High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi

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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. Although AMF are clonal, they have evolved to contain a surprisingly high within-species genetic variability, and genetically different nuclei can coexist within individual spores. These traits 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 Glomus 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 two individual spores, <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, which might be because of the multigenomic nature of AMF, where considerable genetic redundancy could buffer the effects of changes in the genetic content of phenotypic traits. These results have direct implications for ecological research and for studying AMF genes, improving commercial AMF inoculum, and understanding evolutionary mechanisms in multigenomic organisms.

Arbuscular mycorrhizal fungi (AMF) are widely distributed and extremely successful symbionts that colonize the roots of up to 80% of terrestrial plant species (1). They are known to improve plant growth by increasing phosphate uptake (2), and species diversity of AMF has been shown to increase plant species diversity and productivity (3). 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 (3–5). Therefore, there was no replication of the species. So far, to our knowledge there have been no studies combining molecular genetic variation with variation in quantitative genetic traits (QGTs) in an AMF field population. This lack of data is surprising, given that a remarkably high amount of genetic variability exists in an AMF species and within individual spores (6–10). AMF are coenocytic, with many nuclei coexisting in a common cytoplasm, and one species of AMF has recently been shown to be multigenomic, harboring genetically different nuclei (11).

It has already been shown that single-spore isolates of an AMF species of different geographical origin differentially affect plant growth and physiology (12–14). However, in two of those studies, the isolates were not kept for several generations under identical conditions; therefore, observed differences could also be due to environmental differences. Furthermore, only one 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 (15), 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 color indicate that heritable variation exists within AMF populations (16, 17). Although AMF grow clonally by either random or nonrandom processes, the nuclei could become unevenly distributed in the hyphal network or during spore formation, which would create both genetic and possibly phenotypic heterogeneity. Uneven distribution of nuclei could also cause a spatial structure to form among different parts of the population, a possibility that has been described as a particular type of drift (15). The third possibility, and not necessarily exclusive of the second, is that selection acts locally on nuclear genotypes because of environmental heterogeneity, thereby also creating genetic variation (15).

There have been several attempts to use molecular techniques to demonstrate qualitative genetic differences between isolates within a population by picking out different sequences of rRNA-encoding DNA (rDNA) from spores (6, 18). However, this approach is complicated 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 (19), 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. Despite this probability, variation among AMF spores within a population seems likely at the rDNA level (20). Furthermore, no studies have successfully quantified genetic differences among AMF isolates by using a large number of neutral markers, although these data are essential for knowing to what extent isolates actually differ from each other genetically.

The reason 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 (15). Obtaining such a set of AMF individuals requires successfully producing AMF spores from soil samples by using trap cultures, setting up single-spore cultures, and transferring newly produced spores into sterile laboratory cultures, with subsequent propagation in laboratory conditions (Fig. 1). Because of environmental heterogeneity in the field, each isolate has to be maintained for several generations in identical conditions to remove maternal effects, an important precaution for quantitative genetic studies, because variation in traits may otherwise be due to environment rather than genotype. With subsequent replication of isolates, the
preparation of material for an experiment would take $\approx$3 years (Fig. 1).

Whether genetic and phenotypic variation exists and to what extent it exists in AMF populations 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.

Our study aims to quantify and compare both neutral genetic variation and variation in the QGTs 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 in this work as a clonal culture of an AMF that was started from one spore (single-spore culture); 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. Because of the lack of neutral genetic markers at either the spore or the nuclear level, amplified fragment length polymorphism (AFLP) fingerprinting was chosen for genetic characterization. Because of low amounts of DNA, AFLP of DNA from single spores can suffer from artifacts that cannot be quantified because of the lack of replication (11, 21). Our study allowed the clonal growth of large amounts of hyphae and spores from each isolate, which yielded large amounts of high-quality DNA from each individual, thus negating these problems (22) and allowing tests of AFLP reliability with the possibility of replication to quantify the potential AFLP artifacts. Our study of an AMF population combines 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 was started in 1987 (23). The field site of a size of $90 \times 110$ m 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 Table 2 and Fig. 6, which are published as supporting information on the PNAS web site). Each plot was $6 \times 19$ m.

Isolation and Cultivation of a G. intraradices Population. The G. intraradices isolates were obtained from the field site from each of the four plots. Single-spore isolates were then maintained in identical environments (Fig. 1) (24). Four single-spore isolates were taken from each of the four plots and put into culture with Root morph-inducing plasmid T-DNA-transformed carrot roots. The isolates were transferred two more times in an identical environment. Sixteen replicate plates were inoculated with each isolate with an addition of another 16 two-compartment plates for genetic analysis. AFLP fingerprint analysis of genomic DNA allowed measurement of genetic variation.

