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Can rDNA analyses of diverse fungal communities in soil and roots detect effects of environmental manipulations—a case study from tallgrass prairie

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Abstract: We tested whether fungal communities are impacted by nitrogen deposition or increased precipitation by PCR-amplifying partial fungal rRNA genes from 24 soil and 24 root samples from a nitrogen enrichment and irrigation experiment in a tallgrass prairie at Konza Prairie Biological Station in northeastern Kansas, U.S.A. Obtained fungal sequences represented great fungal diversity that was distributed mainly in ascomycetes and basiodiomycetes; only a few zygomycetes and glomeromycetes were detected. Conservative extrapolated estimates of the fungal species richness suggest that the true richness may be at least twice as high as observed. The effects of nitrogen enrichment or irrigation on fungal community composition, diversity or clone richness could not be unambiguously assessed because of the overwhelming diversity. However, soil communities differed from root communities in diversity, richness and composition. The compositional differences were largely attributable to an abundant, soil-inhabiting group placed as a well-supported sister group to other ascomycetes. This group likely represents a novel group of fungi. We conclude that the great fungal richness in this ecosystem precluded a reliable assessment of anthropogenic impacts on soil or rhizosphere communities using the applied sampling scheme, and that detection of novel fungi in soil may be more a rule than an exception.

Key words: Fungi, soil DNA, environmental change, precipitation, nitrogen, biodiversity

INTRODUCTION

Fungi perform important ecosystem functions as mutualistic symbionts of most land plants (Smith and Read 1997) and as saprotrophic decomposers of detrital organic compounds (Cooke and Rayner 1984). Despite their obvious importance from an ecosystem function perspective, fungal species richness is poorly understood. The number of known fungi is estimated as at least 7.0×10^4 (Hawksworth 2001). The true species richness may, however, be much greater than the number of known fungi. Estimates of the global species richness vary from 0.5 to 9.9×10^6 with a proposed ''working hypothesis'' of 1.5×10^6 species (Hawksworth 2001). Accordingly, it is possible that as many as 1.43×10^6 species (or 95% of all extant taxa) have remained undetected.

In their contribution, Hawksworth and Rossman (1997) proposed that discovery of new taxa would be most probable in tropical forests, which likely are among the most species-rich ecosystems. Furthermore, exploration of fungi inhabiting plant, lichen, and insect tissues would also be likely to yield new and unrecorded taxa. Finally, the application of new techniques to substrates studied previously with traditional techniques would also be likely to yield new taxa, which have escaped detection in previous assessments.

Within this framework of potentially vast undetected fungal species richness, environmental change may alter composition of fungal communities. Human activities have changed biogeochemical cycles globally. For example, human N_2 fixation has added at least as much N to the global N cycle as all natural sources combined (Galloway and Cowling 2002, Vitousek et al 1997a, Vitousek et al 1997b). Human additions into the global N cycles impact terrestrial ecosystems and net primary productivity by changes in plant growth, and in plant species diversity (Fenn et al 1998, Kochy and Wilson 2001, Kohn and Stasovski 1990, Vitousek et al 1997a), therefore also likely impacting heterotrophic communities that are either associated with host plants or that inhabit soil (Bowden et al 2004, Compton et al 2004, Fog 1988, Frey et al 2004). Studies on ectomycorrhizal fungi have shown that some fungal species are favored by N additions whereas others may decline and disappear from the community (Avis et al 2003; Lilleskov et al 2001, 2002b; Peter et al 2001; Wallenda and Kottke 1998). Similarly, arbuscular mycorrhizal communities that are obligately associated with host plant roots often respond to N enrichment, although the results appear incongruent and vary considerably among studies (Bentivenga and Hetrick 1992a, Egerton-Warburton and Allen 2000, Eom et al 1999, Johnson 1993).

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In addition to dramatic changes in global N cycling, another biogeochemical global cycle that is likely affected is the hydrological cycle. Future climates are predicted to include changes in both precipitation variability and quantity (Easterling et al 2000, Groisman et al 1999, Knapp et al 2002, Mearns et al 1997). Changes in precipitation may affect soil biota via direct and indirect means. Precipitation has often been shown to be a key factor controlling primary productivity (Epstein et al 1996, Hooper and Johnson 1999, Lauenroth and Sala 1992) as well as plant community composition (Knapp et al 2002). The changes in function and structure of the primary producer communities are likely to result in altered dynamics and structure of soil microbial communities.

The soil environment hosts diverse fungal communities, which may derive energy from mutualistic association with plants and/or by consuming the abundant organic substrates embedded in the soil matrix. In earlier studies, fungal community composition in soil and rhizosphere environments have mainly been assessed by growing fungi from environmental samples in different pure culture media. These studies have provided some impressive volumes of fungi (Domsch et al 1980, Rambelli et al 1983). However, numerous examples from bacterial systems indicate that culture-dependent methods are inaccurate at best because as many as 99% of microbes possibly escape detection in such studies (Amann et al 1995, Ward et al 1992). Furthermore, most studies applying culture-independent techniques in bacterial systems have detected novel groups of organisms on higher taxonomic levels (Hugenholtz et al 1998, Pace 1997). Only relatively recently, have culture independent techniques been used to survey fungal diversity in environmental samples (Borneman and Hartin 2000, Smit et al 1999, Vandenkoornhuyse et al 2002a, Schadt et al 2003). These studies, similarly, indicate a vast undetected diversity among the soil-inhabiting fungi (Schadt et al 2003, Vandenkoornhuyse et al 2002a).

In this study, we analyzed fungal communities using environmental rDNA from tallgrass prairie soil and therein associated roots from experimental plots with and without either N enrichment or irrigation at Konza Prairie Biological Station near Manhattan, Kansas, U.S.A. We chose the small subunit of the ribosomal RNA gene to allow alignment and analysis of sequences representing all four major phyla of fungi. Partial fungal ribosomal small subunit DNA was PCR-amplified (Borneman and Hartin 2000), amplicons were cloned and screened by PCR and restriction enzyme analysis to estimate clone frequencies and to select different clones for sequencing. Our main goals were to estimate resident fungal diversity and richness as well as to determine whether or not manipulations of environmental conditions would result in observable shifts in fungal community composition. Furthermore, we aimed to screen the obtained soil and root samples for the presence of novel fungal lineages.

METHODS

Study site.—The Konza Prairie Biological Station (39°05′N, 96°35′W) is a 3400 ha tallgrass prairie reserve in the Flint Hills region in northeastern Kansas. Soils are mainly Pachic Argiustolls with silty clay loam structure. July mean temperature is 26.6 C and January mean temperature is −2.7 C. The climate is mesic and annual mean precipitation is 835 mm, approximately 75% of which falls during the growing season. Within the Konza Prairie, we utilized an irrigation and N-enrichment experiment located at a homogeneous lowland site. The area has been burned annually to mimic high fire frequencies typical of the tallgrass prairie ecosystem. The resultant plant communities, dominated by native grasses including *Andropogon gerardii*, *Panicum virgatum*, and *Sorghastum nutans*, are representative of high fire frequency sites.

Experimental plots.—Four irrigation transects, comprised of 6 plots each, were established in 1993. Two transects received no additional water and two received 80–90% of the irrigation necessary to completely alleviate plant water stress. The target level of the additional irrigation was determined based on the plant need as determined by a grass reference evapotranspiration model calculated to the FAO-56 standard using the Penman-Monteith equation (Allen 2000). In 1999, a total of 6 experimental 1 m^2-plots were randomly assigned to the annual N enrichment treatments (no additional N and annual N amendment of 10 g-N m^{-2} a^{-1} as NH_4NO_3 dissolved in distilled water) within each of the irrigation treatments. The N amendment was applied each year during the early growing season in June.

Sampling and DNA extraction.—In May 2001, two soil cores (2.5 cm in diam, 20 cm in depth) were collected at each of the 24 experimental plots and pooled. The rhizosphere samples comprised roots that were manually separated from the soil cores and thoroughly washed with tap water then rinsed with double distilled, deionized water. The total environmental DNA from the rhizosphere samples was extracted using Plant DNeasy (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's protocol. The soil samples were from root-free soils. After homogenization, soil DNA was extracted from a total of 2 g of soil from each of the 24 soil samples according to van Elsas et al (2000).

PCR parameters.—A partial sequence of the small subunit of the fungal ribosomal RNA gene (rDNA) was amplified in 50 µl PCR reaction mixtures contain-

ing final concentrations or absolute amounts of reagents as follows: 400 nM of each of the forward (nu-SSU-0817-5′) and reverse (nu-SSU-1536-3′) fungus-specific primers (Borneman and Hartin 2000), 2 μl of the extracted template DNA, 200 μM of each deoxynucleotide triphosphate, 2.5 mM $MgCl_2$, 2 units of Taq DNA polymerase (Promega, Madison, WI, U.S.A.), and 5 μl of manufacturer's PCR buffer. The PCR reactions were performed in a Hybaid OmniCycler (Hybaid Ltd., Middlesex, U.K.). The PCR parameters consisted of an initial denaturation at 94 C for 3 min, then 35 cycles of denaturation at 94 C for 1 min, annealing at 56 C for 1 min and extension at 72 C for 3 min, followed by a final extension step at 72 C for 10 min.

