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Analysis of ribosomal RNA indicates seasonal fungal community dynamics in *Andropogon gerardii* roots

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Abstract Use of the reverse-transcribed small subunit of the ribosomal RNA (rRNA) was tested for exploring seasonal dynamics of fungal communities associated with the roots of the dominant tallgrass prairie grass, *Andropogon gerardii*. Ribosomal RNA was extracted, reverse-transcribed, and PCR-amplified in four sampling events in May, July, September, and November. Analyses of cloned PCR amplicons indicated that the *A. gerardii* rhizospheres host phylogenetically diverse fungal communities and that these communities are seasonally dynamic. Operational taxonomic units with Basic Local Alignment Search Tool affinities within the order Helotiales were dominant in the rhizosphere in May. These putative saprobes were largely replaced by arbuscular mycorrhizal fungi with likely affinities within Glomerales suggesting that the fungal communities are not only compositionally but also functionally dynamic. These data suggest replacement of functional guilds comprised of saprobic fungi by mutualistic fungi in the course of a growing season.

Keywords Rhizosphere · Ribosomal RNA · Seasonal dynamics · Tallgrass prairie

Introduction

Diverse fungal communities inhabit the soil and rhizophere. Recent studies that used high throughput tools to estimate this species richness concluded that while communities of arbuscular mycorrhizal fungi (AMF) may be enumerable (Öpik et al. 2009), the general soil and rhizosphere-inhabiting communities may be orders of magnitude more diverse (Buee et al. 2009). This high species richness often leads to difficulties in drawing clear conclusions about the composition of the fungal communities.

The spatial and temporal variability further hinders the understanding of fungal communities that vary in small spatial scales (Mummey and Rillig 2008; Wolfe et al. 2007) and across soil depths (Oehl et al. 2005). A few studies have targeted seasonal dynamics of fungal communities, and studies focusing on AMF suggest that their sporulation is seasonal (Gemma and Koske 1988; Lee and Koske 1994; Merryweather and Fitter 1998; Oehl et al. 2009; Pringle and Bever 2002). These studies tend to conclude that many species of *Acaulospora* sporulate early in the season (Oehl et al. 2009) and that these patterns of sporulation correlate with root colonization (Merryweather and Fitter 1998). In contrast, species of *Glomus* show usually no distinct seasonal patterns (Oehl et al. 2009). Similarly to the AMF, other rhizosphere-associated communities seem to fluctuate seasonally (Koide et al. 2007; Walker et al. 2008). These seasonal community dynamics are likely not exclusive to mycorrhizal associations. For example, Jumpponen et al. (2010) observed a seasonal replacement of putative antagonists and saprobes by common ectomycorrhizal fungi in the rhizospheres of *Quercus* spp.

The DNA coding for the ribosomal RNA (rRNA) genes or their spacer regions has proven extremely useful for detection of fungi in complex environmental samples. However, fungi that are not actively functioning may persist in the environmental DNA pools as dormant and inactive community components or as residual naked DNA detectable via PCR-based approaches (Agnelli et al. 2007; Anderson et al. 2008; Ceccherini et al. 2007; Hirsch et al. 2010; Pietramellara et al. 2007). The environmental rRNA

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can be targeted as an alternative to detect community components that are active at the time of sampling (Girvan et al. 2004; Mahmood et al. 2005): rRNA is unstable in the environment, and rRNAs of metabolically active organisms will be detected more frequently than those of inactive organisms (Anderson and Parkin 2007; Prosser 2002). As a result, rRNA likely provides a high fidelity target that allows for resolution of the fungal community dynamics beyond that afforded by the analyses of DNA coding for it (Anderson and Parkin 2007; Bastias et al. 2007; Hoshino and Matsumoto 2007), although this may not always be the case (Ros et al. 2009).

The studies reported here aimed to follow the seasonal dynamics of fungal communities that inhabit the rhizosphere of a dominant grass *Andropogon gerardii* Vitman in a tallgrass prairie ecosystem. To do so, roots were sampled and analyzed for fungal community composition by cloning and sequencing of reverse-transcribed 18S rRNA. The main goals of this study were (1) to test the applicability of rRNA extracted from environmental samples, (2) to characterize the dominant fungal communities in *A. gerardii* rhizosphere, and (3) to determine whether or not intra-seasonal turnover of these communities could be detected based on reverse-transcribed rRNA. The results indicate that the early seasonal communities are dominated by non-mycorrhizal groups, whereas Glomerales that commonly form arbuscular mycorrhizas in this ecosystem establish dominance in the course of a growing season.

Materials and methods

Sampling of the *Andropogon gerardii* rhizosphere tissues

The plant materials were collected at the Konza Prairie Biological Station (KPBS, 39˚05′ N, 96˚35′ W), a Long-Term Ecological Research site that represents a native tallgrass prairie in the Flint Hills of eastern Kansas, USA. The vegetation is dominated by C_4 grasses, and four most dominant species are big blue stem (*A. gerardii*), indian grass (*Sorghastrum nutans* (L.) Nash.), little bluestem (*Schizachyrium scoparium* (Michx.) Nash.), and switch grass (*Panicum virgatum* L). The soil parent material is chert-bearing limestone with the soil bulk density of 1.0 g/cm^3. January mean temperature is –3°C (range, –9 to 3 C), and the July mean temperature is 27 C (range, 20–33 C). Annual precipitation averages 835 mm, 75% of which falls in the growing season.

For a total of 20 samples across the entire experiment, five intact *A. gerardii* plants with their roots attached were excavated in May, July, September, and November from an annually burned watershed (1D) at the KPBS. The focal watershed represents tallgrass prairies under a frequent fire cycle. The five sampling locations within the watershed were recorded, and adjacent plants were sampled at each of the four sampling events to minimize the spatial heterogeneity in the observed fungal communities. The plant roots were shaken free of soil, and ca. 20 cm of the root material still attached to *A. gerardii* tillers were excised into the Lysing Matrix E of the FastRNA Pro Soil-Direct kit (Q-Biogene, Carlsbad, CA, USA) and immediately flash-frozen in a liquid N_2 cryoshipper (Mini-Moover Vapor Shipper, Chart Biomedical, Marietta, GA, USA) to minimize RNA degradation. The samples were maintained frozen in the cryoshipper until the nucleic acid extraction within 8 h.