Experimental Design. The aim of the experiment was to measure genetic and phenotypic variation among isolates, among plots, and between treatments (tillage or no tillage). Because of cultivation in identical environmental conditions for a long time, differences in the observed phenotype are assumed to have a genetic basis and are, therefore, QGTs. The isolates were cultured by transfer from each of four replicate plates of each of the 16 isolates. Material from each replicate was then transferred onto four new plates. Therefore, there were 16 plates per isolate, resulting in a total of 256 plates (Fig. 1). Contaminated plates or those with no root growth were excluded from analysis, which 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 QGTs (Fig. 1). In addition, a G. intraradices
isolate from Canada was used as an outgroup. This isolate (DAOM 181 602) was obtained from G. Bécard (University Paul Sabatier, Toulouse, France). Two-compartment plates allowed proliferation of AMF on one-half of the plate (22). Roots that directed their growth to the fungal side were cut to avoid contamination with plant material. After 15 weeks, the fungal compartment of all plates from each isolate was removed and pooled for extraction of hyphae and spores (26). This compartment was refilled with medium to allow the growth of more fungal material for a second extraction (27).

Measurement of Phenotypes. We measured hyphal growth rate and spore production because they can be related to life history traits and tillage treatments (28, 29). Over a growth period of 15 weeks, the number of spores per cm² of medium and length of hyphae per cm² of medium were recorded every 3 weeks. The measurements allowed calculations of the maximum rate of spore production and the maximum rate of hyphal growth. The ratio of spore number to hyphal length was also calculated. A description of the measurement procedure can be found in Supporting Text.

Measurement of Genotypes. Fresh hyphae and spores were taken for extraction of DNA by using the DNeasy plant mini kit (Qiagen). Extraction of 10 isolates gave enough DNA for AFLP analysis with each of 10 different primer pairs. Further details of AFLP can be found in Supporting Text.

Analysis of Phenotypic Variation and Population Diversity. Variation in QGTs among the 16 Swiss isolates was analyzed with the following 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 was performed on the variables with the following main factors: treatment (four levels, df 1), plot nested in treatment (four 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 (30) (see Table 3, which is published as supporting information on the PNAS web site).

Partitioning of variance of polygenic traits (Qa) for all pairwise combinations of the four plots was calculated for all variables separately and as a combination of all five variables. For comparison with genetic variation, the calculations were 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 subpopulation. The following variance components were calculated for all pairs of plots: plot (Vₚ), isolate within plots (Vᵢ), and the residual error (Vₑ). From these, the Qa values were calculated according to the formula \( Q_a = V_p/(V_p + V_i + 2V_e) \) modified from ref. 31 (see Table 4, which is published as supporting information on the PNAS web site).

Variation in QGTs was analyzed to estimate the relationship between the number of isolates and the phenotypic diversity. Calculation of phenotypic diversity is described in the Supporting Text. From the relationship between the number of isolates and diversity, we estimated how many isolates comprised 90% of the total observed phenotypic diversity.

Analysis of Genetic Variation. Ten isolates from the Swiss population were taken for analysis by using the binary data derived from AFLP with the 10 primer pairs. Analysis of molecular variance (AMOVA) was performed with the software ARLEQUIN (32) 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 two replicate DNA extractions of each isolate. Genetic variation among isolates (Vᵢ) and between extractions of each isolate (Vₑ) was compared. In a second AMOVA, the plot structure was used as a factor with four levels, where isolates originating from the same plot were the replicates of the plots. The amount of genetic variation explained within (Vᵢ) and among (Vₑ) the plots was used to examine whether a spatial genetic structure existed in the AMF population (for results of both AMOVAs see Tables 5 and 6, which are published as supporting information on the PNAS web site). The variance components for the plot structure (Vₑ, Vᵢ) were used to calculate genetic variation (Fₛ) values according to the formula \( Fₛ = (Vₑ)/Vₑ + Vᵢ \) (31) (for Fₛ values, see Table 7, which is published as supporting information on the PNAS web site). 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 treatment and taken as replicates in a third AMOVA with two levels to test whether there were genetic differences because of 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 by using PAUP 4.0 BETA 10 with a heuristic search procedure, which uses 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 (1,000 replicates) (33).

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 can be found in Supporting Text. The test was repeated, including the isolate from Canada, with random sampling of up to 11 isolates.