Small-subunit rDNA clone library construction and analyses.— The mixed populations of PCR products were ligated into a linearized pGEM-T vector (Promega, Madison, WI). The circularized plasmids were transformed into competent JM109 cells (Promega, Madison, WI) by heat shock and the putative positive transformants were identified by α-complementation (Sambrook 1989).

Twenty putative positive transformants from each of the clone libraries were randomly sampled. Presence of an insert was confirmed by PCR in 15 μl reaction volumes under the same reaction conditions as described above. Plasmids for sequencing were selected by an endonuclease (*HinfI*, *AluI*; New England BioLabs, Beverly, MA) digestion of the PCR products. The fragments were resolved on 3% agarose gels. The frequencies of the clones within and among the libraries were estimated based on the restriction fragment length polymorphic (RFLP) phenotypes; identical RFLP phenotypes were considered duplicates. From a representative of each RFLP phenotype, a sequence of approximately 760 bp in length was obtained in using fluorescent dideoxy-terminators (ABI Prism® BigDye™; Applied Biosystems, Foster City, CA) and an automated ABI Prism® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) at the DNA Sequencing and Genotyping Facility at Kansas State University (GenBank accession numbers AF504073–AF504141, AY099404–AY099416, and AY773716–AY773810). Vector contamination was removed using the automated vector trimming function in Sequencher (GeneCodes, Ann Arbor, MI). The similarities to existing rDNA sequences in the GenBank database were determined using Blast (version 2.2.1) at the National Center for Biotechnology Information (Altschul et al 1997).

Sequences obtained from our samples and those from GenBank were aligned at 1014 positions using Sequencher (GeneCodes Inc., Ann Arbor, MI) and manually adjusted. The taxa obtained from GenBank were selected to provide a broad coverage among major groups of fungi; additional taxa were included based on best matches to environmental sequences during the BLAST analyses (TABLE I). Highly variable regions V4–V8 (Borneman and Hartin 2000) contained within our sequences were omitted as unalignable (281 positions). Similarly, the alignment contained several gaps and insertions that were also omitted. Accordingly, the final data set contained 733 characters, 245 of which were parsimony informative. The taxonomic relationships among the fungal sequences were inferred using the neighbour joining (NJ) and maximum parsimony (MP) analyses in PAUP* (Swofford 2001). For the NJ analyses, rates for variable sites were assumed equal and no sites were assumed invariable. Data matrices were corrected using Jukes-Cantor correction. Sites with missing data—ambiguous nucleotides or gaps—were ignored for the affected pairwise comparisons. The most parsimonious trees were obtained via a full heuristic search using a random starting tree option and branch swapping with tree bisection reconnection (TBR). Gaps were treated as missing characters. The number of equiparsimonious trees was expected to be high attributable to several closely related sequences in the clone libraries resulting in poor and incomplete resolution in the terminal clades. As a result, the maximum number of retained trees was restricted to one thousand (option MAXTREES = 1000) and branch swapping was performed only among the best trees. The robustness of the inferred NJ and MP topologies was tested by one thousand bootstrap replicates. For the parsimony analyses, full heuristic searches were replicated one thousand times and groups with frequency greater than 50% retained. Because of the large number of possible equiparsimonious trees in MP analyses, only consensus (50% majority rule and strict) topologies were compared to those obtained by NJ analyses. Although resolution and placement within terminal clades differed, both methods resulted in largely similar topologies, which broadly agreed on the current understanding on the placement of major groups of Fungi. Attributable to the limited data, many terminal clades received no support in parsimony analyses and collapsed to basal polytomies (see FIG. 1).

The occurrence of chimeric sequences was analyzed by the use of two different methods. First, potentially chimeric sequences and their break points were determined by the Chimera Check program and Sequence Match (version 2.7) at the Ribosomal Database Project (Maidak et al 1999). The Chimera Check only suggests the possibility of a chimeric origin of a given sequence. Here, we considered sequences potentially chimeric if they had a distinct chimera break point with a score >20 in Chimera Check. Second, to confirm the chimeric sequences, the data set further tested as described previously by Jumpponen (2005) after omission of data beyond the most likely chimera break points. Briefly, to test for the chimeric environmental sequences, the data matrices were reanalyzed after exclusion of data upstream and downstream of the most commonly encountered chimera break point (position 507 in the final alignment). The obtained topologies were then compared to detect cloned sequences that dramatically changed positions in different analyses. Such sequences with unstable positions in the analyses with partial data sets were considered chimeric and omitted from further analyses. We note that, although this rather elaborate analysis is likely to identify and eliminate chimeras comprised of remotely related organisms, it is unable determine chimeras comprised of closely related taxa.

The effects of rhizosphere vs. soil, irrigation and nitrogen enrichment on the fungal community structure were tested using a phylogenetic test (*P*-test) as described in Martin

TABLE I. Analyses of clone libraries obtained from soil and rhizosphere samples in N enrichment and water addition treatments. Occurrence refers to the number of samples a RFLP-phenotype representing that clone occurred. Chimer column indicates whether a sequence received a score greater than 20 in Chimera Check of the RDP and whether the chimeric nature was confirmed in subsequent analyses that used only 5' or 3' end of the sequences. Percent similarity refers to similarity in BLAST analyses. Frequency refers to the proportion of the clones within a library comprised by that RFLP-phenotype

Clone [Accession] Occurrence	Chimer, Confirm	BLAST Match [Accession], Order (Phylum)	%Similar	Freq
Treatment N = 0 g m^{-2} Water = 0				
Soil_01_01 [AF504073]	Chimer, Yes	*Chaetomium globosum* [AB048285], Sordariales (Asc.)	97	0.05
Soil_01_02 [AF504074] 6	No, No	*Neurospora crassa* [X04971], Sordariales (Asc.)	98	0.17
Soil_01_03 [AF504075]	Chimer, Yes	*Anamylopsora pulcherrima* [AF119501Agyriales (Asc.)	96	0.11
Soil_01_07 [AF504076]	Chimer, No	*Dactylella oxyspora* [AF146537In certae sedis (Asc.)	96	0.11
Soil_01_10 [AF504077]	Chimer, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.06
Soil_01_11 [AF504078]	Chimer, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	94	0.06
Soil_01_12 [AF504079]	No, Yes	*Pseudogymnoascus roseus* [AB015778], Onygenales (Asc.)	94	0.11
Soil_01_14 [AF504080] 2	Chimer, No	*Sporothrix schenckii* [M85053], Ophiostomatales (Asc.)	96	0.11
Soil_01_15 [AF504081]	Chimer, No	*Rhodotorula aurantiaca* [AB030354], Uredinales (Bas.)	90	0.11
Soil_01_16 [AF504082]	Chimer, No	*Cyanodermella viridula* [U86583], Ostropales (Asc.)	95	0.06
Soil_01_19 [AF504083]	No, No	*Paraphaeosphaeria quadriseptata* [AF250826], In certae sedis (Asc.)	91	0.06
Soil_02_02 [AF504080] 2	No, No	*Sporothrix schenckii* [M85053], Ophiostomatales (Asc.)	96	0.15
Soil_02_05 [AF504074] 6	No, No	*Neurospora crassa* [X04971], Sordariales (Asc.)	98	0.08
Soil_02_06 [AF504084]	No, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.31
Soil_02_08 [AF504085]	Chimer, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	94	0.15
Soil_02_11 [AF504086]	Chimer, No	*Trapelia placodioides* [AF119500], Agyriales (Asc.)	95	0.08
Soil_02_17 [AF504087] 2	No, No	*Selenaspora guernisacii* [AF144667], Pezizales (Asc.)	99	0.08
Soil_02_19 [AF504088]	No, No	*Tricholoma myomyces* [AF287841], Agaricales (Bas.)	99	0.15
Soil_03_05 [AF504089]	No, No	*Leptosphaeria bicolor* [U04202], Pleosporales (Asc.)	97	0.18
Soil_03_06 [AF504090]	No, Yes	*Penicillium namyslowskii* [D88319], Eurotiales (Asc.)	97	0.18
Soil_03_09 [AF504087] 2	No, No	*Selenaspora guernisacii* [AF144667], Pezizales (Asc.)	99	0.18
Soil_03_10 [AY099409]	Chimer, No	*Hypomyces chrysospermus* [AB027339], Hypocreales (Asc.)	96	0.09
Soil_03_13 [AF504091] 2	Chimer, No	*Pseudallescheria ellipsoidea* [U43911], Microascales (Asc.)	95	0.18
Soil_03_15 [AF504092]	Chimer, No	*Sporothrix schenckii* [M85053], Ophiostomatales (Asc.)	96	0.18
Soil_04_04 [AF504091] 2	No, No	*Pseudallescheria ellipsoidea* [U43911], Microascales (Asc.)	95	0.29
Soil_04_06 [AF504093]	Chimer, No	*Rhizoctonia solani* [D85636], Ceratobasidiales (Bas.)	95	0.43
Soil_04_13 [AF504094]	No, Yes	*Panellus serotinus* [AF026590], Agaricales (Bas.)	95	0.29
Soil_05_03 [AF504095]	No, No	*Humidicutis marginata* [AF287833], Agaricales (Bas.)	98	0.10
Soil_05_05 [AF504096]	No, No	*Glomus proliferum* [AF213462], Glomales (Zyg.)	98	0.10
Soil_05_07 [AF504097]	Chimer, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	94	0.20
Soil_05_09 [AF504098]	Chimer, Yes	*Coccodinium bartschii* [U77668], Dothidiales (Asc.)	97	0.10
Soil_05_12 [AF504099] 3	Chimer, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.20
Soil_05_18 [AF504100]	Chimer, Yes	*Tapinella atrotomentosa* [M90824], Boletales (Bas.)	93	0.10
Soil_05_19 [AF504101]	Chimer, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.10
Soil_05_20 [AY099410]	Chimer, Yes	*Beauveria bassiana* [AF280633], Hypocreales (Asc.)	98	0.10
Soil_06_05 [AF504102] 6	Chimer, No	*Rhizoctonia solani* [D85636], Ceratobasidiales (Bas.)	95	0.73
Soil_06_14 [AF504098] 3	No, No	*Coccodinium bartschii* [U77668], Dothidiales (Asc.)	97	0.09
Soil_06_17 [AY099411]	Chimer, Yes	*Unidentified eukaryote* [AJ130850], ????	98	0.18
Treatment N = 10 g m^{-2} Water = 0				
Soil_07_02 [AF504103]	Chimer, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.08
Soil_07_04 [AF504099] 3	No, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.17
Soil_07_07 [AF504104]	No, No	*Entoloma strictius* [AF287832], Agaricales (Bas.)	97	0.08
Soil_07_08 [AY099412]	No, No	*Neurospora crassa* [X04971], Sordariales (Asc.)	99	0.08
Soil_07_09 [AF504105]	No, No	*Tricholoma myomyces* [AF287841], Agaricales (Bas.)	98	0.17
Soil_07_10 [AF504106]	No, No	*Sporothrix schenckii* [M85053], Ophiostomatales (Asc.)	96	0.08
Soil_07_19 [AF504107]	No, No	*Tricholoma myomyces* [AF287841], Agaricales (Bas.)	98	0.25
Soil_07_20 [AF504108]	Chimer, Yes	*Spizellomyces acuminatus* [M59759], Spizellomycetales (Chy.)	93	0.17
Soil_09_01 [AF504099] 3	No, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.25