RNA extraction from the *Andropogon gerardii* rhizosphere

The frozen *A. gerardii* rhizosphere samples were combined with 1 ml RNApro soil lysis solution, homogenized in the FastPrep instrument (Q-BioGene, Irvine, CA, USA) at setting 6 for 40 s. The roots and adhering soils were completely homogenized as determined by a visual inspection of the samples. The total RNA was extracted using FastRNA Pro Soil Direct kit following the manufacturer's instructions. The samples were eluted in 200 μl of nuclease-free water with 100 U of RNaseOUT (40 U/μl; Invitrogen, Carlsbad, CA, USA) to inhibit RNA degradation before reverse transcription and first-strand complementary DNA (cDNA) synthesis. The RNA extracts were stored in –80 C until further processed.

Reverse transcription of the *Andropogon gerardii* rhizosphere RNA

The extracted rRNAs and the blank extraction control were reverse-transcribed using Thermoscript reverse transcription PCR (RT-PCR) two-step system (Invitrogen, Carlsbad, CA, USA). For a nearly full-length cDNA of the small subunit (SSU) of the ribosomal RNA, NS8 primer (White et al. 1990) located near the 3′-end of the SSU was used for the cDNA synthesis. The use of this primer provides great flexibility in choosing nested priming sites within the SSU for the PCR amplification. To denature the rRNAs before the cDNA synthesis, 2 μl of each RNA template was combined with 1 μl of nuclease-free 10 μM NS8 primer, 2 μl of 10 mM dNTPs, and 7 μl of nuclease free H_2O and incubated at 65°C for 5 min in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The denatured RNAs were transferred to ice and combined with 4 μl of 5× cDNA buffer, 1 μl of 0.1 M DTT, 1 μl of RNaseOUT (Invitrogen, Carlsbad, CA, USA), 1 μl nuclease free H_2O, and 1 μl ThermoScript Reverse Transcriptase or Platinum Taq polymerase (the control for DNA contamination). The cDNAs were synthesized in the Eppendorf Mastercycler at 55°C for 60 min, and the synthesized cDNAs were returned to ice until PCR amplification.

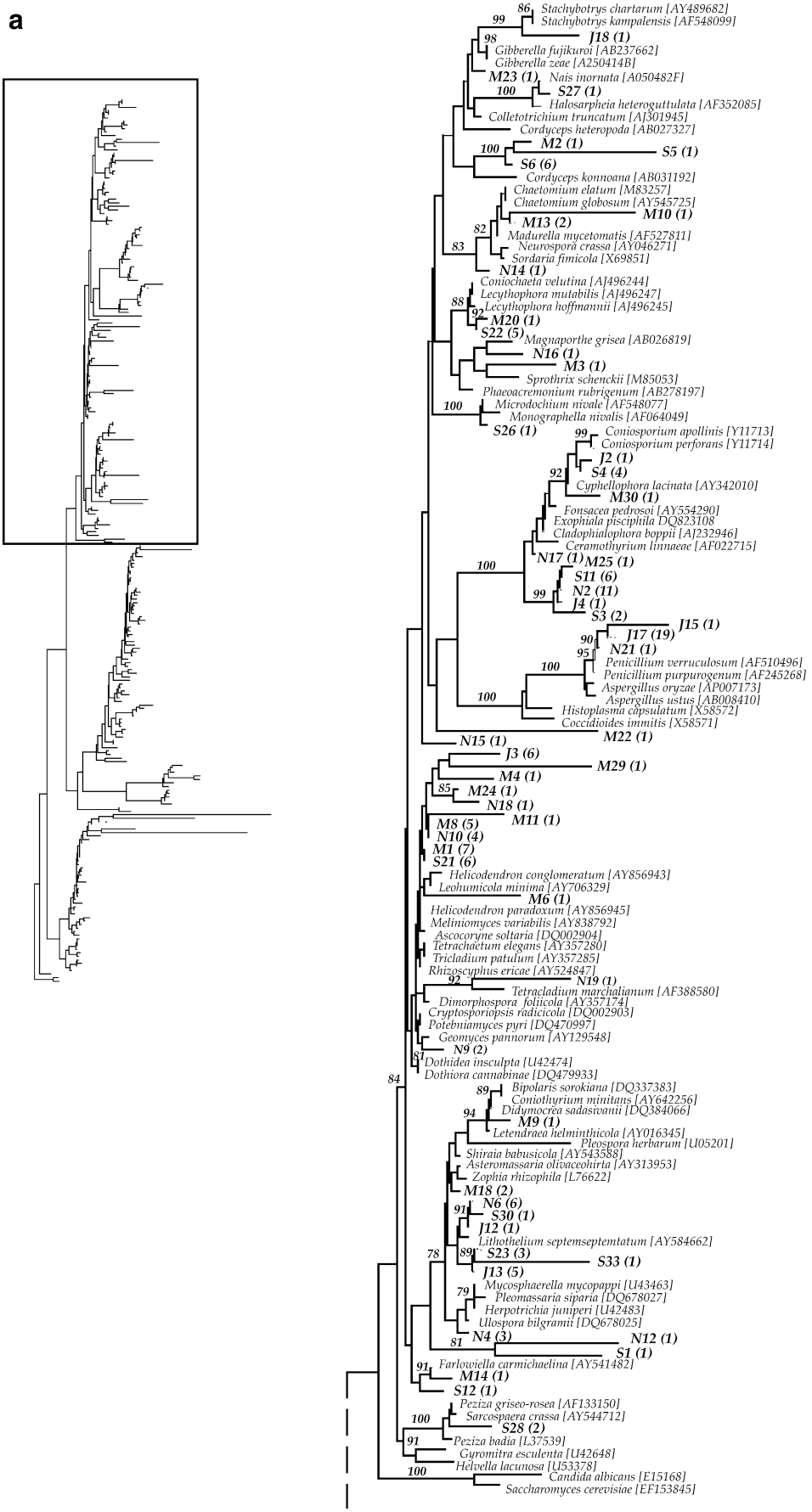
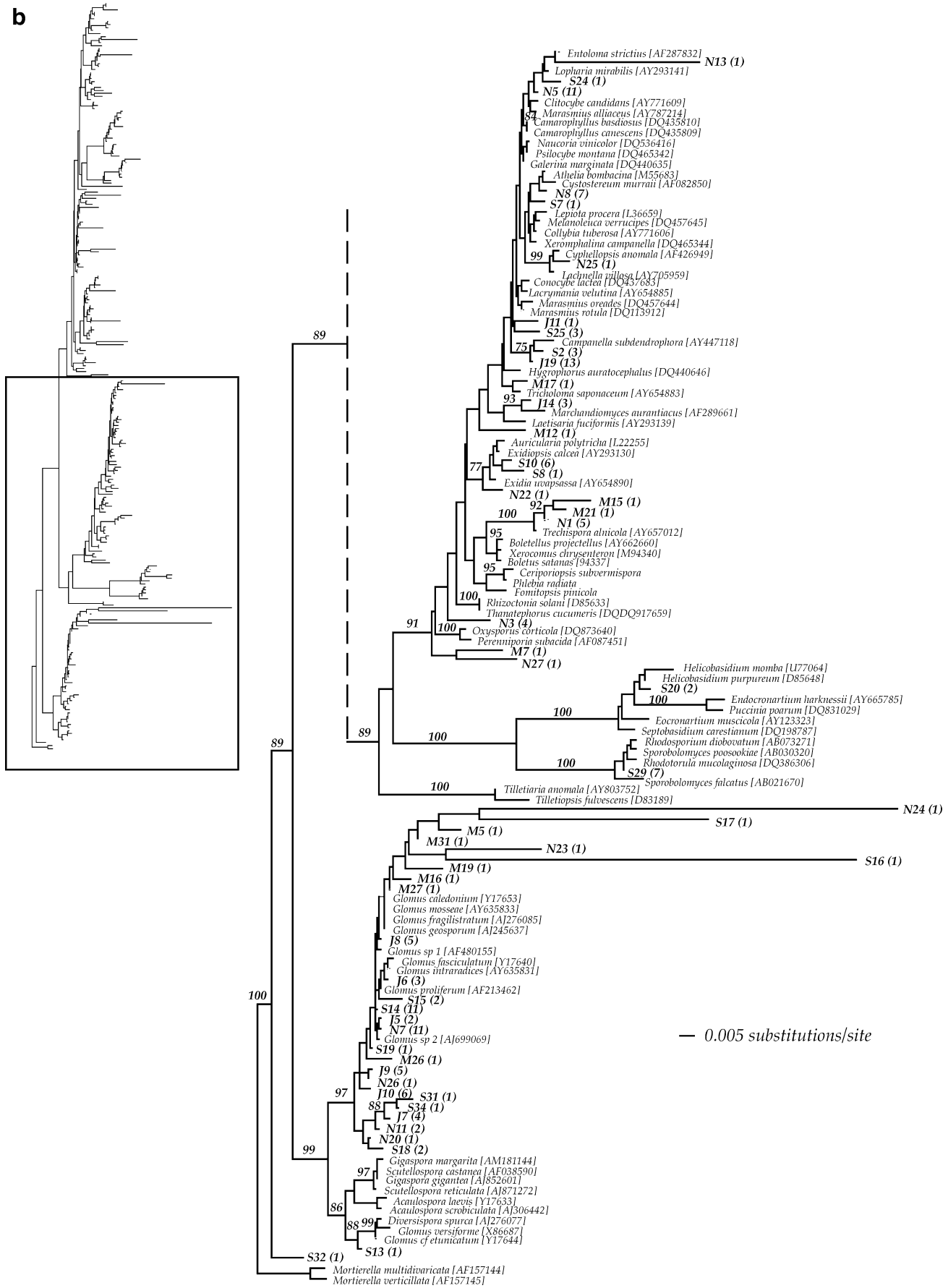


