subjects	Microbiology, Genetics, Photobiology, Immunobiology
1998	Diploma Thesis at Max Planck Institute for terrestrial Microbiology in Marburg
Diploma Title:	“Molekulare Analyse der präsymbiontischen Entwicklungsphase arbuskulärer Mykorrhizapilze”

Graduate studies

since 1999	Graduate studies (troisième cycle) under the supervision of Prof. Ian R. Sanders.
1999 – 2000	PhD thesis started at the University of Basel and
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Thesis Title:	“Organisation of genetic variation in multinucleate arbuscular mycorrhizal fungi”

Publications

Gerrit Kuhn, Alexander Koch, Pierre Fontanillas, Jérôme Goudet, Ian R. Sanders. A multigenomic mycorrhizal population is locally diverse and spatially structured, *PNAS* (submitted)

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Presentations at conferences

Joint meeting of the Cost Action on Mycorrhiza, the E.U. project MYCOREM and the DFG priority program MOLMYK, Cologne, Germany 23.08. – 25.08.02 Oral presentation: Implications and consequences of multiple genomes in AM fungi for studies of functional genes and their ecological role. **Gerrit Kuhn,** Alexander Koch, Mohamed Hijri, Ian R. Sanders.

ICOM 3 (Third international congress on mycorrhiza), Adelaide, Australia, 08.07. - 13.07.01 Poster presentation: Infrequent recombination in the supposedly asexual AM fungi. **Gerrit Kuhn**, Mohamed Hijri, Ian R. Sanders

Evolution 2000, Bloomington, Indiana, USA, 23.06. - 27.06.00. Poster presentation: Is there hidden recombination in arbuscular mycorrhizal fungi? **Gerrit Kuhn,** Mohamed Hijri, Ian R. Sanders.

VAAM (Vereinigung für allgemeine und angewandte Mikrobiologie) Jahrestagung in Göttingen, Germany, 07.03. - 10.03.99. Poster presentation: Molecular analysis of the influence of salicylic acid on the growth of *Gigaspora rosea*. **Gerrit Kuhn**, Ana Menendez, Natalia Requena and Philipp Franken.