TABLE I. Continued

Clone [Accession] Occurrence	Chimer, Confirm	BLAST Match [Accession], Order (Phylum)	%Similar	Freq
Soil_09_13 [AF504109] [2]	No, No	*Entoloma strictius* [AF287832], Agaricales (Bas.)	97	0.25
Soil_09_15 [AF504102] [6]	No, No	*Rhizoctonia solani* [D85636], Ceratobasidiales (Bas.)	95	0.50
Soil_09_17 [AY099412]	No, No	*Neurospora crassa* [X04971], Sordariales (Asc.)	99	0.05
Soil_10_03 [AF504110]	No, No	*Tricholoma myomyces* [AF287841], Agaricales (Bas.)	98	0.25
Soil_10_04 [AF504111]	No, No	*Sporothrix schenckii* [M85053], Ophiostomatales (Asc.)	96	0.17
Soil_10_10 [AF504112]	Chimer, Yes	*Illosporium carneum* [AF289655], Hypocreales (Asc.)	96	0.08
Soil_10_11 [AF504113]	No, Yes	*Tricholoma myomyces* [AF287841], Agaricales (Bas.)	99	0.08
Soil_10_13 [AF504114]	No, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.17
Soil_10_19 [AF504115]	No, No	*Boletus satanas* [M94337], Boletales (Bas.)	94	0.25
Soil_11_10 [AY099413]	No, No	*Illosporium carneum* [AF289655], Hypocreales (Asc.)	99	0.64
Soil_11_14 [AY773764]	No, No	*Illosporium carneum* [AF289655], Hypocreales (Asc.)	99	0.36
Treatment N = 0 g m^{-2} Water = + 80-90%				
Soil_13_01 [AY773765]	Chimer, Yes	*Ajellomyces capsulatus* [AF320009], Onygenales (Asc.)	98	0.06
Soil_13_03 [AY773766]	Chimer, Yes	*Tapinella atrotomentosa* [M90824], Boletales (Bas.)	93	0.06
Soil_13_04 [AF504102] [6]	No, No	*Rhizoctonia solani* [D85636], Ceratobasidiales (Bas.)	95	0.12
Soil_13_06 [AY773767]	No, No	*Boletus satanas* [M94337], Boletales (Bas.)	94	0.18
Soil_13_10 [AY773768]	No, No	*Chaetomium elatum* [M83257], Sordariales (Asc.)	99	0.29
Soil_13_17 [AY773769] [3]	Chimer, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	95	0.29
Soil_14_01 [AF504075] [2]	No, No	*Anamylopsora pulcherrima* [AF119501], Agyriales (Asc.)	96	0.07
Soil_14_02 [AY773770]	No, No	*Rhizoctonia solani* [D85636], Ceratobasidiales (Bas.)	95	0.21
Soil_14_03 [AY773771]	No, No	*Glomus proliferum* [AF213462], Glomales (Zyg.)	98	0.07
Soil_14_06 [AY773772] [6]	No, No	*Rhizoctonia solani* [D85636], Ceratobasidiales (Bas.)	95	0.21
Soil_14_07 [AY773773]	Chimer, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.14
Soil_14_11 [AY773774]	Chimer, Yes	*Cf. Marchandiomyces* [AF289662], ??? (Bas.)	96	0.07
Soil_14_14 [AY773775]	Chimer, No	*Illosporium carneum* [AF289655], Hypocreales (Asc.)	96	0.14
Soil_14_16 [AY773776]	No, No	*Scutellinia scutellata* [U53387], Pezizales (Asc.)	98	0.07
Soil_15_02 [AY773777]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	98	0.33
Soil_15_06 [AY773778]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	98	0.42
Soil_15_13 [AY773779]	Chimer, No	*Pestalotiopsis sp.* [AF346561], Xylariales (Asc.)	94	0.08
Soil_15_14 [AY773780]	Chimer, Yes	*Laccaria pumila* [AF287838], Agaricales (Bas.)	99	0.08
Soil_15_19 [AY773781]	No, No	*Oidiodendron tenuissimum* [AB015787], Onygenales (Asc.)	99	0.08
Soil_16_02 [AY773782]	Chimer, Yes	*Clavaria acuta* [AF184180], Aphyllophorales (Bas.)	93	0.10
Soil_16_05 [AY773783]	Chimer, Yes	*Ceramothyrium linnaeae* [AF022715], Chaetothyriales (Asc.)	97	0.10
Soil_16_06 [AY773784]	Yes, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.20
Soil_16_09 [AY773785]	No, Yes	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	94	0.30
Soil_16_19 [AY773786]	No, No	*Boletus satanas* [M94337], Boletales (Bas.)	95	0.30
Soil_17_03 [AY773787]	No, No	*Leptosphaeria bicolor* [U04202], Pleosporales (Asc.)	96	0.08
Soil_17_07 [AF504102] [6]	No, No	*Rhizoctonia solani* [D85636], Ceratobasidiales (Bas.)	95	0.17
Soil_17_09 [AY773788] [2]	No, No	*Leptosphaeria bicolor* [U04202], Pleosporales (Asc.)	98	0.17
Soil_17_14 [AY773789] [6]	No, No	*Neurospora crassa* [X04971], Sordariales (Asc.)	99	0.08
Soil_17_16 [AY773790] [2]	Chimer, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	94	0.08
Soil_17_18 [AY773791]	No, No	*Chromocleista malachitea* [D88323], Eurotiales (Asc.)	99	0.08
Soil_17_19 [AY773792]	No, No	*Rhizoctonia solani* [D85636], Ceratobasidiales (Bas.)	94	0.08
Soil_17_20 [AY773793]	Chimer, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	92	0.25
Soil_18_01 [AY773788] [2]	No, No	*Leptosphaeria bicolor* [U04202], Pleosporales (Asc.)	98	0.20
Soil_18_03 [AY773789] [6]	No, No	*Neurospora crassa* [X04971], Sordariales (Asc.)	99	0.20
Soil_18_05 [AY773794]	No, No	*Kirschsteiniothelia elaterascus* [AF053728], Pleosporales (Asc.)	98	0.07
Soil_18_08 [AY773795]	No, No	*Trichophaea hybrida* [U53390], Pezizales (Asc.)	98	0.20
Soil_18_09 [AY773796]	Chimer, No	*Cyanodermella viridula* [U86583], Ostropales (Asc.)	94	0.07
Soil_18_14 [AY773797] [2]	Chimer, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	92	0.13
Soil_18_16 [AY773798]	Chimer, No	*Phyllachora graminis* [AF064051], Phyllachorales (Asc.)	96	0.13