Fig. 1 Neighbor-joining (NJ) tree of the fungal 18S sequences obtained by reverse transcription and PCR amplification from *Andropogon gerardii* roots: **a** Ascomycota, **b** Basidiomycota and Glomeromycota including basal lineages and the *Mortierella* (Mortierellales) outgroup. The environmental sequences are identified by M, J, S, and N corresponding to sampling events May, July, September, and November, respectively. *Numbers* that follow identify the sequence (Table 1) and the number of occurrences (in *parentheses*). Bootstrap values >75% are given at the branch points


b
Entoloma strictius [AF287832]
N13 (1)
Lopharia mirabilis [AY293141]
S24 (1)
N5 (11)
Clitocybe candidans [AY771609]
Marasmius alliaceus [AY787214]
Camarophyllus basdiosus [DQ435810]
Camarophyllus canescens [DQ435809]
Naucoria vinicolor [DQ536416]
Psilocybe montana [DQ465342]
Galerina marginata [DQ440635]
Athelia bombacina [M55683]
Cystostereum murrai [AF082850]
N8 (7)
S7 (1)
Lepiota procera [L36659]
Melanoleuca verrucipes [DQ457645]
Collybia tuberosa [AY771606]
Xeromphalina campanella [DQ465344]
Cyphellopsis anomala [AF426949]
N25 (1)
Lachnella villosa [AY705959]
Conocybe lactea [DQ457683]
Lacrymaria velutina [AY654885]
Marasmius oreades [DQ457644]
Marasmius rotula [DQ113912]
J11 (1)
S25 (3)
Campanella subdendrophora [AY447118]
S2 (3)
J19 (13)
Hygrophorus auratocephalus [DQ440646]
M17 (1)
Tricholoma saponaceum [AY654883]
J14 (3)
Marchandiomyces aurantiacus [AF289661]
Laetisaria fuciformis [AY293139]
M12 (1)
Auricularia polytricha [L22255]
Exidiopsis calcea [AY293130]
S10 (6)
S8 (1)
Exidia uvapsassa [AY654890]
N22 (1)
M15 (1)
M21 (1)
N1 (5)
Trechispora alnicola [AY657012]
Boletellus projectellus [AY662660]
Xerocomus chrysenteron [M94340]
Boletus satanas [94337]
Ceriporiopsis subvermispora
Phlebia radiata
Fomitopsis pinicola
Rhizoctonia solani [D85633]
Thanatephorus cucumeris [DQDQ917659]
N3 (4)
Oxysporus corticola [DQ873640]
Perenniporia subacida [AF087451]
M7 (1)
N27 (1)
Helicobasidium mompa [U77064]
Helicobasidium purpureum [D85648]
S20 (2)
Endocronartium harknessii [AY665785]
Puccinia poarum [DQ831029]
Eocronartium muscicola [AY123323]
Septobasidium carestianum [DQ198787]
Rhodosporium diobovatum [AB073271]
Sporobolomyces poosookiae [AB030320]
Rhodotorula mucolaginosa [DQ386306]
S29 (7)
Sporobolomyces falcatus [AB021670]
Tilletiaria anomala [AY803752]
Tilletiopsis fulvescens [D83189]
N24 (1)
S17 (1)
M5 (1)
M31 (1)
N23 (1)
S16 (1)
M19 (1)
M16 (1)
M27 (1)
Glomus caledonium [Y17653]
Glomus mosseae [AY635833]
Glomus fragilistratum [AJ276085]
Glomus geosporum [AJ245637]
J8 (5)
Glomus sp 1 [AF480155]
Glomus fasciculatum [Y17640]
Glomus intraradices [AY635831]
J6 (3)
Glomus proliferum [AF213462]
S15 (2)
S14 (11)
J5 (2)
N7 (11)
Glomus sp 2 [AJ699069]
S19 (1)
M26 (1)
J9 (5)
N26 (1)
J10 (6)
S31 (1)
S34 (1)
J7 (4)
N11 (2)
N20 (1)
S18 (2)
Gigaspora margarita [AM181144]
Scutellospora castanea [AF038590]
Gigaspora gigantea [AJ852601]
Scutellospora reticulata [AJ871272]
Acaulospora laevis [Y17633]
Acaulospora scrobiculata [AJ306442]
Diversispora spurca [AJ276077]
Glomus versiforme [X86687]
Glomus cf etunicatum [Y17644]
S13 (1)
S32 (1)
Mortierella multidivaricata [AF157144]
Mortierella verticillata [AF157145]
— 0.005 substitutions/site

