Applied and Environmental Microbiology, June 2010, p. 3765–3775 Vol. 76, No. 12
0099-2240/10/$12.00 doi:10.1128/AEM.02951-09

TaqMan Real-Time PCR Assays To Assess Arbuscular Mycorrhizal Responses to Field Manipulation of Grassland Biodiversity: Effects of Soil Characteristics, Plant Species Richness, and Functional Traits▽†

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Received 7 December 2009/Accepted 7 April 2010

Large-scale (temporal and/or spatial) molecular investigations of the diversity and distribution of arbuscular mycorrhizal fungi (AMF) require considerable sampling efforts and high-throughput analysis. To facilitate such efforts, we have developed a TaqMan real-time PCR assay to detect and identify AMF in environmental samples. First, we screened the diversity in clone libraries, generated by nested PCR, of the nuclear ribosomal DNA internal transcribed spacer (ITS) of AMF in environmental samples. We then generated probes and forward primers based on the detected sequences, enabling AMF sequence type-specific detection in TaqMan multiplex real-time PCR assays. In comparisons to conventional clone library screening and Sanger sequencing, the TaqMan assay approach provided similar accuracy but higher sensitivity with cost and time savings. The TaqMan assays were applied to analyze the AMF community composition within plots of a large-scale plant biodiversity manipulation experiment, the Jena Experiment, primarily designed to investigate the interactive effects of plant biodiversity on element cycling and trophic interactions. The results show that environmental variables hierarchically shape AMF communities and that the sequence type spectrum is strongly affected by previous land use and disturbance, which appears to favor disturbance-tolerant members of the genus *Glomus*. The AMF species richness of disturbance-associated communities can be largely explained by richness of plant species and plant functional groups, while plant productivity and soil parameters appear to have only weak effects on the AMF community.

Arbuscular mycorrhizae are mutualistic associations between roots of plants and fungi that have been present for more than 400 million years (54). Approximately 80% of examined land plants (71), and almost all fungi of the phylum Glomeromycota (60), are capable of forming such associations. The main benefit of this relationship for plants is that it facilitates their acquisition of nutrients (especially P and N), while the fungus receives photoassimilates (7, 62). About 200 Glomeromycota species have been described to date, based on spore morphology (http://www.lrz-muenchen.de/~schuessler/amphylo/amphylogeny.html), but there is increasing molecular evidence of significantly higher diversity in arbuscular mycorrhizal fungi (AMF) (10, 72).

Diverse AMF communities have been detected in a wide range of plant communities (*inter alia* grasslands, boreal forests, and tropical communities; for an overview, see reference 48). Hence, AMF have been considered to be tolerant of wide ranges of ecological conditions and capable of associating with diverse plant partners. Identifying the factors regulating their community assemblages is challenging, but AMF community composition has been shown to be influenced by plant species diversity (e.g., see references 10, 22, and 33), and conversely, significant effects of AMF species and communities on the diversity and productivity of plant communities have been described (25, 68). Soil physicochemical parameters like phosphorus, nitrogen, and carbon availability (e.g., see references 4, 9, and 31); pH (17); moisture content (53); and disturbance (30) also reportedly influence AMF distribution. Hence, there is some support for niche theory, which presumes that two species of the same trophic level cannot coexist in a limited system and, if two species are present in such circumstances, one should become extinct (21). As a corollary, two cooccurring species must occupy niches that differ in some dimensions, e.g., plant hosts and/or soil properties (28). However, there are also indications that neutral ecological processes, as well as niche-defining parameters, may influence AMF diversity and community composition (17, 39). In contrast to niche theory, neutral theory (27) postulates that all individuals of every species at a given trophic level in a food web have ecological equivalence, and thus, all species within trophically defined communities can be regarded as open nonequilibrium assemblages that are solely shaped by dispersal and distinctions in spatiotemporal dimensions. According to the work of Hubbell (27), neutrality is defined at the level of individual organisms with identical probabilities of birth, death, migration, and speciation and not at the species level. In order to explore AMF

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 23 April 2010.

communities more thoroughly and to test competing hypotheses, such as those raised by the niche and neutral theories, robust methods for high-throughput analyses of the communities are required.

Recent investigations of variables that affect the structure of AMF communities have considered relationships between niche-defining dimensions, such as soil types (39) and pH gradients (17), and spatial variations in AMF community structure but not the role of plant diversity or functional traits of host plants. There have been several plant diversity manipulation experiments designed for coanalyzing multiple sets of ecological variables (e.g., the BIODEPTH and Cedar Creek projects) that would have been ideal for detailed examinations of effects of ecological variables on AMF, but previously reported AMF analyses in these experiments have been limited to counts of spores in a single study (11). However, not all AMF species regularly sporulate, and when present, spores poorly reflect AMF diversity (69), since active AMF occur as mycelia in roots and soils (e.g., see references 12 and 26). PCR-based molecular techniques enable much more rigorous characterization of AMF communities in these compartments (e.g., see references 26, 36, and 72), but assessments of broad spatial (42) and/or temporal (52) variations in AMF communities require analysis of large numbers of samples, which is not feasible using conventional PCR amplification followed by cloning and sequencing. This challenge can be potentially met by real-time PCR-based approaches, in which the AMF sequence types present in compartments of interest are first identified and then sequence type-specific probes are used for large-scale screening in real-time PCR TaqMan assays.

In the study presented here, we explored AMF diversity in plots used in the Jena Experiment, a grassland plant diversity manipulation of 60 plant species representing four functional groups in 81 plots of 400 m^2 (56). The overall AMF diversity and community structure were first assessed by PCR amplification, cloning, and sequencing (55) of internal transcribed spacer (ITS) ribosomal DNA (rDNA) gene sequences in soil samples from 23 representative plots. Using the acquired data, we then developed sequence type-specific probes, which were applied in high-throughput real-time PCR TaqMan assays of samples from all 81 experimental plots, and the effects of 15 plant and soil variables on the AMF community assemblage were investigated.

MATERIALS AND METHODS

Study site. The study was carried out as part of the Jena Experiment, designed to study interactions between plant biodiversity and ecosystem processes in plots established in May 2002 on the floodplain of the Saale River, 130 m above sea level near Jena (11°37′27″E, 50°57′4″N, Thuringia, Germany), on a former arable field site. Plant communities were established with a series of species richnesses (0, 1, 2, 4, 8, 16, and 60 species) in a randomized block design with 81 plots, each 20 × 20 m in size, as follows. The experimental site was systematically divided into four blocks, each containing four plots with 1, 2, 4, and 8 species mixtures, three to four plots with 16 species mixtures, one plot with bare ground, and one (in all except one block) with 60 species. Thus, in total, in the 81 plots there were 15 plant monocultures, 15 two-species compositions, 15 four-species compositions, 17 eight-species compositions, 12 16-species compositions, three 60-species compositions, and four bare-ground plots with no sown species. The plant species were divided into four functional groups (grasses, legumes, and small and tall herbs) based on architecture, phenology, and N fixation. Experimental plots were biannually weeded to maintain the target communities (see references 56 and 57).

Soil sampling. To assess the diversity of AMF in the 81 plots, three soil cores from the first 20 cm of topsoil were sampled in May 2007 using a soil column cylinder auger (Eijkelkamp, Giesbeek, Netherlands) from a 1.5-m-square sector of each plot. The samples were immediately sieved through 2-mm sieves, transported to the laboratory on ice, and stored at −20°C.

Soil chemical analysis. Plant-available phosphorus was extracted and found to range, following the protocol of Olsen et al. (46), from 0.01 to 0.13 mg g (dry weight) $soil^{-1}$. Soil moisture was measured by weighing, drying (105°C overnight), and reweighing 5-g portions of the samples and was found to range from 0 to 16.94%. Inorganic carbon (0.47 to 3.79%), organic carbon (1.34 to 2.91%), and nitrogen (0.14 to 0.26%) were determined in soil samples from all experimental plots in May 2006 (63). Soil pH in H_2O ranged between 7.02 and 7.51, reflecting the carbonate-buffered soil at the Jena Experiment site.