TABLE I. Continued

Clone [Accession] Occurrence	Chimer, Confirm	BLAST Match [Accession], Order (Phylum)	%Similar	Freq
Treatment N = 10 g m^{-2} Water = +80–90%				
Soil_19_05 [AY773790] [2]	No, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	94	0.20
Soil_19_13 [AY773799]	No, Yes	*Mortierella alpina* [AJ271730], Mucorales (Zyg.)	91	0.13
Soil_19_14 [AY773800]	Chimer, No	*Placopsis gelida* [AF119502], Agyriales (Asc.)	92	0.13
Soil_19_15 [AY773801]	No, Yes	*Boletus satanas* [M94337], Boletales (Bas.)	95	0.13
Soil_19_16 [AY773789] [6]	No, No	*Neurospora crassa* [X04971], Sordariales (Asc.)	99	0.07
Soil_19_18 [AY773802]	No, No	*Cyanodermella viridula* [U86583], Ostropales (Asc.)	93	0.20
Soil_19_19 [AY773803]	Chimer, No	*Cyanodermella viridula* [U86583], Ostropales (Asc.)	94	0.13
Soil_20_02 [AY773804]	No, No	*Oidiodendron tenuissimum* [AB015787], Onygenales (Asc.)	99	0.25
Soil_20_04 [AY773805]	No, No	*Trapelia placodioides* [AF119500], Agyriales (Asc.)	95	0.25
Soil_20_12 [AY773789] [6]	No, No	*Neurospora crassa* [X04971], Sordariales (Asc.)	99	0.13
Soil_20_14 [AY773788] [3]	No, No	*Leptosphaeria bicolor* [U04202], Pleosporales (Asc.)	98	0.13
Soil_20_18 [AY773806]	No, No	*Paraphaeosphaeria quadriseptata* [AF250826], In certae sedis (Asc.)	99	0.25
Soil_22_02 [AY773773] [3]	No, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.17
Soil_22_03 [AY773790] [3]	No, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	94	0.33
Soil_22_04 [AY773769] [3]	No, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	95	0.08
Soil_22_07 [AY773807]	No, No	*Glomus proliferum* [AF213462], Glomales (Zyg.)	97	0.25
Soil_23_14 [AY773769] [3]	No, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	95	0.22
Soil_23_15 [AY773773] [3]	No, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.44
Soil_23_20 [AY773808] [6]	No, No	*Neurospora crassa* [X04971], Sordariales (Asc.)	99	0.33
Soil_24_09 [AY773773] [3]	No, No	*Sphaerotheca cucurbitae* [AB033482], Erysiphales (Asc.)	95	0.18
Soil_24_11 [AY773809]	No, No	*Arxula terrestris* [AB000663], Sacharomycetales (Asc.)	94	0.18
Soil_24_19 [AY773810]	No, No	*Graphium calicioides* [AB007655], Chaetothyriales (Asc.)	97	0.18
Soil_24_20 [AY773789] [6]	No, No	*Neurospora crassa* [X04971], Sordariales (Asc.)	99	0.45
Treatment N = 0 g m−2 Water = 0				
Root_01_02 [AF504116]	No, No	*Calvatia gigantea* [AF026622], Lycoperdales (Bas.)	98	0.43
Root_01_07 [AF504117]	Chimer, Yes	*Mortierella alpina* [AJ271729], Mucorales (Zyg.)	92	0.07
Root_01_11 [AF504118]	No, No	*Auricularia polytricha* [L22255], Auriculariales (Bas.)	98	0.07
Root_01_12 [AF504119]	No, No	*Myrothecium leucotrichum* [AJ301992], In certae sedis (Asc.)	99	0.21
Root_01_13 [AF504120] [2]	No, No	*Calvatia gigantea* [AF026622], Lycoperdales (Bas.)	97	0.07
Root_01_14 [AF504121]	No, No	*Boletus satanas* [M94337], Boletales (Bas.)	95	0.07
Root_01_18 [AF504122]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	97	0.14
Root_02_01 [AF504122] [3]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	97	0.18
Root_02_04 [AY099404]	Chimer, Yes	*Poronia punctata* [AF064052], Xylariales (Asc.)	97	0.09
Root_02_05 [AF504123]	Chimer, No	*Leptosphaeria bicolor* [U04202], Pleosporales (Asc.)	97	0.27
Root_02_06 [AF504116] [2]	No, No	*Calvatia gigantea* [AF026622], Lycoperdales (Bas.)	97	0.09
Root_02_08 [AF504124]	No, No	*Leptosphaeria bicolor* [U04202], Pleosporales (Asc.)	97	0.18
Root_02_09 [AF504125]	No, No	*Tricholoma myomyces* [AF287841], Agaricales (Bas.)	99	0.18
Root_03_02 [AF504126]	No, No	*Glomus proliferum* [AF213462], Glomales (Zyg.)	98	0.11
Root_03_08 [AY099405]	Chimer, No	*Hypomyces chrysospermus* [AB027339], Hypocreales (Asc.)	96	0.11
Root_03_14 [AF504121] [3]	No, No	*Boletus satanas* [M94337], Boletales (Bas.)	95	0.22
Root_03_17 [AY099416]	No, No	*Trapelia placodioides* [AF119500], Agyriales (Asc.)	95	0.44
Root_03_20 [AF504127]	No, No	*Graphium calicioides* [AB007655], Chaetothyriales (Asc.)	97	0.11
Root_04_02 [AF504122] [3]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	97	0.25
Root_04_03 [AF504116] [2]	No, No	*Calvatia gigantea* [AF026622], Lycoperdales (Bas.)	97	0.13
Root_04_04 [AF504121] [3]	No, No	*Boletus satanas* [M94337], Boletales (Bas.)	95	0.25
Root_04_14 [AF504128]	Chimer, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	98	0.25
Root_04_19 [AY099408]	Chimer, Yes	*Chondrostereum purpureum* [AF082851], Stereales (Bas.)	98	0.13
Root_06_02 [AF504129] [2]	No, No	*Cystostereum murraii* [AF082850], Aphyllophorales (Bas.)	97	0.85
Root_06_06 [AF504130] [3]	No, No	*Entoloma strictius* [AF287832], Agaricales (Bas.)	97	0.15