◀ **Fig. 1** (continued)

PCR amplification of the *Andropogon gerardii* rhizosphere cDNA

The reverse-transcribed cDNAs were PCR-amplified with Platinum *Taq* polymerase with fungus-specific primers (nu-SSU-0817-5′ and nu-SSU-1536-3′) that target a ca. 760 bp region of the SSU (Borneman and Hartin 2000). The PCR reactions were conducted in a 50 μl volume with 5 μl 10× PCR buffer, 2.5 μl 50 mM $MgCl_2$, 5 μl 2 mM dNTPs, 1 μl of 10 μM forward and reverse primers, 0.4 μl polymerase (5 U/μl), and 2 μl of the cDNA template plus 33.1 μl of nuclease free water. The PCR reactions were carried out with initial 3 min denaturation at 93 C followed by 30 cycles of 1 min at 93 C, 1 min at 56 C, 2 min at 72 C, and a terminal elongation at 72 C for 10 min. Longer extension steps were chosen to minimize the chimeric PCR products (Jumpponen 2007). The amplification of target-sized amplicons was confirmed by horizontal gel electrophoresis. The PCR products were purified with UltraClean PCR Clean-Up kit (MoBio Laboratories, Carlsbad, CA, USA), eluted in 50 μl of nuclease-free water and quantitated with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). A total of 10 ng of each of the five amplicons for each of the four sampling events was pooled for an equal sample representation in the clone libraries.

Control reactions

To account for contaminating nucleic acids in the samples, three controls were included. First, to account for RNA/DNA contamination from the extraction system, a blank extraction without a sample was carried trough the extraction protocol. Second, to account for PCR reagent-borne contaminants, a PCR control without template DNA was included in the PCR. Third, to account for DNA carry-over through the RNA extraction, a control where Thermoscript reverse transcriptase was replaced with Platinum *Taq* polymerase was included. All these controls remained free of contaminants and yielded no visible PCR amplicons, indicating absence of DNA carry-through in the RNA extraction, absence of contaminating DNA in the RNA extraction, and absence of contaminating DNA in the PCR reagents.

Cloning and sequencing of the *Andropogon gerardii* rhizosphere cDNA

The pools of five mixed PCR product populations representing each of the four sampling events were cloned using TOPO-TA cloning system (Invitrogen, Carlsbad, CA, USA). A total of 40 ng of each pool of amplicons was ligated into 10 ng of linearized pCR4 vector (Invitrogen, Carlsbad, CA, USA) for a 4:1 amplicon to vector ratio. The circularized plasmids were transformed into competent TOP10 cells (Invitrogen, Carlsbad, CA, USA) by a 30-s heat shock in a 42°C water bath following the protocol for TOPO-TA cloning of PCR products with an adenosine overhang. Bacteria were grown for an hour at 37°C under 200 rpm agitation in 250 μl of SOC medium. A total of 10 and 100 μl of the incubated bacteria were plated on Luria–Bertani agar amended with 100 μg/ml ampicillin and grown at 37°C overnight to confirm putative positive transformants. A total of 30 random colonies were picked from each of the four libraries to confirm the presence of an insert by PCR in 15-μl reaction volumes using the same conditions as above. The clone libraries were combined with an equal volume of 60% glycerol, flash-frozen in liquid N_2, and shipped for robotic colony picking, plasmid preparation, and sequencing at University of Washington High Throughput Genomics Unit (Seattle, WA, USA). A total of 96 clones from each of the four clone libraries were randomly sampled and sequenced using the vector's T3 and T7 priming sites.

Analyses of the *Andropogon gerardii* rhizosphere sequence data

The sequences were imported into Sequencher (version 4.7; GeneCodes, Ann Arbor, MI, USA). Terminal vector sequences were removed using Sequencher's trimming function. The sequences were assigned to operational taxonomic units (OTUs) at 98% similarity and 100 bp overlap, and a representative of each of the OTUs was selected for further analyses. This resulted in a matrix with 107 environmental OTUs across the four libraries. An example read for each of the OTUs is available at GenBank under accession numbers HM753155–HM753261. The similarities to existing rDNA sequences in the GenBank database were determined on July 3, 2008, using Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (Altschul et al. 1997). A framework of reference sequences representing major clades of higher fungi in James et al. (2006) was aligned in Sequencher with the environmental sequences to approximate their affinities.

The phylogenies were approximated by neighbour joining (NJ) in PAUP* (Swofford 2001). The optimal evolutionary model (GTR+I+G) was selected based on Modeltest 3.8 (Posada and Crandall 1998; Posada 2006), and the empirical base frequencies and estimated rate matrices and distribution parameters were used. Sites with missing data—ambiguous nucleotides or gaps—were ignored for the affected pairwise comparisons. The robustness of the inferred NJ topology was tested by 1,000 bootstrap replicates.