Plant species richness and weeding disturbance. During the Jena Experiment nontarget plant species ("weeds") within target communities were weeded out by hand during biannual weeding campaigns early in the growing season, and aboveground plant biomass was harvested annually during peak standing biomass, by mowing, as described in reference 40. In the part of the study reported here, disturbance by management was estimated in April 2007, before the soil samples were collected, as the number of hours needed to weed out nontarget species (0 to 345 h per plot), and the aboveground plant biomass was harvested in May.

DNA extraction, nested PCR, cloning, restriction fragment length polymorphism (RFLP) typing, and sequencing. Prior to DNA extraction, to remove aromatic hydrocarbons (often encountered as residues of herbicides), extracellular DNA, and other organic compounds, a 5-g portion of each soil sample was washed with a solution containing 50 mM Tris-HCl, 0.005% (vol/vol) Triton X-100, and 200 mM NaCl (pH 8.2) and then twice with a Triton-free but otherwise identical solution. The subsamples were then homogenized in 10 ml of the Triton-containing solution by vortex mixing using an MS 2 Minishaker (IKA, Staufen, Germany) at maximum speed for 2 min and centrifuged at 3,000 × *g* for 3 min, and the supernatant was discarded. The second and third washing steps were then repeated (for details, see the protocol described by Fortin et al. [19]). Genomic DNA was extracted from ca.-0.7-g portions of the washed soil samples by using a FastDNA Spin kit for soil (MP Biomedicals/Q-BIOgene, Heidelberg, Germany) following the manufacturer's protocol, except that an additional DNA extract cleaning step using 5.5 M guanidine thiocyanate was included to remove phenolic compounds that could inhibit *Taq* polymerases and quench nuclease enzymatic activities (51). The resulting DNA concentrations, measured spectrophotometrically using a NanoDrop 8000 V1.1 spectrometer (Thermo Fisher Scientific., Wilmington, DE), were 8 to 20 ng DNA per μl.

PCR amplification, cloning, and sequencing. To establish a reference DNA sequence library, the AMF diversity was initially analyzed in a subset of 23 plots, including four monoculture plots (one per plant functional group), four four-species plots, four eight-species plots, eight 16-species plots, and three 60-species plots. The DNA extracts (undiluted and at both 10-fold and 100-fold dilutions) were then used as template in nested PCR to amplify the internal transcribed spacer (ITS) sequence of the nuclear ribosomal DNA, as described in the work of Renker et al. (55). The primer pair LSU-Glom1/SSU-Glom1, used in the first amplification, is Glomeromycota specific, and the amplicons obtained using these primers are here referred to as "first PCR products." The primer pair ITS5/ITS4, used in the second amplification, is universal, and its products are here referred to as "nested PCR products." For a detailed description of clone library screening and phylogenetic analysis, see text S1 in the supplemental material.

Real-time TaqMan PCR probe design. Separate alignments of each AMF sequence type were used to identify convenient target regions representing at least 80% of the sequences in each sequence type in designing TaqMan probes. The probes met the following criteria: (i) a 5- to 10°C-higher melting temperature than that of the associated primer pair; (ii) a low tendency to form hairpin and/or looped secondary structures, defined as ΔG values (*m*-fold) of <1.5 (73); and (iii) no cytosine or guanine at the 5′ end. The resulting target regions were virtually adjusted for sequence type specificity in comparison to the remaining sequences using Sequencher version 4.8 (Gene Codes Corporation, Ann Arbor, MI). To minimize costs, in a first step of the TaqMan probe generation, we used sequence type-specific parts (18 to 22 bp long) of every probe as nonlabeled forward primers in cross-reactions with single clone reference templates (referring to each of the sequence types). The general eukaryotic primer ITS4 (28S rDNA) served as the reverse primer in all of this conventional PCR (using the PCR conditions described above). AMF sequence type-specific probes (29 to 33 bp long to ensure that they had a melting temperature between 59 and 70°C, with ΔG values of −2 to +1.3) were designed and also assessed in cross-reference tests using the general ITS3 and ITS4 primers. The resulting fragments of

amplified rDNA varied between 200 and 400 bp in length, an appropriate range for real-time PCR-based TaqMan assays (24).

Further cross-amplification tests were conducted to optimize the annealing temperatures and concentrations of TaqMan probes and primers (which were varied in ranges of 53 to 63°C, 10 to 800 nm, and 400 nM to 20 μM, respectively) and dyes for probe labeling to be used in the real-time TaqMan PCR assays. The optimal annealing temperature and TaqMan probe and forward and reverse primer concentrations were found to be 56.9°C, 800 nM, 10 μM, and 20 μM, respectively. Fluorescent dyes with nonoverlapping emission spectra were chosen in accordance with the *Real-Time PCR Applications Guide* (Bio-Rad, Munich, Germany) (see Table S1a in the supplemental material). To assess the detection accuracy of the multiplex real-time TaqMan PCR assays for underrepresented targets, we conducted further cross-amplification tests using a set of reference DNA mixtures that contained vector DNA of each AMF sequence type in a "cross-dilution series" of 10^1- to 10^6-fold dilutions in relation to the others.

Further, to assess the potential of the TaqMan assays to reveal AMF types not detected by nested PCR, two additional probes were designed for sequence types that were not detected at the Jena Experiment site but have previously been found elsewhere in central Germany (see footnote 1 in Table S1a in the supplemental material). Due to the lack of reference DNA material from the Jena Experiment site, these two probes were generated from sequence information obtained from analyses of samples from a meadow in the Thüringer Schiefergebirge (Thuringia, Germany) published by Hempel et al. (26) (*Gigaspora* sp. [DQ400249] and *Acaulospora* sp. [DQ400133]), but the linearity of their amplifications has not yet been tested.

Performance of TaqMan assays. Real-time TaqMan PCR assays were carried out using a Bio-Rad IQ5 real-time PCR detection system (Bio-Rad Laboratories, Munich, Germany), starting with an initial denaturation step coupled with HotStar *Taq* activation at 95°C for 15 min followed by 45 cycles of 94°C for 1 min and 56.9°C for 1.5 min using a QuantiTect Multiplex PCR NoROX kit according to the manufacturer's (Qiagen, Hilden, Germany) recommendations. To enhance the throughput, TaqMan probes were arranged in 3- to 5-fold multiplex PCR batches according to phylogenetic relationships, shared semispecific forward primers, minimal tendencies to form heterodimers, and similarity of melting temperatures, as presented in Table S1a in the supplemental material. First, all AMF sequence type-specific probes were successfully tested in single-plex TaqMan assays for their specificity in cross-amplifications with various linear dilutions of single reference clone DNA. No signals were detected from either the soil DNA extracts or the first PCR products of the nested PCR when the general ITS3/ITS4 fungal primers were used, due to predominant coamplification of non-AMF taxa, predominantly *Cryptococcus aerius* (AF444376), *Cryptococcus podzolicus* (AJ581036), *Cryptococcus rajasthanensis* (AM262981) and *Trechispora alnicola* (DQ411529). Consequently, forward primers specific for up to five related sequence types (*Glomus* sp. sequence type 1 [ST1] to ST5) were designed to avoid interference by non-AMF taxa (see Table S1a). In tests using SYBR green and TaqMan real-time PCR reagents, including probes, and conditions, these primers successfully amplified targets from both single template clone DNA references and mixtures including non-AMF sequences.

Preanalysis of oligonucleotides showed that three pairs of probes and primers used in the multiplex approach (see Table S1a in the supplemental material) formed heterodimers: (i) probes of *Glomus caledonium* and *Glomus* sp. "Bad Sachsa" (which have 3 complementary bp), (ii) probes of environmental *Glomus* sequence type 3b and *Glomus* sequence type 2 (5 complementary bp), and (iii) *Paraglomus laccatum* probes and *Glomus intraradices* (1a) primer (5 complementary bp). However, linear regression of results obtained from amplifications with variations of at least 7 orders of magnitude in template abundance (100 to 10^9 DNA molecules) showed that the heterodimer formation had no significant impact on the multiplex real-time TaqMan PCR assay results, since the R^2 values were consistently very high (0.974 to 0.996) and standard errors were low (see Table S1b). Average PCR amplification efficiencies, calculated from standard curves obtained from the three independent serial template dilutions, ranged from 71.0 to 99.6% (Tables S1a and S1b and Fig. S3a to d). These results validate the reliability of the TaqMan probes used in the multiplex PCR for both reference and environmental DNA samples under the applied reaction conditions (i.e., using the QuantiTect Multiplex PCR NoROX kit, with an annealing temperature of 56.9°C, and TaqMan probes and primers at the concentrations shown in Table S1a). In addition, the results from the cross-reference dilution series indicate that the multiplex real-time PCR TaqMan assays are capable of detecting rare sequence types, with DNA concentration ratios up to 10^6-fold lower than those of the most abundant types (data not shown).