TABLE I. Continued

Clone [Accession] Occurrence	Chimer, Confirm	BLAST Match [Accession], Order (Phylum)	%Similar	Freq
Treatment N = 10 g m^{-2} Water = 0				
Root_07_06 [AF504131]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	98	0.60
Root_07_19 [AF504132]	No, No	*Chromocleista malachitea* [D88323], Eurotiales (Asc.)	99	0.40
Root_08_01 [AF504131] [2]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	98	0.50
Root_08_13 [AF504133]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	98	0.17
Root_08_18 [AY099416] [2]	No, No	*Trapelia placodioides* [AF119500], Agyriales (Asc.)	95	0.33
Root_09_10 [AF504134]	Chimer, No	*Arxula terrestris* [AB000663], Saccharomycetales (Asc.)	94	0.09
Root_09_11 [AF504130] [3]	No, No	*Entoloma strictius* [AF287832], Agaricales (Bas.)	97	0.18
Root_09_15 [AF504135]	No, No	*Dark septate endophyte* [AF178177], Unknown (Asc.)	98	0.36
Root_09_16 [AY099415]	Chimer, Yes	*Neurospora crassa* [X04971], Sordariales (Asc.)	98	0.09
Root_09_17 [AF504136]	Chimer, Yes	*Selenaspora guernisacii* [AF144667], Pezizales (Asc.)	97	0.09
Root_09_20 [AF504137]	Chimer, No	*Leptosphaeria bicolor* [U04202], Pleosporales (Asc.)	98	0.18
Root_10_02 [AF504130] [3]	No, No	*Entoloma strictius* [AF287832], Agaricales (Bas.)	97	0.60
Root_10_03 [AF504138] [2]	No, No	*Paraphaeosphaeria quadriseptata* [AF250826], In certae sedis (Asc.)	97	0.20
Root_10_15 [AF504139]	Chimer, Yes	*Oidiodendron tenu* [AB015787], Onygenales, (Asc.)	95	0.20
Root_11_09 [AF504140]	Chimer, Yes	*Claviceps purpurea* [AF281178], Clavicipitales (Asc.)	96	0.20
Root_11_10 [AY099414]	Chimer, Yes	*Magnaporthe grisea* [AB026819], In certae sedis (Asc.)	90	0.20
Root_11_11 [AF504135]	Chimer, Yes	*Gibberella pulicaris* [AF149875], Hypocreales (Asc.)	97	0.20
Root_11_20 [AF504129] [2]	No, No	*Cystostereum murraii* [AF082850], Aphyllophorales (Bas.)	97	0.40
Treatment N = 0 g m^{-2} Water = +80–90%				
Root_14_01 [AY773716]	No, No	*Cordyceps ophioglossoides* [AB027321], Clavicipitales (Asc.)	96	0.30
Root_14_08 [AY773717]	No, No	*Auricularia polytricha* [L22255], Auriculariales (Bas.)	97	0.30
Root_14_09 [AY773718]	No, No	*Glomus fasciculatum* [Y17640], Glomales (Zyg.)	99	0.10
Root_14_10 [AY773719]	No, Yes	*Torulaspora delbrueckii* [X98120] Saccharomycetales (Asc.)	94	0.10
Root_14_14 [AY773720]	Chimer, Yes	*Cyanodermella viridula* [U86583], Ostropales (Asc.)	94	0.10
Root_14_17 [AY773721]	Chimer, Yes	*Cordyceps ophioglossoides* [AB027321], Clavicipitales (Asc.)	93	0.10
Root_14_20 [AY773722]	No, No	*Sporothrix schenckii* [M85053], Ophiostomatales (Asc.)	96	0.10
Root_16_03 [AY773723]	No, No	*Glomus geosporum* [AJ245637], Glomales (Zyg.)	98	0.50
Root_16_09 [AY773724]	No, No	*Cordyceps ophioglossoides* [AB027321], Clavicipitales (Asc.)	96	0.50
Root_17_01 [AY773725]	No, Yes	*Myrothecium inundatum* [AJ302005], Hypocreales (Asc.)	99	0.17
Root_17_09 [AY773726]	Chimer, Yes	*Cordyceps ophioglossoides* [AB027321], Clavicipitales (Asc.)	96	0.33
Root_17_11 [AY773727]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	98	0.17
Root_17_19 [AY773728]	Chimer, Yes	*Myrothecium inundatum* [AJ302005], Hypocreales (Bas.)	99	0.33
Root_18_01 [AY773729]	No, No	*Trapelia placodioides* [AF119500], Agyriales (Asc.)	94	0.07
Root_18_04 [AY773730]	No, Yes	*Oidiodendron tenuissimum* [AB015787], Onygenales (Asc.)	95	0.53
Root_18_05 [AY773731] [2]	No, No	*Auricularia polytricha* [L22255], Auriculariales (Bas.)	97	0.13
Root_18_12 [AY773732]	No, No	*Cordyceps ophioglossoides* [AB027321], Clavicipitales (Asc.)	96	0.13
Root_18_14 [AY773733]	No, Yes	*Oidiodendron tenuissimum* [AB015787], Onygenales (Asc.)	97	0.07
Root_18_19 [AY773734]	No, No	*Arxula terrestris* [AB000663], Saccharomycetales (Asc.)	95	0.07
Treatment N = 10 g m^{-2} Water = +80–90%				
Root_19_05 [AY773735]	Chimer, Yes	*Mycoarthris corallinus* [AF128439], Helotiales (Asc.)	97	0.10
Root_19_08 [AY773736]	No, No	*Monographella nivalis* [AF064049], Xylariales (Asc.)	99	0.30
Root_19_10 [AY773737]	No, No	*Tricholoma myomyces* [AF287841], Agaricales (Bas.)	98	0.10
Root_19_12 [AY773738]	No, No	*Sporothrix schenckii* [M85053], Ophiostomatales (Asc.)	97	0.50
Root_20_03 [AY773739]	Chimer, No	*Lycoperdon sp.* [AF026619], Lycoperdales (Bas.)	98	0.73
Root_20_11 [AY773740]	No, No	*Rhizoctonia solani* [D85636], Ceratobasidiales (Bas.)	95	0.18
Root_20_20 [AY773741]	Chimer, Yes	*Chondrostereum purpureum* [AF082851], Stereales (Bas.)	97	0.09
Root_21_04 [AY773742]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	97	0.67
Root_21_09 [AY773743]	No, Yes	*Calvatia gigantea* [AF026622], Lycoperdales (Bas.)	97	0.08
Root_21_11 [AY773744]	No, No	*Laccaria pumila* [AF287838], Agaricales (Bas.)	97	0.08

TABLE I. Continued

Clone [Accession] Occurrence	Chimer, Confirm	BLAST Match [Accession], Order (Phylum)	%Similar	Freq
Root_21_15 [AY773745]	No, No	*Glomus mossea* [U96141], Glomales (Zyg.)	97	0.08
Root_21_18 [AY773746]	Chimer, No	*Leptosphaeria bicolor* [U04202], Pleosporales (Asc.)	98	0.08
Root_21_20 [AY773747]	No, No	*Monographella nivalis* [AF064049], Xylariales (Asc.)	99	0.08
Root_22_01 [AY773748]	No, No	*Tricholoma myomyces* [AF287841], Agaricales (Bas.)	99	0.42
Root_22_06 [AY773749]	Chimer, Yes	*Daedalea quercina* [AF026600], Aphyllophorales (Bas.)	93	0.17
Root_22_07 [AY773750]	Chimer, Yes	*Oidiodendron tenuissimum* [AB015787], Onygenales (Asc.)	98	0.08
Root_22_13 [AY773751]	No, No	*Phyllachora graminis* [AF064051], Phyllachorales (Asc.)	97	0.17
Root_22_15 [AY773752]	No, No	*Gibberella pulicaris* [AF149875], Hypocreales (Asc.)	99	0.17
Root_23_01 [AY773753]	Chimer, No	*Petriella setifera* [U43908], Microascales (Asc.)	90	0.18
Root_23_02 [AY773754]	Chimer, No	*Penicillium freii* [AJ005446], Eurotiales (Asc.)	91	0.18
Root_23_03 [AY773755]	Chimer, No	*Glomus fasciculatum* [Y17640], Glomales (Zyg.)	95	0.09
Root_23_04 [AY773756]	Chimer, No	*Laodelphax striatellus* [AF267232], In certae sedis (Asc.)	90	0.09
Root_23_06 [AY773757]	Chimer, Yes	*Graphium calicioides* [AB007655], Chaetothyriales (Asc.)	94	0.18
Root_23_12 [AY773758]	No, No	*Trapelia placodioides* [AF119500], Agyriales (Asc.)	95	0.18
Root_23_17 [AY773759]	No, Yes	*Cordyceps konnoana* [AB031192], Clavicipitales (Asc.)	96	0.09
Root_24_02 [AY773760]	No, No	*Kirschsteiniothelia elaterascus* [AF053728], Pleosporales (Asc.)	97	0.38
Root_24_03 [AY773761]	No, Yes	*Cystostereum murraii* [AF082850], Aphyllophorales (Bas.)	97	0.38
Root_24_04 [AY773762]	No, No	*Cordyceps ophioglossoides* [AB027321], Clavicipitales (Asc.)	96	0.13
Root_24_13 [AY773763]	No, No	*Monographella nivalis* [AF064049], Xylariales (Asc.)	99	0.13

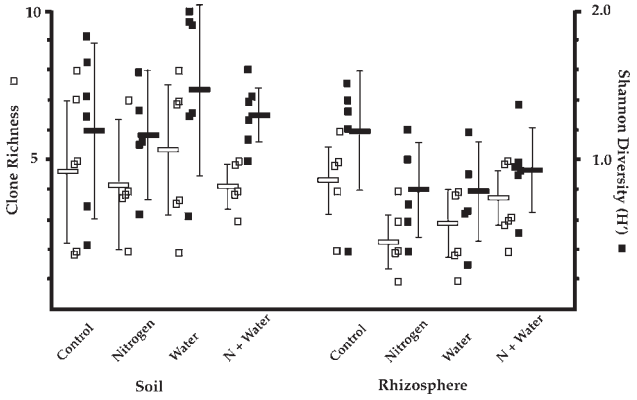

FIG. 1. Observed OTU richness in the clone libraries (left Y-axis, open squares) and Shannon diversity index (right Y-axis, filled squares) among the four treatments in soil and roots. The open (OTU richness) and closed rectangles (Shannon diversity index) indicate the means and the associated bars indicate standard deviations (mean ± 1 SD). Note that the richness and diversity did not differ among the four treatments, whereas both richness and diversity were higher in soil than in the roots.

(2002). We tested whether the distributions of sequences covaried with the obtained NJ and MP topologies. Presence of sequences obtained from each treatment were optimized on the topologies in MacClade (Maddison and Maddison 2001) and analyzed separately. The NJ and MP topologies provided an estimate of the minimum number of changes in the rhizosphere vs. soil, irrigation and N treatments to explain their observed distribution in the obtained topologies. The significance of this observed covariation with the topologies was established by determining the expected number of changes under the null hypothesis that no covariation exists (Martin 2002). The expected distribution was obtained using one thousand randomly generated topologies (Maddison and Slatkin 1991). Fewer changes in observed distributions for the treatments than in random distributions indicated the community level differences among the treatments.

Estimation of the fungal diversity.—Richness and diversity of OTUs (Operational Taxonomic Units—here, cloned sequences that are <99% similar) were estimated based on sequence or RFLP phenotype frequencies after omission of all chimeric data. We used Shannon diversity index H′ (Shannon and Weaver 1949) to estimate diversity within each clone library obtained from a plot. Two different estimates were used to assess the proportion of the taxa that were detected in our analyses. First, species area curves were estimated using PC-ORD for Windows (McKune 1991) and potential richness extrapolated using first and second order jackknife estimators. The species area curves indicated that the diversity increased linearly within the number of samples analyzed in this study (data not shown). Second, the proportion of taxa detected was estimated using a coverage metric (Mullins et al 1995) that provides an estimate of how well the

sample sizes reflect the apparent diversity among the samples.

