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Simulated nitrogen deposition affects community structure of arbuscular mycorrhizal fungi in northern hardwood forests

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Abstract

Our previous investigation found elevated nitrogen deposition caused declines in abundance of arbuscular mycorrhizal fungi (AMF) associated with forest trees, but little is known about how nitrogen affects the AMF community composition and structure within forest ecosystems. We hypothesized that N deposition would lead to significant changes in the AMF community structure. We studied the diversity and community structure of AMF in northern hardwood forests after more than 12 years of simulated nitrogen deposition. We performed molecular analyses on maple (*Acer* spp.) roots targeting the 18S rDNA region using the fungal-specific primers AM1 and NS31. PCR products were cloned and identified using restriction fragment length polymorphism (RFLP) and sequencing. N addition significantly altered the AMF community structure, and *Glomus* group A dominated the AMF community. Some *Glomus* operational taxonomic units (OTUs) responded negatively to N inputs, whereas other *Glomus* OTUs and an *Acaulospora* OTU responded positively to N inputs. The observed effect on community structure implies that AMF species associated with maples differ in their response to elevated nitrogen. Given that functional diversity exists among AMF species and that N deposition has been shown to select less beneficial fungi in some ecosystems, this change in community structure could have implications for the functioning of this type of ecosystem.

Keywords: arbuscular mycorrhizal fungi, community structure, forest, nitrogen, phylogeny, ribosomal DNA, sugar maple (*Acer saccharum*)

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Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous and have a very broad host range (Smith & Read 2008). However, it is clear that species can vary in abundance in response to different hosts (e.g. Helgason *et al.* 1998, 2002) and environmental conditions (e.g. Helgason *et al.* 1998, 2002; Treseder & Allen 2002; Wirsel 2004; Egerton-Warburton *et al.* 2007). It is also clear that both AMF diversity (Van der Heijden *et al.* 1998) and the identity of mycorrhizal fungal symbionts can have a large impact on host function (e.g. Johnson 1993; Corkidi *et al.* 2002; Helgason *et al.* 2002). Given rapidly changing environmental conditions (increased CO_2, N deposition, warming), it is important to study how these plant–fungal relationships are changing. To do this, we first need to identify the AMF species present in a broad range of ecosystem types and across a variety of environmental gradients to be able to understand the distribution, response to environmental change and function of mycorrhizal fungal species and communities (Lilleskov & Parrent 2007).

Increased nitrogen (N) deposition has generally been found to negatively affect the abundance of AMF

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(Treseder 2004; Van Diepen *et al.* 2007, 2010). Decrease in AMF abundance could be associated with (i) a decrease in all species present, (ii) a change in relative abundance of the associated species or (iii) a complete change in community structure.

Studies of AMF community response to N additions have had varying results often depending on ecosystem type, amount of nitrogen addition and number of years of fertilizer application. Several have shown increased nitrogen deposition, leading to a change in AMF community structure via analysis of asexual spores (e.g. Johnson 1993; Eom *et al.* 1999; Egerton-Warburton & Allen 2000; Egerton-Warburton *et al.* 2007), and more recently community changes have been detected via molecular techniques (Jumpponen *et al.* 2005; Porras-Alfaro *et al.* 2007). However, most of the N deposition studies looking at AMF community structure in plant roots have been performed in grassland or chaparral ecosystems. To our knowledge, no published studies have examined changes in forest ecosystems, so the generality of N deposition effects on AMF community structure across ecosystem types is yet to be determined. Effects of N deposition on AMF communities in nitrogen-limited northern hardwood ecosystems could be substantial if functional groups adapted to low N availability are negatively affected.

Identifying AMF species is a prerequisite for elucidating the functional significance of changes in AMF community composition and structure. We performed molecular analyses on maple roots from northern hardwood forests of Michigan, USA, in a study system in which an N deposition gradient had been combined with a fertilizer experiment for 12 years. At these sites, a myriad of changes have already been demonstrated (Table 1). In particular, the significant increase in ANPP combined with reduced soil respiration and lower AMF abundance in roots and soil (Table 1) incited our study of the AMF community structure at these sites. We hypothesized that increased chronic nitrogen deposition would decrease AMF species diversity and change AMF community structure.