Labeling dyes were selected that should, theoretically, provide emission signals that do not spectrophotometrically overlap at the transmission bands of the five fluorescence detection filters used in the Bio-Rad IQ5 fluorescence detection system. However, we observed overlapping interference in the signals from the fluorescence reporter dyes 5′-tetrachloro-fluorescein phosphoramidite (TET) and 6-carboxytetramethylrhodamine (TAMRA), where TET was replaced by 5′-hexachloro-fluorescein (HEX). TAMRA exhibited the weakest fluorescence signals of the fluorescence reporter dyes that we used (in accordance with information in the GeneScan reference guide [Applied Biosystems]), but they were interpretable in all cases.

Threshold cycles (C_T) for the DNA soil extract dilution series (undiluted, 10-fold diluted, and 100-fold diluted) and both first and nested PCR products were calculated using Bio-Rad IQ5 optical system software (Bio-Rad Laboratories, Munich, Germany). Important parameters of the real-time PCR-based TaqMan assays (sensitivity, reproducibility, amplification efficiency, and the range of template concentrations that could be reliably quantified) were evaluated as described in the work of Gamper et al. (20) (this information is also available from the *Real-Time PCR Application Guide*). The reproducibility and efficiency of the PCR amplifications were assessed by TaqMan assays with three separate serial dilutions for each multiplex AMF sequence type combination (as used in the last experiment mentioned above). The initial DNA template numbers were calculated in absolute terms, which require a reliable source of template of known concentration. Therefore, DNA concentrations of undiluted single clone references (as vectors) were measured spectrophotometrically using the NanoDrop 8000 V1.1 spectrometer, and DNA template numbers were calculated using the formula $NC = (K \times N_a)/(660 \times L)$, where L is the (known) average DNA fragment length for each fungal sequence type (see Table S1b in the supplemental material), K is the DNA concentration determined from its absorbance at 260 nM (g liter^{-1}), 660 is the molecular mass of DNA in Da per base pair, and N_a is Avogadro's constant (6.023×10^{23}) (32). Based on the measured DNA (pure vector) concentrations, which ranged between 6 and 13 ng per μl, the calculated initial DNA template numbers (NC) of the serial dilutions (1 to 10^{-7}) ranged from 107 to 1.1×10^9 molecules of AMF DNA templates. The mean PCR cycle thresholds (C_T) calculated independently for each of the primer-probe combinations and each dilution of the respective DNA templates were used to estimate linear standard curves. Initial DNA molecule numbers in field samples were then calculated in absolute terms using the mean cycle thresholds obtained from three independent real-time PCR TaqMan assays for each AMF sequence type and exponential formulas derived from linear regression of standard curves obtained by plotting non-logarithmically transformed DNA template molecule numbers against cycle numbers.

Comparison of clone library screening and TaqMan assay results. The AMF species diversity and richness values obtained by screening clone libraries from nested PCR products of undiluted soil DNA extracts and the TaqMan assays of soil DNA extracts (undiluted, 10-fold-diluted, 100-fold-diluted, first PCR, and nested PCR products) were compared. This was done by calculating noniterative first-order jackknife estimators of species richness and species saturation values for each plot for each approach (and each DNA dilution for TaqMan assays) using functions implemented in PC-Ord version 4 (MjM Software Design, Gleneden Beach, OR). The species saturation curves were derived using a presence/absence matrix transformed by the Sørensen (Bray-Curtis) distance measure to estimate species area-curves for total AMF sequence type numbers per plot.

Mantel tests were also performed, using PC-Ord, to test congruency in AMF sequence type composition data obtained by the two approaches and the different DNA sources. Such tests are based on resemblance matrices and measure the extent to which variations in distances in one matrix are correlated with distance variations in a second matrix. The statistical significances of the similarities are evaluated by Monte Carlo permutation tests with (in our analyses) 1,000 randomized permutations, which yield standardized Mantel coefficients (r_M) indicating the correlation between the two matrices (37). In addition, analysis of variance (ANOVA) was carried out using R, version 2.7.2 (R Development Core Team, 2008), to assess the effects of measured variables on AMF species richness using the saturation analysis data, initial DNA template numbers, and the reproducibility of TaqMan assays. The data were log transformed (base 10) to improve the approximation to normality prior to ANOVA of the numbers of individual AMF sequence types detected in the 23 clone libraries and DNA template numbers in the real-time PCR TaqMan approach.

Ecological factors related to the AMF community structure. Based on the outcome of the preliminary TaqMan analysis of samples from the subset of 23 plots, the AMF communities in the other 58 plots were analyzed using TaqMan probes and undiluted soil extracts. The AMF diversity in all 81 experimental plots was then estimated in terms of Shannon-Wiener diversity indices, based on AMF sequence type richness and initial DNA template numbers per plot. For the environmental variable analyses, we performed a multiple regression with stepwise backward model selection (13) to investigate the effects of plant func-

tional group (FG) levels, FG composition, real observed plant richness, experimental design, soil nutrient status (soil nitrogen, phosphorus, and inorganic and organic carbon contents), pH, and soil moisture on AMF species richness, AMF diversity, and the abundance of AMF sequence types. All data (except presence-absence data for the canonical correspondence analysis [CCA]; see below) were log transformed to meet assumptions of normality and variance homogeneity. The significance and importance of the measured variables were quantified using ANOVA of the final model.

To investigate the responses of the AMF community to the measured environmental variables, we applied CCAs (45) to the AMF sequence type presence-absence data, focusing on the effects of the presence of individual plant functional groups rather than plant functional group richness. The significance and importance of each environmental parameter in the CCA were assessed using permutation-based ANOVA (45).

To examine whether spatial processes affected the AMF community composition or AMF sequence type distribution in the plots, e.g., dispersal limitations as postulated by the neutral theory (27, 38), especially for AMF (17), we computed correlograms (i.e., plots of Moran's *I* versus distance between plots) for the residuals of our models using the function correlog from Bjornstad (6). Models revealing significant residual spatial autocorrelation (SAC) patterns were subjected to spatial eigenvector mapping using the ME function from Bivand (5), based on the spatial coordinates of the plots (for reviews of applications of SAC, see references 15 and 16). Reexamination of the correlograms indicated satisfactory reduction of residual SAC.

RESULTS

PCR clone library screening. In a preliminary step, the AMF diversity was explored in 23 of the 81 plots by RFLP screening and sequencing of clone libraries constructed from nested PCR amplicons of the ITS region. After RFLP screening, 978 of 1,725 obtained clones were sequenced, 548 of which belonged to the Glomeromycota. Based on a 92% identity threshold, 13 AMF sequence types were inferred from 5.8S (see Fig. S1 in the supplemental material) and 5.8S plus part of ITS2 phylogeny within the *Glomus* group Ab sensu (61) (Fig. S2). Ten sequence types belonged to the *Glomeraceae* (including *Glomus intraradices*, *Glomus mosseae*, *Glomus caledonium*, and *Glomus claroideum*), and three belonged to the *Archaeosporaceae* (*Archaeospora trappei*), *Diversisporaceae* (*Glomus versiforme*), and *Paraglomeraceae* (*Paraglomus laccatum*). The sequence type accumulation curve reached a plateau (Fig. 1), indicating exhaustive screening and sequencing, which was corroborated by the first-order jackknife estimate of saturation at 14.9 sequence types. On average, 3.8 sequence types per plot were detected (Fig. 2a).