The pooling of samples for each of the sampling events precluded traditional statistical inference using the taxon

Table 1 Inferred BLAST affinities and number of occurrences of the reverse-transcribed and PCR-amplified fungal 18S ribosomal RNA sequences

Sample/OTU	Match (Order)	Accession	Similarity (%)	Coverage (%)	Number of .reads
May					
M1 [HM7533182]	*Helicodendron paradoxum* (Helotiales)	AY856945	99	99	7
M2 [HM7533192]	*Cordyceps konnoana* (Hypocreales)	AB031192	96	99	1
M3 [HM7533195]	*Sporothrix schenckii* (Ophiostomatales)	M85053	96	99	1
M4 [HM7533196]	*Pezicula carpinea* (Helotiales)	DQ471016	97	99	1
M5 [HM7533197]	*Glomus proliferum* (Glomerales)	AF213462	97	100	1
M6 [HM7533198]	*Chloroscypha* cf. *enterochroma* (Helotiales)	AY544700	99	96	1
M7 [HM7533199]	*Tricholoma saponaceum* (Agaricales)	AY654883	96	99	1
M8 [HM7533200]	*Helicodendron paradoxum* (Helotiales)	AY856945	99	99	5
M9 [HM7533201]	*Letendraea helminthicola* (Pleosporales)	AY016345	99	99	1
M10 [HM7533172]	*Chaetomium globosum* (Sordariales)	AY545725	96	97	1
M11 [HM7533173]	*Helicodendron paradoxum* (Helotiales)	AY856945	99	96	1
M12 [HM7533174]	*Exidia uvapsassa* (Auriculariales)	AY654890	97	99	1
M13 [HM7533175]	*Madurella mycetomatis* (incertae sedis)	AF527811	98	99	2
M14 [HM7533176]	*Farlowiella carmichaeliana* (incertae sedis)	AY541482	99	99	1
M15 [HM7533177]	*Trechispora alnicola* (Trechisporales)	AY657012	98	99	1
M16 [HM7533178]	*Glomus mosseae* (Glomerales)	AY635833	97	100	1
M17 [HM7533179]	*Tricholoma saponaceum* (Agaricales)	AY654883	99	99	1
M18 [HM7533180]	*Asteromassaria olivaceohirta* (Pleosporales)	AY313953	99	99	2
M19 [HM7533181]	*Glomus proliferum* (Glomerales)	AF213462	97	96	1
M20 [HM7533183]	*Lecythophora mutabilis* (Coniochaetales)	AJ496247	99	99	1
M21 [HM7533184]	*Trechispora alnicola* (Trechisporales)	AY657012	97	99	1
M22 [HM7533185]	*Flammispora bioteca* (incertae sedis)	AY22100	96	90	1
M23 [HM7533186]	*Gibberella pulicaris* (Hypocreales)	AF149875	99	99	1
M24 [HM7533187]	*Pezicula carpinea* (Helotiales)	DQ471016	98	99	1
M25 [HM7533188]	*Ceramothyrium linnaeae* (Chaetothyriales)	AF022715	97	99	1
M26 [HM7533189]	*Glomus proliferum* (Glomerales)	AF213462	97	100	1
M27 [HM7533190]	*Glomus mosseae* (Glomerales)	AY635833	98	100	1
M28 [HM7533191]	*Oidiodendron tenuissimum* (incertae sedis)	AB015787	95	99	1
M30 [HM7533193]	*Cyphellophora laciniata* (Chaetothyriales)	AY342010	98	99	1
M31 [HM7533194]	*Glomus proliferum* (Glomerales)	AF213462	97	100	1
				Total	42
July					
J2 [HM7533164]	*Coniosporium* sp. (incertae sedis)	AJ972863	98	99	1
J3 [HM7533165]	*Dimorphospora foliicola* (Helotiales)	AY357274	97	97	6
J4 [HM7533166]	*Exophiala pisciphila* (Chaetothyriales)	DQ823108	97	99	1
J5 [HM7533167]	*Glomus* sp. (Glomerales)	AJ699069	98	99	2
J6 [HM7533168]	*Glomus intraradices* (Glomerales)	AY635831	99	99	3
J7 [HM7533169]	*Glomus* sp. (Glomerales)	AJ699064	98	100	4
J8 [HM7533170]	*Glomus* sp. (Glomerales)	AF480155	98	99	5
J9 [HM7533171]	*Glomus mosseae* (Glomerales)	AY635833	98	100	5
J10 [HM7533155]	*Glomus mosseae* (Glomerales)	AY635833	98	100	6
J11 [HM7533156]	*Hygrophorus auratocephalus* (Agaricales)	DQ440646	98	99	1
J12 [HM7533157]	*Lithothelium septemseptatum* (Pyrenulales)	AY584662	98	99	1
J13 [HM7533158]	*Lithothelium septemseptatum* (Pyrenulales)	AY584662	99	99	5
J14 [HM7533159]	*Marchandiomyces aurantiacus* (incertae sedis)	AF289661	98	98	3
J15 [HM7533160]	*Penicillium verruculosum* (Eurotiales)	AF510496	98	99	1
J17 [HM7533161]	*Penicillium verruculosum* (Eurotiales)	AF510496	99	99	19

Table 1 (continued)