Table 1 Summary of main effects of simulated N deposition on northern hardwood forest ecosystems (Michigan gradient study sites)

Variable	N-addition effect	References
Root & soil AMF biomass	↓	Van Diepen *et al.* 2007, 2010
Soil microbial biomass	↓	Van Diepen *et al.* 2010
Organic matter (Oa, Oe horizon)	↑	Zak *et al.* 2008
Organic matter (mineral soil 0–10 cm)	↑	Pregitzer *et al.* 2008
ANPP & litter N	↑	Pregitzer *et al.* 2008
Soil N content	↔	Pregitzer *et al.* 2008
Leaf litter biomass & area	↔	Pregitzer *et al.* 2008
Soil respiration	↓	Burton *et al.* 2004
Root biomass & respiration	↔	Burton *et al.* 2004
Leached DON, DOC	↑	Pregitzer *et al.* 2004

↓, significant decrease; ↔, no significant change; ↑, significant increase.
AMF, arbuscular mycorrhizal fungi; ANPP, aboveground net primary production; DON/DOC, dissolved organic nitrogen and carbon.

Materials and methods

Site description and sampling

Roots were sampled in October 2006 from four sugar maple (*Acer saccharum* Marshall)–dominated forest sites throughout Michigan, USA (Van Diepen *et al.* 2010). Three untreated and three N-amended plots (30 × 30 m) are located at each site. To experimentally simulate atmospheric nitrogen (N) deposition, N has been applied annually since 1994 in six equal increments of $NaNO_3$ during the growing season, for a total of 30 kg N/ha per year. Ambient wet N deposition at the sites ranged from 3.0 kg N/ha per year near site A to 6.8 kg N/ha per year near site D in 2006 (NADP 2006). All sites have similar soil development (Kalkaska sand, Typic haplorthod), stand age (94 ± 3 years), stand composition (82 ± 4% basal area in sugar maple) and stand structure, but differ in mean annual temperature and growing season length. More detailed information about the sites can be found in Burton *et al.* (1991).

For root sampling, each plot was divided into six equal (10 × 15 m) subplots, and one soil core (2 cm in diameter and 10 cm deep) was taken randomly in each subplot. Each soil core was washed through a 2- and 1-mm screen until most soil particles were washed out. Maple (*Acer* spp.) roots, easily recognized by their unique beaded structure (Pregitzer *et al.* 2002), were handpicked from the screens and cleaned more thoroughly with DI water and by sonication for 5 min. The maple roots were divided up into two diameter classes, fine roots (<0.5 mm) and larger roots (>0.5 mm), and weights of both classes were recorded. For each core, the fine maple roots were divided into two equal subsamples. One subsample was composited at the plot level and used for phospholipid fatty acid (PLFA) analysis to estimate AMF intraradical abundance (Van Diepen *et al.* 2010), similarly to the previous year (Van Diepen *et al.* 2007, 2010). The other subsample was

frozen, freeze dried and used for molecular analyses. Each subsample (144 in total) was analysed separately in all the steps of the molecular analyses to ensure maximum sensitivity to infrequent and rare taxa.

Molecular analysis

The lyophilized fine sugar maple roots were cut up in ~1 cm pieces, mixed, and DNA was extracted from a 5 mg subsample using a DNeasy 96 Plant Kit (Qiagen, USA) according to manufacturer's instructions. Extracted DNA was amplified according to Helgason *et al.* (1999) PCR cycling parameters, in a 25 μL reaction mixture of 0.5 μL Easy-A high fidelity PCR cloning enzyme (Stratagene), 2.5 μL manufacturer's Easy-A reaction buffer, 2.5 μL dNTP's (2 mM), 0.25 μL (20 μM) each of a eukaryotic primer AM1 (Simon *et al.* 1992) and a fungal primer NS31 (Helgason *et al.* 1998). This combination preferentially amplifies AM fungi, although a small percentage of certain other fungal taxa are amplified. PCR products were cloned into TOPO TA pCR2.1 vector (Invitrogen) and transformed into *Escherichia coli* (One Shot TOP10 Chemically Competent) according to manufacturer's instructions.

From each of the 144 samples, 15 positive clones were randomly selected and amplified using the same reaction mixture as in the first PCR, but replacing the cloning enzyme with Paq500 DNA polymerase (Stratagene). Up to 12 positive PCR products per sample were digested with restriction enzymes *Nla*III and *Dpn*II (New England Biolabs Inc.). Representatives of each restriction fragment length polymorphism (RFLP) type were then re-amplified, cleaned with StrataPrep PCR purification kit (Stratagene) according to manufacturer's instructions, and sequenced. Sequencing was performed by Nevada Genomics Center (Reno, Nevada, USA) on an ABI Prism 3730 DNA analyzer using primers NS31 and AM1 to obtain forward and reverse sequences, respectively.