Comparison of clone library screening and real-time PCR-based TaqMan assay results. As in the clone library screening, the sequence type accumulation curve approached saturation in the real-time PCR-based TaqMan assays, but with a significantly higher efficiency in the case of undiluted ($P < 0.01$) and 10-fold-diluted ($P < 0.05$) soil DNA extracts (Fig. 1). With the TaqMan assays, a *Gigaspora* species (DQ400249) was detected in three replications of the undiluted sample extracts which was not found by clone library screening. Analysis of variance showed that the TaqMan assays detected significantly greater AMF sequence type richness than did the clone library screening, with all DNA templates, and the detected richness was always significantly greater ($P < 0.001$) when undiluted soil DNA extracts or 10-fold-diluted extracts were used in the TaqMan assays (Fig. 2a). Slightly but nonsignificantly greater richness was detected when undiluted extracts rather than 10-fold dilutions were used. Compared to clone library screening, significantly higher Shannon diversity indices were also recorded

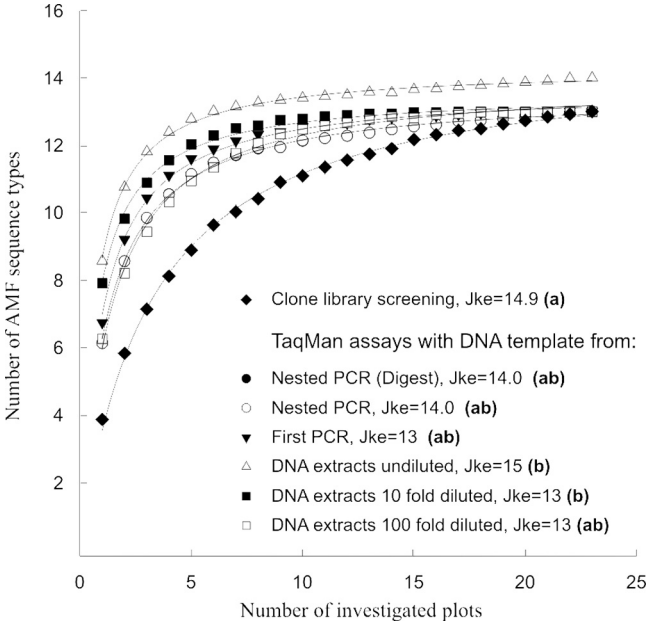

FIG. 1. Rarefaction curves, based on first-order jackknife estimators for the detected AMF sequence types based on nested PCR [open circles; $y = 13.5811x/(1.1687 + x)$, $R^2 = 0.9981$], DNA templates obtained from the first PCR step in the nested PCR [closed triangles; $y = 13.9442x/(1.3316 + x)$, $R^2 = 0.9971$], undiluted DNA extracts [open triangles; $y = 13.5177x/(0.5162 + x)$, $R^2 = 0.9657$], 10-fold dilutions [closed squares; $y = 13.5820x/(0.7108 + x)$, $R^2 = 0.9915$], 100-fold dilutions [open squares; $y = 13.7244x/(1.0172 + x)$, $R^2 = 0.9971$], and clone library screening [diamonds; $y = 14.6821x/(3.1653 + x)$, $R^2 = 0.9981$]. First-order jackknife estimators (Jke) and significant differences between the clone library and real-time PCR TaqMan results, according to Tukey honestly significant difference tests ($P < 0.05$), are indicated in bold letters in parentheses in the curve descriptions. Closed circles are covered by open circles since they indicate the same AMF distribution in the presence/absence matrix-based analysis.

using TaqMan assays with nested PCR products ($P < 0.05$) and undiluted ($P < 0.01$) and 10-fold-diluted ($P < 0.05$) extracts (Fig. 2b). However, despite these differences, the Mantel tests indicated that there were significant positive correlations between the clone library screening results and the results of the TaqMan assays with all types of nested PCR products ($r_M \geq 0.17$; $P < 0.05$) except those with the soil DNA extract dilution series (see Table S2 in the supplemental material).

In the TaqMan assays, the DNA dilution level, PCR steps, and initial DNA template numbers all influenced the detected AMF diversity (Fig. 2c and d). Using the number of repeated detections of AMF types as an indicator, the reproducibility and reliability of the PCR in the TaqMan approach were significantly lower when the 10- and 100-fold dilutions of DNA were used than when amplification products were used (Fig. 2c). DNA template numbers were highest in nested PCR and successively, significantly lower ($P < 0.01$) in first PCR products than in undiluted and 10-fold- and 100-fold-diluted extracts (Fig. 2d). The number of repeatedly detected records significantly increased with increasing initial DNA template numbers ($P < 0.01$). We also found significant positive relationships between mean numbers of individual AMF sequence type clones per clone library, DNA template numbers in the nested PCR products as detected by TaqMan assays, and se-