Relationship Between Phenotypic Variation and Genetic Variation. The combined binary datasets of 10 primer pairs were used, and Fₛ values between all pairs of plots was recalculated based on the plot structure of the second AMOVA. Plot Fₛ values were plotted against plot Qa values for all five variables separately and for a Qa within where all five variables were combined. This pairwise comparison of the amount of Qa with the amount of Fₛ allowed us to test the null hypothesis that a given trait evolves by genetic drift, in which case Fₛ will be equal to Qa (34). Presence of selection on either the genetic or phenotypic level is expected to lead to deviation from this assumption.

Results

Variance Analyses (ANOVA and AMOVA). Isolates differed significantly in their final hyphal length and spore number (Figs. 2 A and B). The hyphal length ranged from 10 cm² to 52 cm² and spore number ranged from 5 spores per cm² to 75 spores per cm². 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 (Fig. 2 C, D, and E). 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 five variables.

Genetic differences among isolates were large, and this was true for all primer pairs (Table 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 1). AMOVA with plot as factor showed that 68.3% of the total variation was explained by differences among the four plots; whereas 31.7% was explained by differences within the plots (Table 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 three
monophyletic groups that were well supported with bootstrap values (100%, 100%, and 100%) (Fig. 3A). The analysis was repeated with the Canadian isolate, which did not greatly change the bootstrap values (100%, 100%, and 98%) or the position of the other isolates in the tree (Fig. 3B). One group was represented by isolate C2. The second and third groups included isolates B1, B3, D1, D2, D3, and A1, A2, C3, C4, respectively (Fig. 3). The presence of three 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 amount of diversity in the population (Fig. 4A). Two isolates chosen at random accounted for 50% of the total observed genetic variation. After the selection of seven or more isolates at random, the curve leveled off and accounted on average for 90% of the total observed variation. Thus, adding more isolates after this point did not greatly

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<th>Table 1. Results of two AMOVAAs</th>
<th>Isolates, %</th>
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<td>Eco</td>
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<td>AGG</td>
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<td>AGG</td>
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<td>AA</td>
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<td>Mean</td>
<td>94.2</td>
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Shown are the percentage of variation for within-isolate variation (between DNA extractions of the same isolate) and among-isolate variation and 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*.
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 <50% of the total observed phenotypic diversity, and this was true for all models of phenotypic diversity. By using the most conservative assumptions of the model (two classes), the asymptotic curve started leveling off after six or more isolates were selected at random, and the curve accounted on average for >90% of the observed total phenotypic diversity (Fig. 4B). Again, adding more isolates did not greatly increase the total observed variation. Increasing the number of classes in the model (three or four) shifted the point where the curve started leveling off to 10 or more isolates.

Comparison of $Q_{st}$ to $F_{st}$. $Q_{st}$ values of each pair of plots were compared to the corresponding $F_{st}$ values, giving six data points representing each possible combination. $F_{st}$ was generally $>$ $Q_{st}$ for all five variables (Fig. 5A–E). This pattern was also true for the relationship between the combined values of phenotypic differentiation and genetic differentiation (Fig. 5F).

Discussion

Differences Between AMF Individuals. In this study, we have shown that large genetic differences exist between individuals in an AMF population in an area of 90 m x 110 m. 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 (35). 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 ~150 morphologically distinct taxa in 400 million years of terrestrial evolution (36, 37).

The variation that we show could either be created by drift, as described by ref. 15, or by selection because of 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 (31). 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 (31, 34). Our data are in contrast to these studies and, therefore, suggest that there could be selection on similar QGTs in the presence of great genetic diversity. Given that 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 se-
quences 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. These minor changes could, however, lead to considerable changes in the genetic content. The observed variation in QGTs 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 that there could be a spatial genetic structure within the population. This means that AMF from different parts of a 90- × 110-m field are likely to be genetically different and that genetic differences are not randomly distributed. Because of 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 three major groups within the population. Evidently, a link between the patterns of variation in QGTs and the phylogenetic structure in the population could exist, given that one monophyletic group was found to comprise the isolates that exhibited the highest hyphal growth. Indeed, a Mantel test comparing genetic relatedness 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 level but also on a functional level.

Anastomosis, the fusion of hyphae, has been shown in AMF (38), allowing possible exchange of nuclei within the hyphal network, which should reduce the effects of drift and selection by allowing remixing of nuclear genotypes after their distribution has been rendered heterogeneous. However, to date, experimental studies have only revealed anastomoses between hpysa of spores coming from the same isolate (38, 39) and not between isolates of different geographical origin (40). 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 <50% of the diversity in the population. Despite the large genetic differences among isolates found within one field, the Canadian isolate was not genetically distant, and, phylogenetically, it fitted into one of the main branches of the population, which 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 finding, however, is in contrast to the findings that geographically distant isolates do not anastomose (40).

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. 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 (11), genetic differences in a population might lead to segregation of alleles of genes among individual spores, which could lead to the 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.

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