Statistical analyses.—Observed richness, Shannon diversity index, and coverage were compared among the treatments (N enrichment and irrigation) and among the soil type (rhizosphere vs. non-rhizosphere soil) using PROC GLM in SAS (SAS 1989). Nested models were used so that the treatment effects could be analyzed within each of the two soil types (soil vs. root). When significant treatment effects were found differing means were identified using Tukey-Kramer pairwise comparisons at $\alpha = 0.05$.

RESULTS

We obtained 90 fungal sequences from the root samples and 119 fungal sequences from the soil samples (TABLE I). The sequences represented all four fungal phyla (Ascomycota—48, Basidiomycota—36 and Zygomycota—6 in the root samples; Ascomycota—85, Basidiomycota—29, Chytridiomycota—1 and Zygomycota—4 in the soil samples). Chimeric sequences were relatively frequent, 21 chimeric sequences (17.6%) were detected and confirmed in the soil clone libraries and 24 (26.6%) in the root clone libraries (TABLE I). The Chimera Check of RDP seemed more sensitive than the testing of stability of sequences in the inferred topologies. The Chimera Check detected 42 possible chimeras in the soil samples and 30 in the root samples, whereas 21 and 24 were confirmed by partial analyses of 5′- and 3′-ends of the obtained sequences. Chimera Check and reanalyses agreed in 14 and 18 cases in the soil and root libraries (TABLE I).

Fungal richness in the cloned communities was high and our sampling did not reach saturation in species accumulation (data not shown). Among the 98 non-chimeric sequences from soil, 71 were unique (<98% similarity within the conserved 18S of the rDNA), while among the 66 non-chimeric sequences from the root samples, 53 were unique. Only sixteen sequences or RFLP phenotypes were detected more than once among the soil samples and only ten among the rhizosphere samples. The most frequently detected sequences among the soil clone libraries were similar (95% similarity) to *Rhizoctonia solani* and (99% similarity) to *Neurospora crassa* (both detected in six samples). Most frequently detected sequences among the root clone libraries were similar (97% similarity) to *Entoloma striatus*, to (97% similarity) *Laccaria pumila* , and to (95% similarity) *Boletus satanas* (all detected in three samples).

Clone richness ($F_{[1,33]} = 6.61$, $P = 0.0176$) and Shannon diversity index ($F_{[1,33]} = 8.71$, $P = 0.0058$) per sample were greater in soil than in the root samples, while there were no differences among the treatments ($F_{[6,33]} = 0.87$, $P > 0.10$) (FIG. 1). Jackknife estimators were nearly twice the observed richness (TABLE II). The extrapolated jackknife estimates of the true richness were supported by the coverage estimates, which indicated that less than half of the resident taxa were detected in either the soil or root samples (TABLE II). While soil and root samples did not differ in coverage ($F_{[1,33]} = 1.64$, $P > 0.10$), root samples from treatments with additional irrigation had lower coverage than other treatments (Tukey-Kramer pairwise comparison of means, $P < 0.05$).

Many of the cloned sequences may represent novel taxa. A total of 35 (35.7%) non-chimeric sequences in the soil libraries and 11 (16.6%) in the rhizosphere libraries were less than 96% similar to sequences available in the GenBank. Approximately half of these putatively novel sequences (ca. 15%; 22 sequences from 15 different soil samples and 2 from different root samples) formed a well-supported clade (100% and 99% bootstrap support in NJ and MP analyses; FIG. 2). Although the placement of this group could not be unambiguously resolved in our analyses, our NJ analysis (FIG. 2) and MP analyses (data not shown) consistently supported a placement as a sister group to other ascomycetes in the inferred topologies. It is likely that this group represents a novel but frequent, higher-level taxon (order or above) that has escaped detection in earlier studies focusing on fungal communities in soil and rhizosphere. The remaining novel sequences were nested among the basidiomycetous hymenomycetes and filamentous ascomycetes, reflecting likely the limited sampling of soil- and root-inhabiting fungi in GenBank.

Ascomycetes dominated the fungal communities. In soil, more than 70% of the sequences were ascomycetous, while in the roots ca. 50% of the sequences were ascomycetous. A wide array of filamentous ascomycetes (including Chaetotheriomycetes, Dothideomycetes, Eurotiomycetes, Leotiomycetes, Pezizomycetes and Sordariomycetes; FIG. 2) were detected in addition to the novel group mentioned above. However, no Taphrinomycetes or Saccharomycetes were observed. Basidiomycetous fungi from our samples were mainly nested within Hymenomycetes, although representatives of Urediniomycetes and Ustilaginomycetes were also detected exclusively in the soil samples. All zygomycetous sequences were nested within the Glomeromycota (arbuscular mycorrhizal fungi); soil and rhizosphere sequences represented members of Glomaceae, whereas no other arbuscular mycorrhizal families (e.g. Scutellosporaceae and Gigasporaceae) were detected. The only Chytridiomycetous sequence was

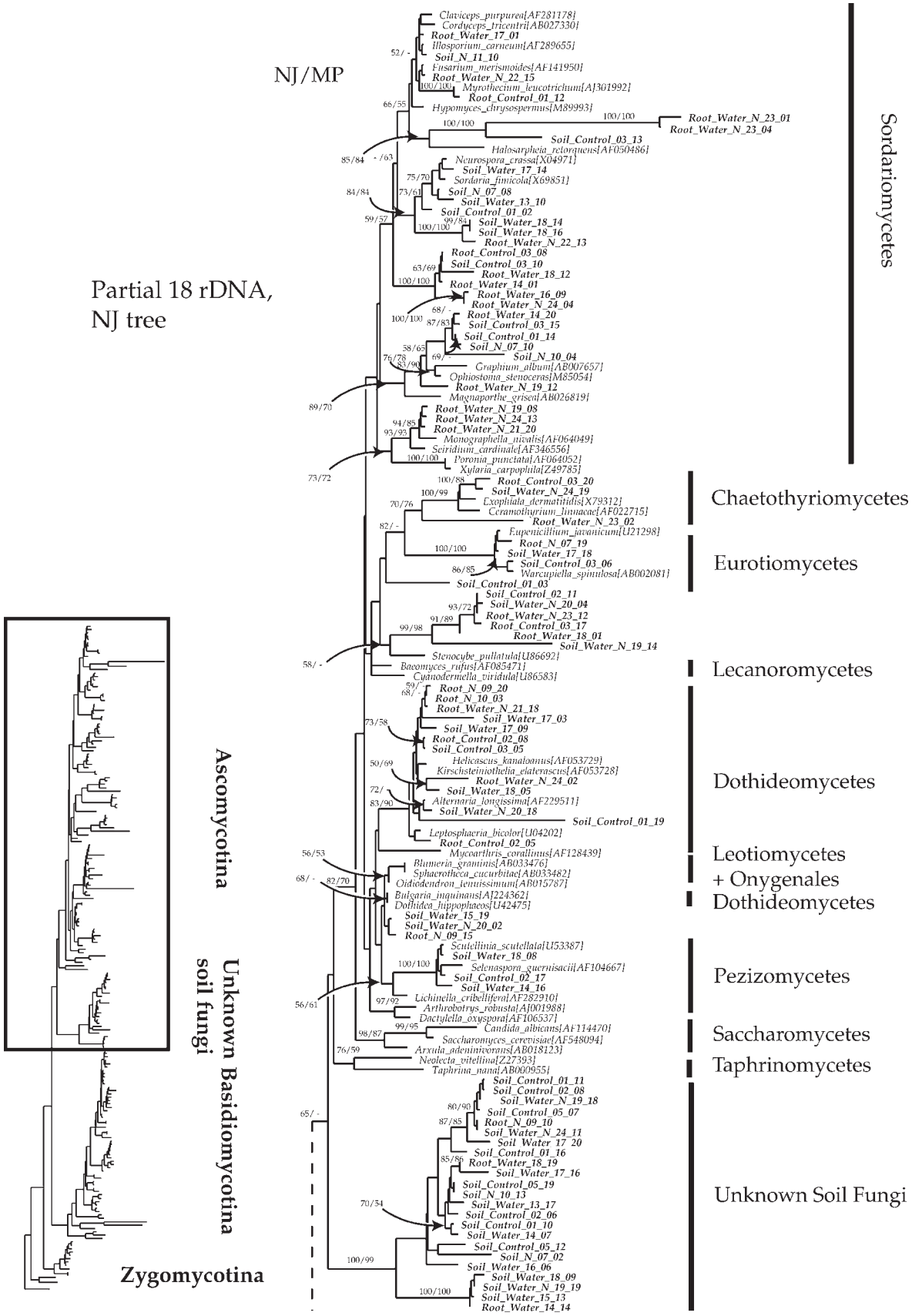

FIG. 2. Continued on next page.