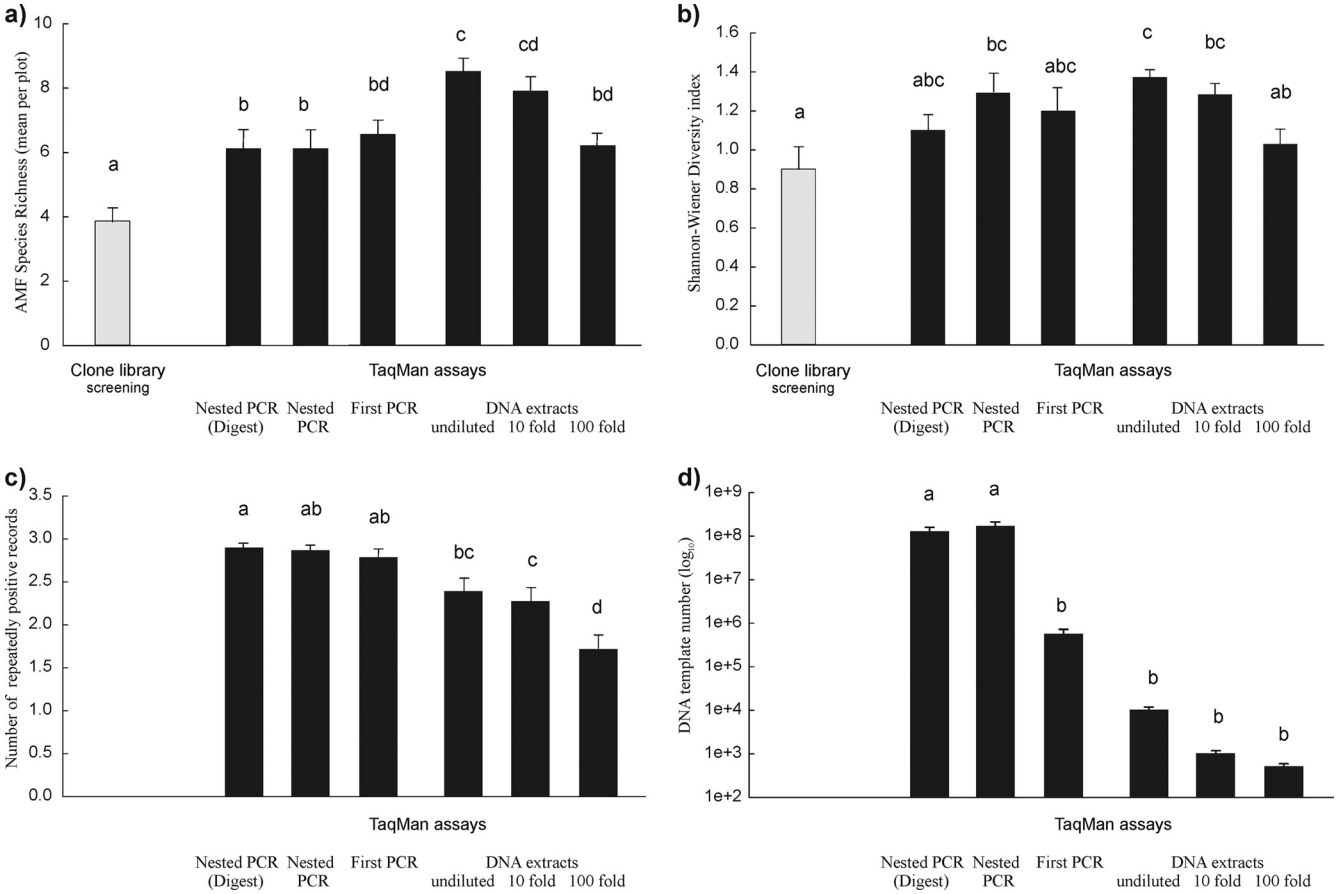

FIG. 2. (a) Mean species richness per plot according to analyses of samples from 23 plots using the TaqMan approach (black bars) with different sources of DNA template and the clone library screening based on undiluted soil DNA extracts and subsequent nested PCR, cloning, and Sanger sequencing (gray bars) + standard error (SE; $P = 1.285e{-}11$). (b) Mean Shannon-Wiener diversity indices per plot according to analyses of samples from 23 plots using the TaqMan approach with different DNA sources (black bars) and clone library screening (gray bar) + SE ($P = 0.0014$). (c) Mean numbers of repeatedly positive detected TaqMan multiplex PCR records (in 3-fold independently replicated PCR) in the samples from 23 plots with different sources of DNA + SE ($P = 3.893e{-}10$). (d) Mean number of detected initial DNA templates (NC) using the TaqMan approach in samples from the 23 plots + SE ($P = 3.098e{-}8$). Different letters above bars indicate significant differences according to Tukey honestly significant difference tests ($P < 0.05$). Data for *Gigaspora* sp. were excluded from panels b and c due to the lack of reference DNA material for determining the linearity of standard curves generated by the TaqMan approach for this sequence type.

quence type richness detected by both approaches ($P < 0.01$). A significant positive correlation was also detected between log-transformed individual sequence type richness per clone library and mean initial DNA template number of nested PCR products ($P < 0.01$, $R^2 = 0.47$) (see Table S3 in the supplemental material).

Interrelationships between environmental parameters. Spearman rank correlation analyses of soil, plant, and disturbance-related parameters revealed interdependencies between the parameters and enabled them to be grouped into four clusters (see Fig. S4 in the supplemental material). Within the widest cluster, the presence of legumes, plant species richness, and plant functional group richness were correlated positively with total plant biomass but negatively correlated with soil phosphorus content (in both cases, $P < 0.001$). The soil moisture was positively correlated with nitrogen ($P < 0.01$) and organic carbon ($P < 0.05$) contents. Soil moisture increased with plant species richness ($P < 0.001$) and plant biomass production ($P < 0.05$) but not with plant functional group richness or any individual plant functional group. The presence of grasses as a plant functional group was negatively correlated with disturbance by weeding ($P < 0.001$), reflecting the production of lateral shoots by many grasses, which limits invasion by nonsown plants (see also reference 40). The soil inorganic carbon content and pH increased concomitantly with reductions in sand proportions, which were related to the distance of the plots from the Saale River (56).

AMF diversity and factors influencing the AMF community structure. Analysis of the richness and distribution of 15 AMF sequence types using the optimized real-time PCR TaqMan assays procedure revealed the occurrence of 3 to 11 AMF sequence types per plot. *Glomus claroideum* and *G. versiforme* were detected in all plots, whereas *Gigaspora* sp. appeared in samples from only four plots. *Acaulospora* sp. was not detected at all. Shannon-Wiener diversity indices for the plots ranged between 0.11 and 1.89.

Analyses of variance based on multiple regression analysis indicated that there were significant effects of four plant-re-

TABLE 1. ANOVA table of effects of the indicated factors on (log-transformed) AMF species richness and diversity[a]

Source	Log (AMF species richness)					Log (Shannon-Wiener diversity index)				
	df	SS	MS	*F*	*P*	df	SS	MS	*F*	*P*
Block	3	0.259	0.086	3.085	**0.034**	3	0.564	0.188	2.615	0.059
Log (soil nitrogen)	1	0.000	0.000	0.002	0.963	1	0.000	0.000	0.001	0.974
Log (soil phosphorus)	1	0.002	0.002	0.087	0.769	1	0.117	0.117	1.630	0.206
Log (soil inorganic carbon)	1	0.003	0.003	0.090	0.765	1	0.160	0.160	2.221	0.141
Log (soil organic carbon)	1	0.046	0.046	1.649	0.204	1	0.003	0.003	0.039	0.845
Log (pH)	1	0.006	0.006	0.220	0.641	1	0.003	0.002	0.034	0.854
Log (soil water content)	1	0.178	0.178	6.358	**0.014**	1	0.319	0.319	4.433	**0.039**
Log (disturbance by weeding)	1	0.093	0.093	3.329	0.073	1	0.020	0.020	0.277	0.601
Log (plant species richness)	1	0.227	0.227	8.118	**0.006**	1	0.681	0.680	9.470	**0.003**
Log (plant biomass)	1	0.071	0.071	2.522	0.117	1	0.080	0.080	1.118	0.294
Plant functional group richness	1	0.085	0.085	3.019	**0.037**	1	0.170	0.170	2.365	0.129
Grasses	1	0.054	0.054	1.916	0.171	1	0.010	0.010	0.141	0.708
Legumes	1	0.194	0.194	6.935	**0.011**	1	0.224	0.223	3.110	0.083
Small herbs	1	0.019	0.019	0.688	0.410	1	0.012	0.012	0.169	0.683
Tall herbs	1	0.296	0.296	10.554	**0.002**	1	0.043	0.043	0.599	0.442
Significant two-way interactions										
Log (plant biomass) × legumes	1	0.347	0.347	12.390	**<0.001**	NS	NS	NS	NS	NS
Residuals	62	1.736	0.028			63	4.527	0.072		

[a] Model summary representing R^2, F, and P statistics for the complete models. Abbreviations of depicted ANOVA output: df, degrees of freedom; SS, sum of squares; MS, mean sum of squares; NS, not significant. The summary model is as follows: for AMF species richness, R^2, F, and P are 0.5199, 3.73, and <0.001 (significant), respectively; for the Shannon-Wiener diversity index, R^2, F, and P are 0.347, 1.968, and 0.030 (significant), respectively. Boldface indicates statistical significance.

lated parameters on AMF sequence type richness: species richness ($P < 0.01$), functional group richness ($P < 0.05$), the presence of tall herbs ($P < 0.001$), and the presence of legumes ($P < 0.05$). The presence of grasses, small herbs, and biomass production did not significantly affect AMF richness. Soil moisture was the only measured soil parameter that significantly affected AMF sequence type richness ($P < 0.01$). Plant biomass was affected by AMF richness only in two-way interaction with legumes, and only two parameters had a significant effect on AMF diversity (expressed as Shannon-Wiener indices): the plant species richness and the soil moisture ($P < 0.01$ and 0.05, respectively; Table 1).

ANOVA also revealed positive effects of plant functional group richness, plant species richness, and soil moisture on AMF sequence type richness (Fig. 3a to c). There was a negative effect of disturbance by weeding, but no significant effects of either the experimental block design or plant biomass were detected (Fig. 3d to f). The remaining soil parameters also had no significant influence on AMF species richness. To assess the influence of the four plant functional groups, ANOVAs were performed using both AMF species richness and diversity, as expressed by Shannon-Wiener indices (Fig. 4). The species richness was again the most responsive of these AMF community variables, being significantly affected by small herbs, tall herbs, and grasses, while the AMF diversity indices were affected only by tall herbs.