Sample/OTU	Match (Order)	Accession	Similarity (%)	Coverage (%)	Number of .reads
J18 [HM7533162]	*Stachybotrys chartanum* (Hypocreales)	AY489682	98	100	1
J19 [HM7533163]	*Campanella subdendrophora* (Agaricales)	AY445118	99	99	13
				Total	77
September					
S1 [HM7533239]	*Gremmeniella abietina* (Helotiales)	AF548076	92	100	1
S2 [HM7533250]	*Campanella* sp. (Agaricales)	AY916675	99	100	3
S3 [HM7533256]	*Ceramothyrium linnaeae* (Chaetothyriales)	AF022715	96	99	2
S4 [HM7533257]	*Coniosporium* sp. (incertae sedis)	AJ972863	99	99	4
S5 [HM7533258]	*Cordyceps konnoana* (Hypocreales)	AB031192	94	99	1
S6 [HM7533259]	*Cordyceps konnoana* (Hypocreales)	AB031192	97	99	6
S7 [HM7533260]	*Dendrocollybia racemosa* (Agaricales)	DQ825432	99	99	1
S8 [HM7533261]	*Exidiopsis calcea* (Auriculariales)	AY293130	98	99	1
S10 [HM7533229]	*Exidiopsis calcea* (Auriculariales)	AY293130	98	99	6
S11 [HM7533230]	*Exophiala pisciphila* (Chaetothyriales)	DQ823108	97	99	6
S12 [HM7533231]	*Farlowiella carmichaeliana* (incertae sedis)	AY541482	99	100	1
S13 [HM7533232]	*Glomus* cf. *etunicatum* (Glomerales)	Y17644	97	100	1
S14 [HM7533233]	*Glomus proliferum* (Glomerales)	AF213462	99	100	11
S15 [HM7533234]	*Glomus proliferum* (Glomerales)	AF213462	98	100	2
S16 [HM7533235]	*Glomus* sp. (Glomerales)	AJ699069	89	99	1
S17 [HM7533236]	*Glomus* sp. (Glomerales)	AJ699069	94	98	1
S18 [HM7533237]	*Glomus* sp. (Glomerales)	AF480155	98	98	2
S19 [HM7533238]	*Glomus intraradices* (Glomerales)	AY635831	98	99	1
S20 [HM7533240]	*Helicobasidium purpureum* (Helicobasidiales)	D85648	99	99	2
S21 [HM7533241]	*Helicodendron paradoxum* (Helotiales)	AY856945	99	99	6
S22 [HM7533242]	*Lecythophora mutabilis* (Coniochaetales)	AJ496247	99	99	5
S23 [HM7533243]	*Lithothelium septemseptatum* (Pyrenulales)	AY584662	99	99	3
S24 [HM7533244]	*Lopharia mirabilis* (Russulales)	AY293141	98	99	1
S25 [HM7533245]	*Marasmius oreades* (Agaricales)	DQ457644	98	99	3
S26 [HM7533246]	*Microdochium nivale* (Xylariales)	AF548077	99	99	1
S27 [HM7533247]	*Nais inornata* (Microascales)	AF050482	99	100	1
S28 [HM7533248]	*Peziza griseo-rosea* (Pezizales)	AF133150	98	99	2
S29 [HM7533249]	*Rhodotorula mucilaginosa* (Sporidiobolales)	DQ832199	99	99	7
S30 [HM7533251]	Uncultured ascomycete isolate (N/A)	AF504089	99	99	1
S31 [HM7533252]	Uncultured soil zygomycete (N/A)	AY773807	99	100	1
S32 [HM7533253]	*Endogone lactiflua* (Endogonales)	DQ536471	95	100	1
S33 [HM7533254]	*Lithothelium septemseptmtatum* (Pyrenulales)	AY584662	96	99	1
S34 [HM7533255]	*Glomus proliferum* (Glomarales)	AF213462	97	99	1
				Total	87
November					
N1 [HM7533212]	*Trechispora alnicola* (Trechisporales)	AY803753	98	99	5
N2 [HM7533211]	*Ceramothyrium linnaeae* (Chaetothyriales)	AF022715	98	99	11
N3 [HM7533222]	*Thanatephorus cucumeris* (Cantharellales)	DQ917659	97	100	4
N4 [HM7533223]	*Mycosphaerella mycopappi* (Capnodiales)	U43463	99	99	3
N5 [HM7533224]	*Camarophyllus canescens* (Agaricales)	DQ435810	98	99	11
N6 [HM7533225]	Uncultured ascomycete (N/A)	AF504089	99	100	6
N7 [HM7533226]	*Glomus* sp. (Glomerales)	AJ699069	99	99	11
N8 [HM7533227]	*Cystostereum murraii* (Corticiales)	AF082850	99	99	7
N9 [HM7533228]	*Potebniamyces pyri* (Rhytismatales)	Q4770997	98	99	2
N10 [HM7533202]	*Helicodendron paradoxum* (Helotiales)	AY856945	99	99	4

Table 1 (continued)

Sample/OTU	Match (Order)	Accession	Similarity (%)	Coverage (%)	Number of .reads
N11 [HM7533203]	*Glomus* sp. (Glomerales)	AJ699069	98	99	2
N12 [HM7533204]	*Asteromassaria olivaceohirta* (Pleosporales)	AY313953	93	99	1
N13 [HM7533205]	*Entoloma strictius* (Agaricales)	AF287832	95	100	1
N14 [HM7533206]	*Chaetomium globosum* (Sordariales)	AY545725	98	99	1
N15 [HM7533207]	*Helicodendron paradoxum* (Helotiales)	AY856945	98	99	1
N16 [HM7533208]	*Magnaporthe grisea* (Magnaporthales)	AB026819	98	100	1
N17 [HM7533209]	*Exophiala pisciphila* (Chaetothyriales)	DQ823108	99	100	1
N18 [HM7533210]	*Pezicula carpinea* (Helotiales)	DQ471016	98	99	1
N19 [HM7533211]	Uncultured rhizosphere ascomycete (N/A)	AY7737754	97	99	1
N20 [HM7533213]	*Glomus* sp. (Glomerales)	AF480155	98	99	1
N21 [HM7533214]	*Penicillium verruculosum* (Eurotiales)	AF510496	99	100	1
N22 [HM7533215]	Uncultured rhizosphere basidiomycete (N/A)	AJ506024	98	100	1
N23 [HM7533216]	*Glomus* sp. (Glomerales)	AJ699069	91	99	1
N24 [HM7533217]	*Glomus* sp. (Glomerales)	EF033125	98	96	1
N25 [HM7533218]	*Lachnella villosa* (Agaricales)	AY705959	99	99	1
N26 [HM7533219]	*Glomus* sp. (Glomerales)	AF480157	94	100	1
N27 [HM7533220]	Uncultured fungus (N/A)	AF504752	95	100	1
N28 [HM7533221]	Uncultured fungus (N/A)	AF504752	95	99	1
				Total	82
				Grand Total	288

abundance data. Accordingly, the differences in the fungal communities across the four sampling events were tested using principal coordinates analyses (PCoA) available in Fast UniFrac (Hamady et al. 2010). To account for changes in organismal abundances within the NJ-inferred lineages, the abundance weighting option was selected. The number of sequences within each of the OTUs was used for the "sample mapping" file for Fast UniFrac (Hamady et al. 2010). Furthermore, to account for evolutionary rates that likely differ across the fungal phyla, PCoA, and subsequent UniFrac tests of significance were conducted using the normalization option. Using 1,000 Monte Carlo permutations, the sequence distributions across the four sampling events were analyzed and inference based on UniFrac significance after a conservative Bonferroni correction. Organismal coverage among the sampling events was