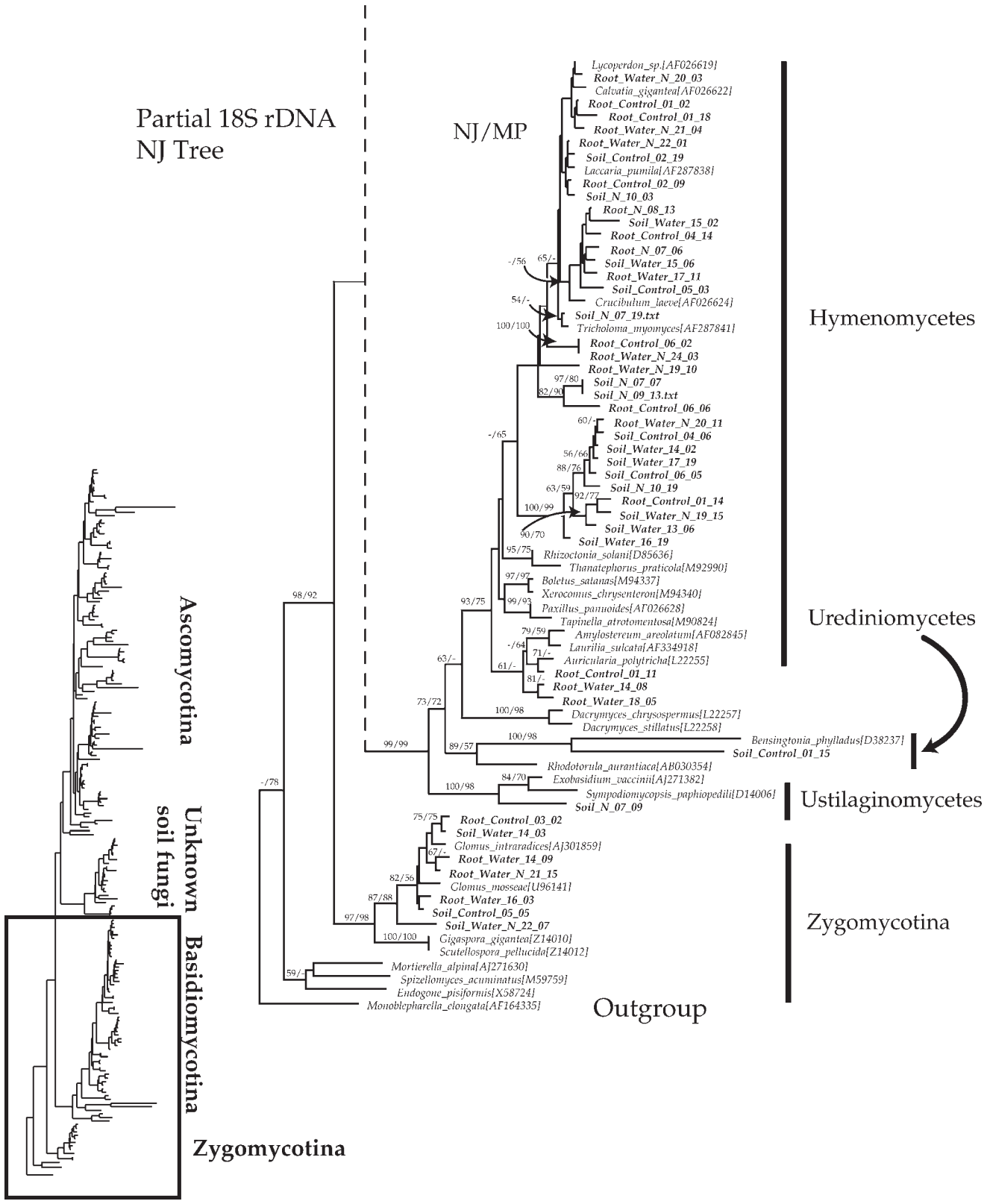

Fig. 2. Inferred neighbor joining topology of 18S rDNA sequences obtained from soil and rhizosphere in a tallgrass prairie ecosystem as well as representation of fungal taxa obtained from the GenBank. Numbers above the nodes indicate support (%) from 1000 neighbor joining (NJ) or maximum parsimony (MP) bootstrap replicates (NJ/MP). Soil or root in the sample labels refer to soil or rhizosphere samples. Control, water and N refer to treatments that did not receive additional irrigation and nitrogen, were irrigated or N amended, respectively.

TABLE II. Observed (total and mean ± 1 standard deviation) and extrapolated jackknife species richness estimates, coverage and estimated diversity among the four N enrichment and irrigation treatments in soil and rhizosphere environments. N0 = no additional N, N10 = N enrichment (10 g m^{-2} a^{-1}), Water0 = no additional water, Water80 = Irrigation (80–90% of the necessary irrigation to alleviate water stress)

Treatment	Total Species Richness	Mean Species Richness	1st Order JackKnife	2nd Order JackKnife	Coverage (%)	Shannon H′
Soil						
N0, Water0	24	4.83 ± 2.48	39.8	49.8	41.4	1.24 ± 0.54
N10, Water0	14	4.25 ± 2.06	24.0	30.9	29.4	1.15 ± 0.39
N0, Water80	28	5.33 ± 2.34	48.8	64.4	31.3	1.46 ± 0.51
N10, Water80	14	4.20 ± 0.84	22.3	27.9	52.4	1.3 ± 0.19
Overall Soil	71	4.65 ± 1.98	126.6	174.5	40.4	1.30 ± 0.43
Rhizosphere						
N0, Water0	15	4.40 ± 1.52	25.0	33.0	54.5	1.20 ± 0.44
N10, Water0	10	2.40 ± 1.14	14.8	19.0	50.0	0.78 ± 0.33
N0, Water80	11	2.75 ± 1.50	20.2	27.5	9.1	0.77 ± 0.37
N10, Water80	20	3.50 ± 1.22	35.8	48.0	4.8	0.93 ± 0.26
Overall Rhizosphere	53	3.26 ± 1.45	97.1	136.6	33.3	0.93 ± 0.37

omitted from further analyses because of its possibly chimeric origin.

Phylogenetic tests (*P*-tests) did not indicate any covariance between the obtained topologies and N enrichment or irrigation treatments (FIG. 3a, b) suggesting that these treatments did not change the fungal community composition. However, soil and root samples were distributed in the topologies differently (FIG. 3c). The covariance in the significant *P*-tests was largely attributable to the novel ascomycetous group of sequences that were nearly unique to soil samples. With the exception of the Urediniomycetes and Ustilaginomycetes, the rest of the sequences did not show a detectable pattern in their distribution, but the soil and root samples were nested (FIG. 2).

DISCUSSION

The most important finding of our study may be the abundant, novel soil-inhabiting fungi placed as a well-supported sister group to other ascomycetes. This group is placed conspicuously similarly to an unknown group of ascomycetes detected earlier in alpine tundra soil (Schadt et al 2003). Unfortunately, direct comparisons are not possible because we sequenced the small subunit of the ribosomal RNA gene, whereas Schadt et al (2003) used a partial sequence of the large subunit of the ribosomal RNA gene and the internal transcribed spacer region for their analyses. Our data as well as those by others (Schadt et al 2003, Vandenkoornhuyse et al 2002b) strongly suggest that discovery of undetected taxa on the ordinal or higher taxonomical level may be a rule rather than an exception. The soil environment likely hosts abundant fungi that have escaped detection by fruiting body surveys or culture-dependent and microscopic methods thus far.

In addition to the probable novel group of ascomycetes, several sequences less than 96% similar to those available in the GenBank were detected. Because the small subunit of the rDNA is conserved at least on the species level, our choice of 96% similarity threshold to distinguish taxa should provide a conservative estimate of species richness (Kowalchuk et al 1997, Smit et al 1999). Regardless of the chosen level for estimating the number of novel taxa, these observations support the hypothesized presence of a large number of filamentous fungi that have not been detected previously (Hawksworth 2001). However, these observations may be exaggerated and largely indicate the limited sampling of soil-inhabiting fungi in GenBank. GenBank accessions are most likely to include charismatic macromycetes, microfungi of economical importance, or abundant pathogens of plants and animals. Our results emphasize the importance of developing and employing culture-independent methods for estimating fungal diversity and for studying fungal community composition in new or previously explored habitats and substrates as well as continuing accessioning of a broad selection of fungi into the available databases.