To analyze factors affecting the distribution of each of the 14 AMF types (the 13 detected by both PCR clone library screening and the TaqMan assays plus the *Gigaspora* sp. [DQ400249] detected only by the latter), canonical correspondence analysis (CCA) was performed, which detected significant effects of legumes ($P < 0.01$) and small herbs ($P < 0.05$). The CCA also revealed two AMF clusters: one displaying high affinity to legumes (*Glomus mosseae*, *G. caledonium*, *Glomus* sp. "Bad Sachsa," and *Archaeospora trappei*) and one displaying affinity to the other functional groups (*Glomus* sp. sequence type 3 [ST3] and ST4, and *Paraglomus laccatum*) (Fig. 5). In order to investigate the effects of each plant functional group on AMF sequence type distribution, a set of one-way ANOVAs was performed, which confirmed the marked influence of legumes (Fig. 6). While the occurrence of sequence types corresponding to *Glomus* group Aa (*G. mosseae*, *G. caledonium*, and *Glomus* sp. "Bad Sachsa") and *Archaeospora trappei* were positively correlated with the presence of legumes, *Paraglomus laccatum* ($P < 0.001$) and all types of *Glomus* group Ab (except *Glomus* sp. ST5 and *G. intraradices*) were negatively affected. The abundance of the remaining AMF sequence types was not significantly affected and was lower in plots with legumes than in legume-free plots. Significant effects (revealed by univariate ANOVA) between AMF sequence types and remaining plant functional groups were rare (*P. laccatum* and grasses, $P < 0.05$). Univariate ANOVA of relationships between soil parameters and AMF distribution indicated that there were positive effects of soil moisture on *Glomus* sp. "Bad Sachsa" and *Glomus* sp. ST2 and ST5 ($P < 0.05$), a positive correlation between soil nitrogen and *G. intraradices* ($P < 0.05$), and negative correlations between plant-available phosphorus content and both *G. caledonium* ($P < 0.05$) and *Glomus* sp. ST5 ($P < 0.05$). As shown in Table S4 in the supplemental material, univariate ANOVA also revealed significant positive effects of plant diversity, plant functional group richness, soil moisture, and nitrogen on several individual AMF sequence types and negative effects of soil phosphorus, disturbance by weeding, and soil pH. In contrast, plant biomass had a significant positive effect on *Glomus* sp. ST1 but a significant negative effect on *Glomus* sp. ST5 (Table S4).

No significant spatial autocorrelation (SAC) between AMF species richness and Shannon-Wiener diversity was observed.

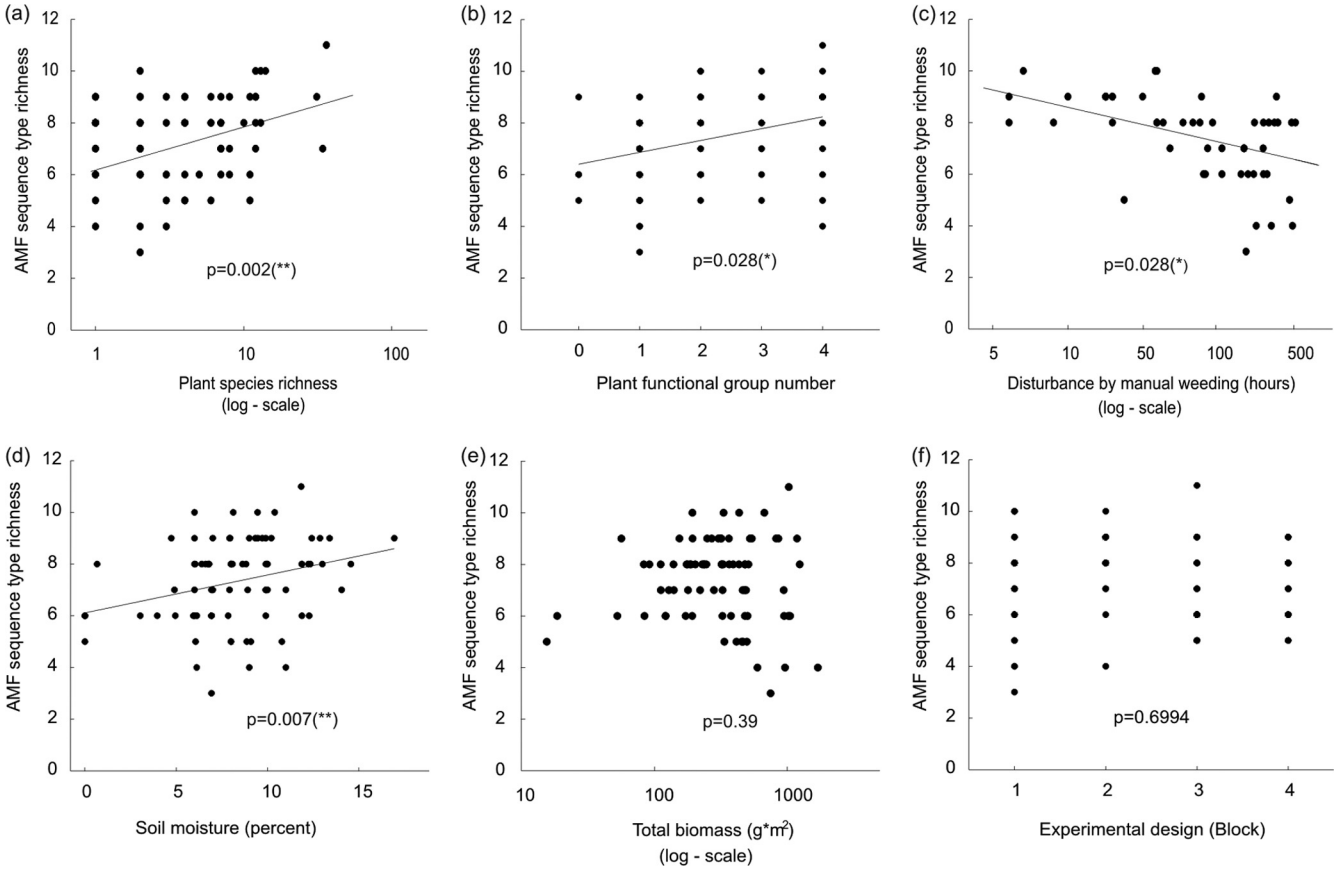

FIG. 3. Relationships between AMF richness and plant species richness (log scale) (a), plant functional group richness (b), disturbance by manual weeding (log scale) (c), soil water content (d), plant biomass (log scale) (e), and experimental design (f). Only significant regression lines are displayed, with *P* values.

Among the AMF sequence types investigated using presence-absence data, we found only one sequence type, *Glomus* sp. "Bad Sachsa," to be spatially autocorrelated. However, for this sequence type the eigenvector-adjusted model (Moran's *I*) fits better than the nonspatial model. The number of significant parameters increased by plant functional group ($P < 0.05$) and by the spatial eigenvector itself, where the respective R^2 increased from 0.3 to 0.43 and model significance decreased from 0.09 to 0.004. The SAC associated with *Glomus* sp. "Bad Sachsa" could be removed by adding the single significant eigenvector (see Fig. S5 in the supplemental material). This eigenvector encodes a fine-scaled pattern representing part of the plots along block 1 of the experimental site (data not shown). Unlike the AMF sequence type presence-absence data, analysis of SAC using sequence type abundance in the neutral theory sense indicated that there was no significant spatial autocorrelation.

DISCUSSION

Application of real-time PCR-based TaqMan assays. Real-time PCR TaqMan assays have been previously used in AMF research to trace 1 to 5 AMF inoculates in pot cultures (20, 32, 49) and quantify root colonization (3, 29). Their quantification power is known to be poor, due to the coenocytic character of AMF (20) and the variable rDNA copy numbers in different nuclei (66), but they can provide valuable qualitative data. Therefore, we developed a new qualitative real-time PCR-based TaqMan assay approach for broad-field sample analysis to investigate AMF diversity and the impact of plant- and soil-related variables on AMF community structure.