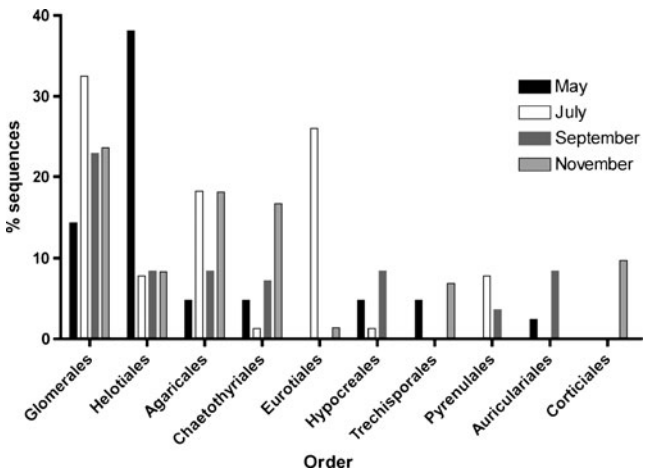


Fig. 2 Rank abundance of the orders detected via reverse transcription and PCR in the roots of *Andropogon gerardii* in May, July, September, and November. Note that while Helotiales dominate the fungal communities in the first sampling in May, Glomerales dominate these communities in all subsequent sampling occasions

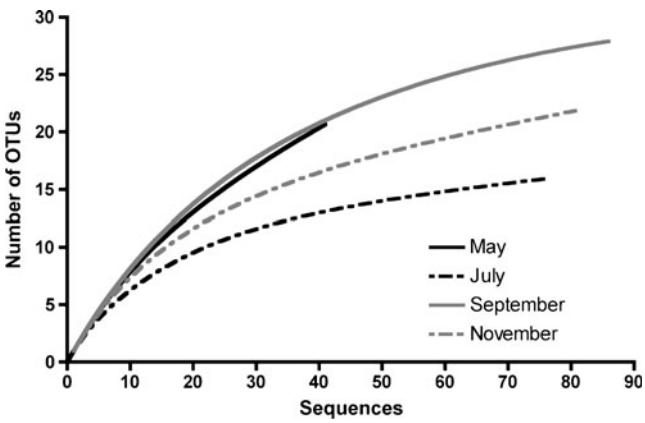


Fig. 3 OTU accumulation (Cole rarefaction) curves for the four clone libraries representing the reverse-transcribed and PCR-amplified fungal communities in *Andropogon gerardii* roots in May, July, September, and November

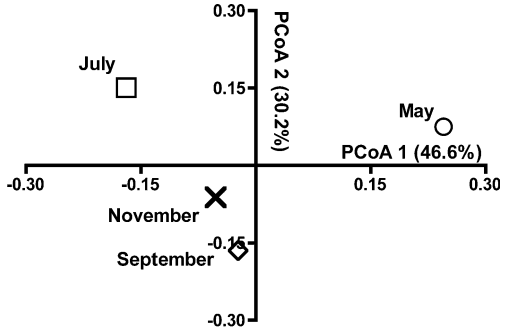


Fig. 4 Principal coordinates analysis (PCoA) of the fungal communities inhabiting the *Andropogon gerardii* rhizosphere. The two PCoA axes represent 46.6% and 30.2% (76.8% in total) of the variation. Each of the sampled communities in May, July, September, and November is distinct based on UniFrac test of significance with 1,000 Monte Carlo permutations

explored using species accumulation (rarefaction) curves in EstimateS (version 8; Colwell 2006).

Results

Acquisition of rRNA from *Andropogon gerardii* rhizosphere

The RNA extraction and cDNA synthesis from *A. gerardii* rhizosphere were successful. The control reactions indicated that the RNA extraction protocol and the cDNA syntheses were robust and not contaminated by DNA carry-through. In other words, DNAse treatment of the extracts was deemed unnecessary but would provide an additional safeguard if concerns emerge.

The conserved NS8 priming site (White et al. 1990) near the 3′-end of the SSU was chosen for flexible PCR amplification with various nested primers. Use of fungus specific primers (Borneman and Hartin 2000) facilitated a broad assay across the Kingdom Fungi, including Ascomycota (Fig. 1a), Basidiomycota, and Glomeromycota (Fig. 1b). In all, the sampled rRNA represented a total of 22 orders in addition to mitosporic taxa whose taxonomic position is uncertain (Table 1). In contrast, primers strongly biased toward Glomeromycota (Helgason et al. 1998) did not produce amplicons, although Glomerales were the most abundant taxon in this study (Table 1).

Taxon distribution of the fungal communities in *Andropogon gerardii* rhizosphere

The environmental rRNA sampling covered a large number of fungi that were presumably active at the time of sampling (Fig. 1a, b). As is typical to environmental analyses relying exclusively on a relatively small fragment of the small subunit of the ribosomal RNA gene, many nodes received only poor support in NJ analyses. To exemplify, the abundant Helotiales with BLAST affinities to genus *Helicodendron* clustered as a basal polytomy within the ascomycetes. Similarly, the various glomeromycotan sequences with affinities within the Glomerales did not produce resolved terminal clades, such as those observed in analyses focusing exclusively on Glomeromycota (e.g., Stockinger et al. 2010).

Many of the observed OTUs occurred only in one sampling event and in low abundances in those libraries (Table 1). This resulted in typical rank-abundance curves with a majority of the observed orders present in low frequencies (Fig. 2) and poor saturation in the rarefaction analyses for most clone libraries (Fig. 3). Based on BLAST affinities, four orders were detected at all four sampling events: Agaricales, Chaetothyriales, Helotiales, and Glomerales. The most frequent OTUs within Agaricales showed affinities to *Camarophyllus canescens* (11 occurrences) and *Campanella subdendrophora* (13 occurrences); within Helotiales to *Helicodendron paradoxum* (20 occurrences) and *Dimorphospora foliicola* (six occurrences); within Chaetothyriales to *Ceramothyrium linnaeae* (14 occurrences) and *Exophiala pisciphila* (eight occurrences); and within Glomerales to environmental *Glomus* sequences (32 occurrences) and also to *Glomus intraradices* (four occurrences), *Glomus mosseae* (13 occurrences), and *Glomus proliferum* (17 occurrences).