Our data present a conservative estimate of the potential fungal species richness in tallgrass prairie soil and associated rhizosphere environment. Our sample of 24 soil and root clone libraries was clearly inadequate to capture the resident diversity. The

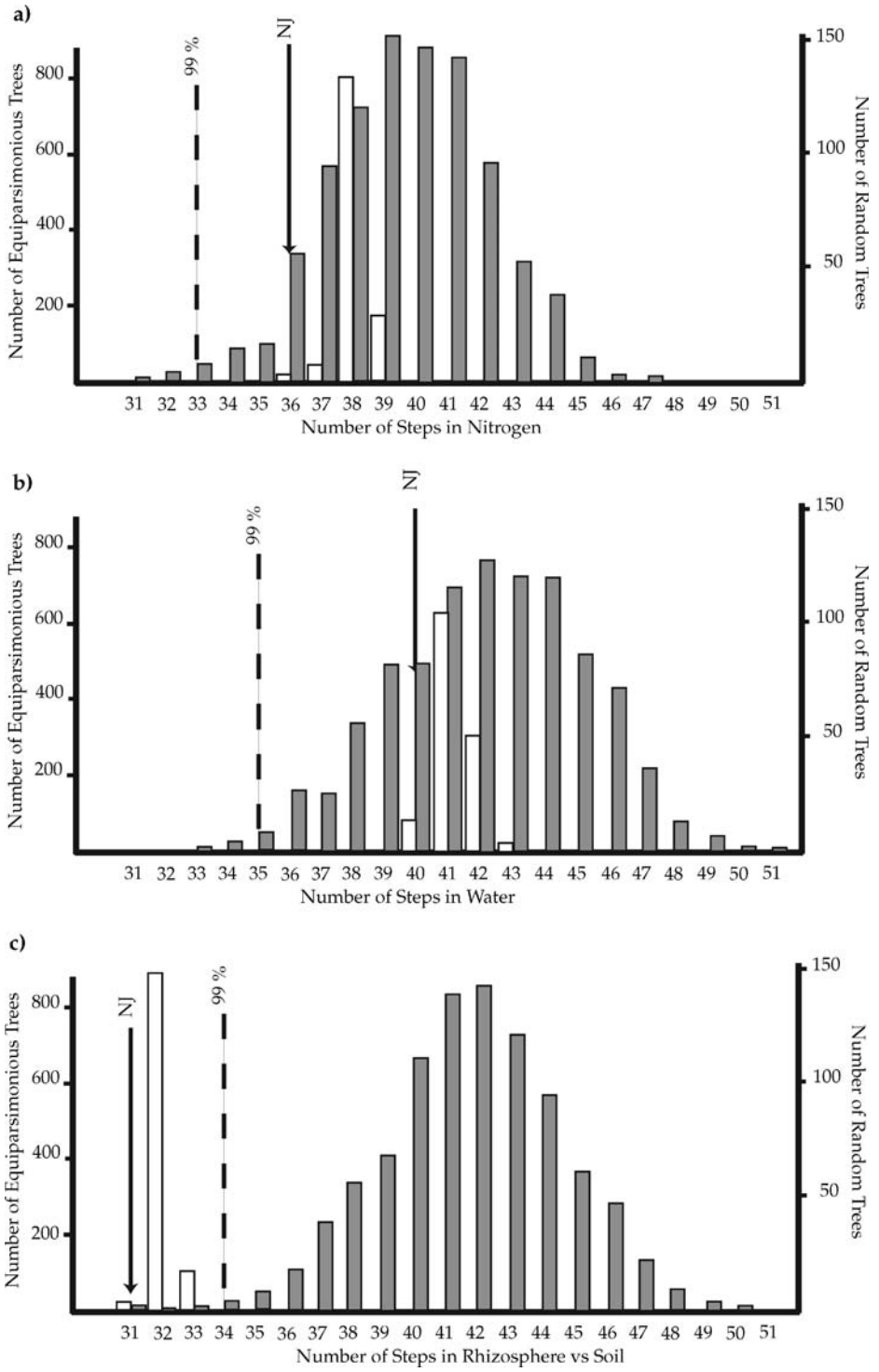

FIG. 3. Frequency distributions of required steps to describe covariation between obtained topologies and N enrichment treatment (a), irrigation treatment (b), or rhizosphere vs. soil (c). Observed frequency distributions are based on topologies of 1000 equiparsimonious trees (open bars, left Y-axis). Number of changes in one neighbor joining tree is indicated by an arrow. Frequency distributions in randomly generated topologies (filled bars, right Y-axis) were obtained from MacClade (Version 4.06; Maddison and Maddison 2001). Dashed lines indicate the 99% lower confidence limit for the randomized data. N enrichment or irrigation treatments did not differ significantly, whereas the rhizosphere and soil communities were different at $\alpha = 0.01$.

extrapolated jackknife estimates provided here also likely represent an underestimate: jackknife estimators tend to be conservative and negatively biased when true diversity substantially exceeds the observed diversity and when the sampling does not provide adequate representation of the diversity (Baltanas 1992, Palmer 1990). The coverage estimates (Mullins et al 1995) agree as they indicated that less than 50% of the resident taxa may have been observed in these soil and rhizosphere samples. Our data support high spatial heterogeneity in soil and the microbial communities dwelling therein (Franklin and Mills 2003, Trevors 1998). Accordingly, much greater sampling effort is clearly required to obtain more reliable estimates of the fungal diversity in the soil matrix as was also found by Kirk et al (2004).

Although our analyses of the conserved 18S rDNA may suffer from limited support to terminal branches, broad general inferences about the most likely affinities of our environmental sequences are possible. The communities were clearly dominated by ascomycetes. More than 60% of all cloned and analyzed sequences represented the Ascomycota. Although in contrast with earlier reports (Hunt et al 2004), this finding is not unexpected. Ascomycetes include a wide variety of saprobic taxa and may also represent numerous root-associated endophytes (Jumpponen and Trappe 1998). Similarly, the detected basidiomycetes likely represent taxa that are either saprobic or common plant root symbionts. The latter is exemplified by the frequently detected putative taxon similar to *Rhizoctonia* sp. (Sneh et al 1991). Surprisingly, obligately mutualistic zygomycetous arbuscular mycorrhizal fungi that form associations with a majority of the tallgrass prairie grasses and forbs (Smith and Read 1997, Wilson and Hartnett 1998) were detected infrequently and represented less than 10% of all detected taxa. This may be attributable to seasonal dynamics of root and soil colonization that have been suggested to include low colonization early in the growing season (Bentivenga and Hetrick 1992b, Lugo et al 2003, Lutgen et al 2003, Sanders and Fitter 1992) when our sampling was conducted. While the rDNA-based analyses are presumably free of investigator biases and those associated with culturing target organisms, variety of other, technique-related biases may compromise assessment of the community composition. Such biases may include extraction, primer, amplification, ligation and cloning biases (Chandler et al 1997, Farrelly et al 1995, Gray and Herwig 1996, Jumpponen 2005, Reysenbach et al 1992, Suzuki and Giovannoni 1996, von Wintzingerode et al 1997, Ward et al 1992, Zheng et al 1996).

We were unable to detect any responses to N enrichment or irrigation within the fungal communities—their richness, diversity or composition. This is in contrast with numerous other reports that have shown community shifts in rhizosphere and soil communities in response to N enrichment (Avis et al 2003, Egerton-Warburton and Allen 2000, Eom et al 1999, Frey et al 2004, Johnson 1993, Lilleskov et al 2002a, Peter et al 2001, Saiya-Cork et al 2002, Sinabaugh et al 2002, Wallenda and Kottke 1998). However, the overwhelming imbalance between the number of sampled soil cores or number of clones sampled from each library and species richness precluded reliable analyses of any community responses because of the lack of reoccurring taxa and the resultant minimal statistical power. While many of the previous studies have explicitly focused on either ectomycorrhizal or arbuscular mycorrhizal fungi, our sampling detected only occasional biotrophic symbionts belonging to arbuscular mycorrhizal Glomales. It is possible that mutualistic fungus communities that are directly dependent on plant photosynthates are more vulnerable to environmental alterations than are soil and rhizosphere fungi that are able to rely on various detrital carbon sources.

The irrigation effects on fungal communities have received less attention than N enrichment. However, moisture and water availability are critical factors in determining occurrence of macrofungal species across environmental gradients (Claridge et al 2000, Trudell et al 2003, Wiklund et al 1995) as well as biomass and community composition of mycorrhizal, soil-inhabiting and saprobic fungi (Cornejo et al 1994, Jensen et al 2003, Koske et al 1997, McCulley and Burke 2004, Wilkinson et al 2002). Although it is likely that availability of water is a major driver for the composition of fungal communities, the sampling conducted here did not adequately capture the resident species richness compromising our ability to detect any community level shifts.

While the limited sampling precluded reliable testing of the experimental treatment effects on diversity, richness or community composition, the soil fungal communities hosted greater richness and diversity than the root-inhabiting communities. Furthermore, the soil- and root-inhabiting fungal communities were comprised of different taxa. We point out here that our limited sampling was unlikely to detect rare or infrequent species: we were unlikely to exhaust diversity in the clone libraries and our extrapolative estimates clearly indicated that a much greater effort would have been necessary for saturation of the species accumulation curves. Accordingly, our discussion is largely limited to dominant taxa. Nonetheless, it is not surprising that root and soil communities were dominated by different fungi.

Roots and surrounding rhizosphere present a unique environment with readily available root secretions, lysates and exudates as well as sloughed off cells, whereas soil matrix beyond the rhizosphere may be an environment with different available carbon sources (Bowen and Rovira 1991, Curl and Truelove 1986, Kent and Triplett 2002, Lynch and Whipps 1990, Söderberg et al 2004). Although the differences in richness and diversity observed among the soil and root samples may partially be due to the different volumes of samples used for the DNA extraction, the different extraction methods unlikely explain the observed differences in the community composition.

In summary, our data lack the statistical power to reliably address community level shifts due to the great diversity of organisms residing in the soil and root environments. Our rDNA analyses confirm that the two contrasting environments—soil and the roots—host distinct fungal communities. A significant proportion of the fungi in soil environment may represent novel taxa, possibly including novel higher level groups of fungi.

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