The ITS rDNA was chosen as a target sequence since it displays higher variation and species-level discrimination than do both ribosomal subunits of the rDNA gene loci (43, 64) and other potential loci. Semispecific primers and AMF sequence type-specific TaqMan probes were combined in four multiplex real-time TaqMan PCR assays to detect 15 AMF targets. Comparison of the results obtained with the TaqMan assays and clone library screening using first and nested PCR products and a DNA soil extract dilution series showed that TaqMan assays with undiluted soil DNA extracts detected higher numbers of sequence types than did assays with the nested PCR products, reflecting the tendency of general primer pairs used in nested PCR to discriminate between some sequence types (35). Soil organic compounds coextracted with DNA are also known to inhibit PCR amplifications, and diluting DNA extracts reduces this effect (70). In the TaqMan assays, however, no PCR inhibition was observed when the undiluted DNA extracts were used, and the reliability was similar to that obtained using undiluted extracts or first or nested PCR products

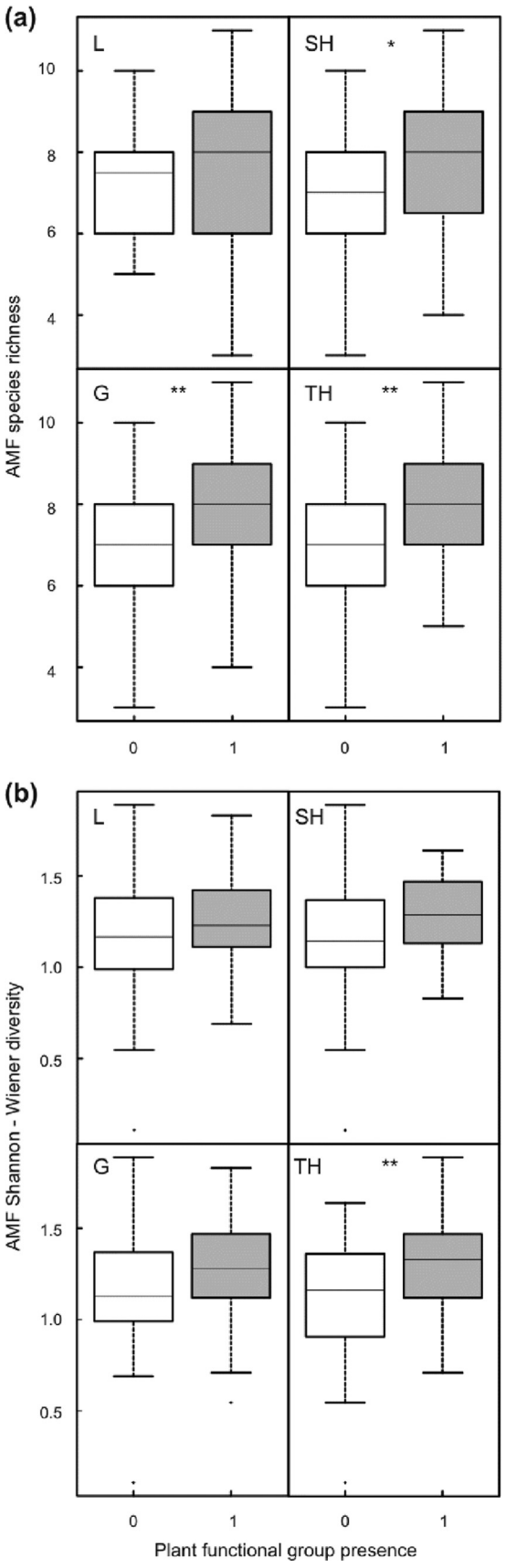

FIG. 4. AMF richness (a) and AMF diversity (b) values obtained for experimental plant communities with one to four plant functional groups, showing median values ± 25th percentiles (boxes) and ± 75th percentiles (whiskers) obtained for experimental plots with plant species of the indicated functional groups present (gray) and absent (white). ANOVA revealed no significant differences between the different functional groups in responses of AMF richness and diversity. Levels of significance of differences in AMF richness and diversity related to FG presence and absence are indicated by asterisks in the panels: **, $P < 0.01$; *, $P < 0.05$. L, legumes; G, grasses; SH, small herbs; TH, tall herbs.

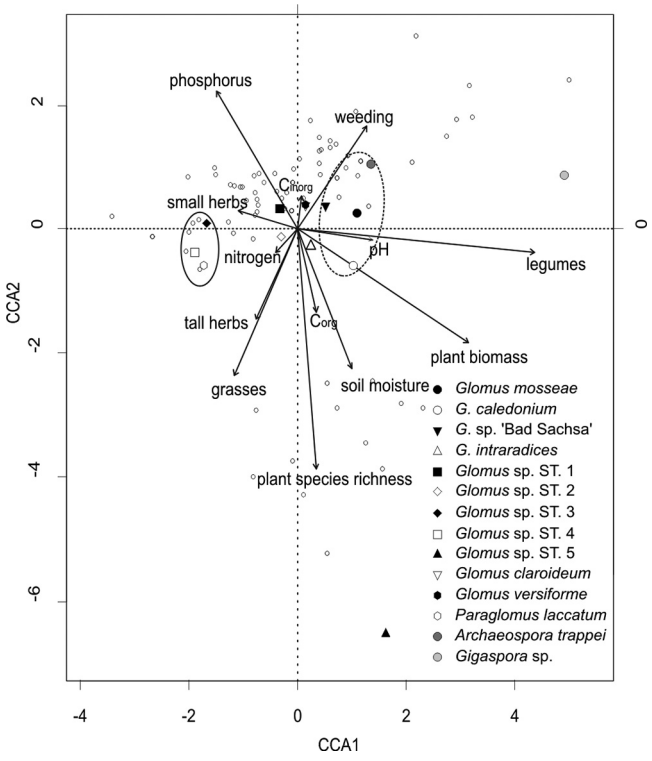

FIG. 5. Results of canonical correspondence analysis (CCA) of factors affecting AMF distribution, showing significant effects of the presence of legumes ($P < 0.01$) and small herbs ($P < 0.05$). Ellipses separate AMF sequence types with high (dashed line) and weak (solid line) affinity with legumes. *Glomus versiforme* and *G. claroideum* generally occurred on all plots.

as templates (Fig. 2c). This high reproducibility indicates that there was little (if any) primer binding bias (65) or primer GC content and degeneration effects (50). Furthermore, clone library screening showed that insignificant formation of chimeric products occurred (affecting <2% of 548 sequences).

TaqMan assays also yielded higher species richness values and more consistent AMF sequence type distribution patterns, possibly due to the amplification of a substantial proportion of non-AMF sequences in the ITS nested PCR (55) used to construct the clone libraries, as reported in analyses of AMF in mountain meadows in central Germany (10, 26) and tropical rainforests (2). This interpretation is supported by the fact that the TaqMan analysis of the first PCR products detected a significantly higher number of AMF sequences. Despite this

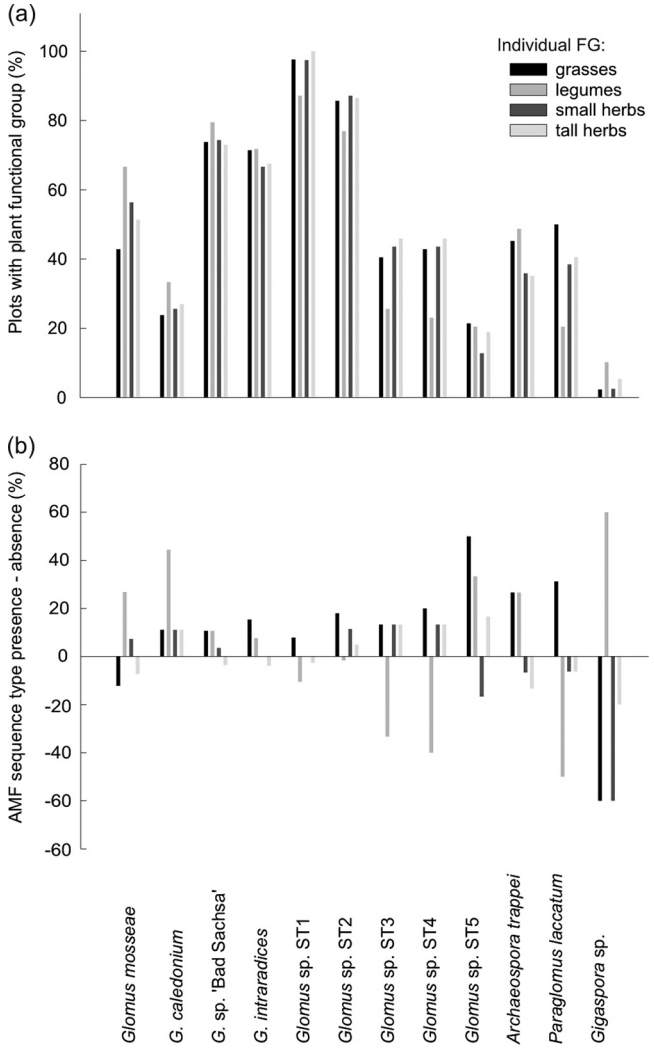

FIG. 6. (a) Absolute percentages of plots with indicated plant functional groups inhabited by the detected AMF sequence types. (b) Relative abundances of AMF sequence types in plots with indicated functional groups, expressed as a percentage of the number of plots in which they were found minus the number in which they were not detected. (a and b) ANOVA revealed significant correlations between grasses and both *Glomus* sp. ST2 and *P. laccatum* ($P < 0.05$); legumes and *P. laccatum* ($P < 0.001$), *G. mosseae* ($P < 0.01$), and *G. caledonium*, *Glomus* sp. "Bad Sachsa," *Glomus* sp. ST1, *Glomus* sp. ST3, *Glomus* sp. ST4, and *A. trappei* ($P < 0.05$); and between both small and tall herbs and *Glomus* sp. ST2 ($P < 0.05$). Data for *Glomus versiforme* and *G. claroideum* are not shown because they generally occurred in all plots.

bias, the clone library screening approach provided results consistent with those of the TaqMan assays, as shown by the Mantel tests and the correlation between the detected AMF sequence types and the measured DNA template numbers.