Seasonal trends of fungal communities in *Andropogon gerardii* rhizosphere

The PCoA separated the four libraries on the first two principal coordinate axes (Fig. 4) that represented 46.6% and 30.2% of the variation, 76.8% in total. The UniFrac tests of significance after a conservative Bonferroni correction indicated that the *A. gerardii* associated fungal communities were seasonally distinct ($P \leq 0.001$ for all pairwise comparisons across the four libraries).

Glomerales were always either the most or the second most abundant order in the *A. gerardii* rhizophere, indicating their dominance and significant activity across the growing season. Helotiales were the most abundant order in the early season sampling in May but substantially declined in the subsequent sampling events. Much of this abundance was attributable to the OTUs with a high sequence similarity to *H. paradoxum* in BLAST analyses (Table 1). However, this affinity was not supported in the NJ analyses (Fig. 1a, b). A single Eurotiales OTU with an affinity to *Penicillium verruculosum* dominated the July sampling. This OTU was nearly as abundant as all Glomerales from that sampling combined. The high frequency of this OTU likely represents one of the five separate RNA extracts producing mainly this

amplicon. However, beyond that sampling event, Eurotiales were near absent.

Discussion

Analysis of environmental rRNA provides a high fidelity tool for assessing fungal communities and their dynamics. The rRNA markers and their abundances have been often interpreted as an indication of organismal activities (Girvan et al. 2004; Poulsen et al. 1993). While it is often assumed that RNA turnover is rapid and its degradation quick after decreased cellular activity, it remains unclear how well the rRNA detection relates to ribosome number and activity (Anderson and Parkin 2007). Regardless, the rRNA targets may suffer less from issues related to DNA present either as dormant cells or residual DNA preserved free in the soil matrix (Ostle et al. 2003) and therefore provide a more accurate view of the active microbial community at the time of sampling. One avenue to improve the detection of active organisms is to use the precursors for the rRNA gene operons (Anderson and Parkin 2007), but acquisition and reverse transcription of these ephemeral transcripts from environmental samples may prove problematic.

This study targeted the fungal rRNA from the rhizosphere of a dominant C4 grass in a tallgrass prairie ecosystem to elucidate seasonal dynamics in the rhizosphere-associated fungal communities. Fungal sequences were successfully obtained following reverse transcription with NS8 primer (White et al. 1990) and PCR amplification with fungus-specific primers (Borneman and Hartin 2000). Surprisingly, primers targeting Glomeromycota (Helgason et al. 1998) did not produce amplicons from the reverse-transcribed DNA, although Glomerales were frequently detected in the examined clone libraries. The reasons for this are unclear but may relate to folding of the single-stranded DNA template.

The RT-PCR application adopted for this study detected diverse fungal communities at each of the four sampling events. A majority of the OTUs were detected in only one sampling event and only at low abundances—a pattern that is typical to environmental sequencing studies (see Buee et al. 2009; Jumpponen et al. 2010). In a sampling and design that pools a number of samples into one clone library, the low frequency of most resident organisms unavoidably leads to stochastic detection of organisms except those that are the most dominant. It is interesting to note, however, that nearly all these observed taxa are most likely potential saprobes, whereas the AMF tended to occur in greater frequencies. This is likely related to the low diversity of the AMF (Öpik et al. 2009).

The AMF represented exclusively Glomerales, an observation consistent with a previous molecular assay of mycorrhizal communities inhabiting the roots of common plants in tallgrass prairie (Jumpponen et al. 2005). It is somewhat surprising that no other Glomeromycotan orders were detected, as their spores are a frequent component in this prairie ecosystem (Egerton-Warburton et al. 2007; Eom et al. 1999, 2000, 2001). The absence of these orders in a molecular survey such as this may be partly attributable to the PCR biases that favor the most abundant taxa, making detection of subordinate taxa difficult without an extensive sampling effort. The strong bias toward Glomerales may also be a result of management practices. The watershed sampled in this survey was annually burned—frequent burning has been suggested to lead to decline in AMF species richness and increase in the abundance of the dominant taxa (Eom et al. 1999).

The primary goal of this study was to test whether or not the use of environmental rRNA targets would permit detection of seasonal patterns in fungal communities associated with a dominant C4 grass. PCoA provided strong support for seasonal dynamics in the fungal communities. While the ecological roles and function of the AMF in the *A. gerardii* rhizosphere are predictable, the ecological functions of the remaining OTUs are less clear. For example, the OTUs representing Helotiales were largely comprised of those with affinities to species of *Helicodendron*. These fungi are best characterized by their coiled conidiospores (helicospores) and considered aero-aquatic because they are commonly collected in slow-running streams and ponds, and they sporulate when exposed to air (Goos 1987; Tsui et al. 2000; Tsui et al. 2001). The presence of fungi with possible affinities to aquatic taxa is surprising. However, these taxa are common decomposers of submerged plant tissues and include a variety of plant parasites and biotrophs (Goos 1987). Their prevalence in these soils and the *A. gerardii* rhizosphere may best be explained by the saturation or near saturation of the soil in the beginning of the growing season making it likely that fungi with adaptations to decomposition of submerged substrates might occur. Detection of the OTUs with affinities to helicosporous hyphomycetes across the later sampling events suggests that these fungi are a persistent but seasonally dynamic component of the rhizosphere. A comparison with another recent study that focused on the seasonal dynamics of *Quercus macrocarpa* rhizosphere communities (Jumpponen et al. 2010) suggests that partial replacement of saprotrophic communities by mutualists in the course of a growing season may be a common, if not universal, pattern in the rhizosphere environment.

This study explored the use of ribosomal RNA as a potential marker for tracing seasonal dynamics in root- and rhizosphere-associated fungal communities. Most of the adopted protocols are based on commercially available kits

and, therefore, are easily adoptable. The acquired data provide support for temporal turnover in fungal communities that includes partial replacement of saprobic communities by AMF. Further investigations to test these patterns in greater detail are warranted.

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