AMF communities in the Jena Experiment plots. The number of AMF sequence types found in the preliminary analysis is similar to the number found by Jansa et al. (30), who identified 14 species in samples from a long-term field tillage experiment. The prevalence of *Glomus* spp. that we observed is also consistent with former studies of managed field sites and correlations between these small-spored AMF types and tillage intensity (1, 8). Jansa et al. (30) also found *G. caledonium*, *G. mosseae*, and *G. claroideum* to be highly correlated with tillage intensity. In contrast, we found the large-spored AMF *Gigaspora* sp. to be restricted to five plots close to the border of the field site, in accordance with its previously reported low capacity for hyphal regeneration following disturbance (14). Interestingly, disturbance by weeding negatively affected both the species richness of these *Glomus* species-dominated communities and the presence of *Glomus* species ST1.

After the preliminary screening, validation, and optimization procedures, the optimized TaqMan assay was used to assess the presence and distribution of the 15 AMF target sequence types in all 81 experimental plots. The results clearly show that high plant diversity promoted an increase in AMF diversity and that plant species richness influences AMF sequence type richness more strongly than does plant functional group diversity (Table 1 and Fig. 3a and b). These findings corroborate converse findings that increasing the AMF diversity resulted in more diverse plant communities in pot culture experiments (68). However, our results (Fig. 3e) conflict with the cited authors' general conclusion that increases in plant biomass production are triggered by increases in AMF diversity and their recommendation that this link should be considered in detail. For example, grasses have been found to have minor effects on plant community productivity as a plant functional group at the Jena Experiment site (40), but our data indicate that this functional group favors AMF species richness (Fig. 4). In contrast, tall herbs and legumes have been shown to have strong effects on plant community productivity (40), but only the presence of tall herbs was positively correlated with AMF species richness and diversity, while the presence or absence of legumes had insignificant effects (Fig. 4). Effects of legumes were, however, detected at the level of AMF sequence type composition. The most abundant types, *Glomus* sp. ST1 to ST4 and *P. laccatum*, were less frequent in plots with legumes, where members of the *Glomus* group Aa were favored. This could be due to the differential selection by root nodules of legumes for nitrogen-adapted AMF (59). Although tall herbs and grasses enhanced the AMF diversity, they had weaker effects on the occurrence of specific AMF types. Only *P. laccatum* displayed a higher occurrence on plots harboring grasses (Fig. 5 and 6). In general the observed differences in AMF community structure between plots with legumes and plots with no legumes are coherent with the results of Vandenkoornhuyse et al. (67), although our data are based on analyses of rhizosphere soil, while the cited authors examined roots (67).

Soil moisture was the only soil parameter found to be positively correlated with AMF type richness and Shannon-Wiener diversity. It was also positively correlated with plant species richness, and a previous study has shown that intermediate levels of soil moisture may promote higher P mobilization from AMF to the plant host (41). In contrast to the report of Dumbrell et al. (17), soil pH had no impact on sequence type richness and AMF community composition, but there is only a narrow range of soil pHs at the Jena Experiment site. N and organic carbon (C_{org}) in soils were poor predictors of AMF species richness and Shannon-Wiener diversity (Table 1); however, the CCA detected an effect (albeit nonsignificant) of P on the distribution of the AMF sequence types (Fig. 5). This might have been partly due to differences in P contents associated with the presence of legumes and nonlegumes, since plant-available P contents were lower in plots

containing legumes and tall herbs than in other plots (see Fig. S4 in the supplemental material). In addition, nitrogen contents decreased with increase in plant species richness and the presence of grasses but were enhanced in plots with legumes (44). Organic carbon displayed similar variations (Fig. S4). While the distribution of most AMF types was not affected by any of the soil chemical parameters (Table S4), the presence of *G. caledonium* and *Glomus* species ST5 in plots with legumes or tall herbs was correlated with low levels of plant-available phosphorus (Table S4). This general observation of weak soil nutrient effects on AMF community assemblages conflicts with results of a wide range of studies (e.g., references 18, 23, and 34), which have documented strong effects. However, nearly all of these studies focused on monocultures of agricultural plants along strong fertilization gradients, with minor consideration of the effects of plant diversity and plant functional groups, which might have a stronger hierarchical effect on AMF diversity.

Earlier studies that have considered potential effects of niche-predicting environmental parameters and random dispersal limitations on AMF distribution have focused on closely located undisturbed habitats (17) or widely separated managed arable field sites (39). In contrast, the habitats examined here were managed grassland plots of the Jena Experiment site, separated by intermediate distances. All the factors found to be influential contribute to distinctions between ecological niches, indicating that the AMF distribution is consistent with niche theory. However, we found spatial effects only for the presence/absence of one AMF sequence type (*Glomus* sp. "Bad Sachsa"), in general accordance with the neutral theory. The indications that aspects of the AMF distributions have consistency with both theories, depending on the AMF sequence type, may reflect the variety of propagation strategies within AMF. However, the unique neutral theory (27) is difficult to apply to AMF since it is based on the effects of stochastic biogeographic processes related to ecological drift on discrete individuals, whereas AMF are coenocytic organisms, fungal rDNA is a multicopy marker, and the entity of a single individual AMF in the field is difficult to define (see reference 58). Hence, there is no clear criterion for AMF abundance, and frequency values derived from PCR-based molecular data like clone numbers, RFLP patterns, and DNA template numbers are poor predictors of abundance. Thus, there are severe problems in applying Hubbell's abundance-dependent neutral theory in AMF molecular ecological studies.

General conclusions. We have demonstrated the suitability of a real-time PCR-based TaqMan assay approach for high-throughput analysis of the presence and distribution of targeted AMF sequence types in the field. Following advances in sequencing technology, there is an increasing tendency to employ tagged and targeted amplicon (454) sequencing (e.g., see reference 47), but TaqMan assays would be the best high-throughput approach for following shifts in the community composition of a specific group of AMF in natural or experimental conditions. Compared to conventional PCR amplification, cloning, and sequencing, TaqMan assays are cheaper by a factor of five when using the manufacturers' protocols for both methods (i.e., approximately $40 versus $205 per sample), and they provide ca.-6-fold time savings.

We found evidence that a strong hierarchical gradient of factors shaped the AMF communities that we examined (which were largely composed of disturbance-tolerant species), in terms of both species richness and composition. The presence/absence-based diversity of the species could be largely explained by the richness of plant species and plant functional traits and did not appear to be related to either plant productivity or soil parameters. Plant functional group identity was the most important variable shaping AMF community assemblages. Generally, niche-describing parameters were found to explain most of the AMF diversity and community structuring processes. The results indicate that AMF abundance data detected by molecular techniques should be interpreted carefully in AMF diversity and community analyses.

ACKNOWLEDGMENTS

Special thanks are due to Sabine Jarzombski and Jeanette Keim for generating baseline sequences for assay development, Armin Michel for introduction to real-time PCR, Maike Habekost and colleagues for collecting soil samples, and three anonymous referees and colleagues of our department for very helpful comments and advice.

This work was funded by the German Research Foundation (DFG) (BU 941/14-2 and BU 941/14-3) extended to the Jena Biodiversity Experiment (FOR 456).

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