Sequence and community structure analyses

In order to identify the obtained sequences, the sequences were compared with all known sequences in GenBank using Basic Local Alignment Search Tool (BLAST; Altschul *et al.* 1990) at the NCBI website. Only sequences matching unambiguously identified AMF taxa (first 100 BLAST matches are AMF taxa and have >95% sequence similarity) from GenBank were used for alignment in BioEdit (Hall 1999), using ClustalW (Thompson *et al.* 1994). The ClustalW alignment was checked and improved manually where needed.

To define operational taxonomic units (OTUs), a distance matrix was computed of our sequences using DNADIST version 3.5c (J. Felsenstein, University of Washington, Seattle, WA, USA). To understand the effects of N-amendment at different taxonomic levels, OTUs were defined at three different levels of RFLP type sequence similarity: 100%, 97% and 95% (Appendix).

Taxon accumulation curves (Mao Tau) were constructed per treatment and site and for all pooled data using a sample-based rarefaction method in EstimateS version 7.5 (Colwell 2005) using the analytical formulas of Colwell *et al.* (2004). Furthermore, the estimated total species richness by functional extrapolation was calculated in EstimateS using the Michaelis-Menten function (Colwell & Coddington 1994).

Shannon diversity index was calculated at each OTU level, and effects of treatments were determined using a two-way ANOVA with N treatment ($n = 2$) and site ($n = 4$) as factors.

The effects of N-amendment on AMF community structure for all three levels of OTUs were examined using permutational multivariate analysis of variance (PERMANOVA; Anderson 2005) based on the Bray–Curtis distance measure. We used PERMANOVA because it can handle statistical models with interaction terms. It does not provide graphical data display, so the community data were visualized using biplots of Canonical Analysis of Principal Coordinates (CAP; Anderson 2004) output. CAP was performed with each treatment at each site as a separate group (total of eight groups). Furthermore, effects of treatment on the most abundant OTUs were analysed using a two-way ANOVA with N treatment ($n = 2$) and site ($n = 4$) as factors.

To understand potential differences in AMF community structure among treatment and sites in more detail, we performed nonmetric multidimensional scaling (NMDS) using Bray–Curtis distance measure and fitted available environmental variables, e.g. soil N content, pH, temperature, precipitation, ANPP, foliar and soil nutrients using the Vegan package version 1.17-3 (Oksanen *et al.* 2010) in R version 2.10.0 (Team RDC 2010). Environmental variables were tested for significance with 1000 permutations, and the 95% confidence ellipses were calculated for the factors site and treatment.

An additional sequence alignment was made in BioEdit combining our sequences, AMF taxa from GenBank with high similarity (>97%) with our sequences and known AMF species. The full alignment was used to create phylogenetic trees in PAUP 4.0b10 (Swofford 1998), using maximum parsimony or neighbour-joining algorithm with 1000 bootstrap replicates to check support for the tree. The tree was rooted with a *Paraglomus occultum* sequence. Phylogenetic trees were visualized using Treeview (Win32 version 1.6.6; R.D.M. Page 2001). Sequences obtained from this study

were registered in GenBank under accession numbers GU322373 to GU322410.

A phylogenetic tree (maximum parsimony) of all sequences obtained in our study was also analysed in Unifrac (Lozupone *et al.* 2006) to understand if the differences in AMF communities between treatment and/or sites are related to specific lineages or clusters within the phylogenetic tree. Unifrac significance test was performed to study if AMF communities among treatments or sites differed based on unique branch lengths or unique clustering within the tree. All analyses were carried out using abundance data and were corrected using Bonferroni correction. Furthermore, the calculated Unifrac metrics were used for principal coordinated analysis (PCoA) and in environmental cluster analysis with Jackknife support to find clusters of treatments or sites and to test the confidence of the clustering, respectively.

Results

AMF taxa and diversity

Within the study, a total of 2160 clones (15 clones per sample) were amplified and resulted in an average of 12 positive PCR products of ~550 bp per sample (~1700 clones). Digestion of the ~550 bp positive PCR products resulted in 45 different RFLP patterns of which the sequences of 27 were matched with confirmed AMF taxa. Over 94% of all clones analysed within this study represented AMF taxa. Four RFLP patterns sequenced to Basidiomycetes (1 clone in 4 different samples; <1%) and six to Ascomycetes (<4%) based on BLAST matches in GenBank. Five of the Ascomycetes found only represented 1–2 clones within six samples. Ascomycete *Menispora tortuosa* was found in 32 of the 144 samples (3.3% of all clones). The remainder of the sequenced RFLP types (~0.5% of clones) did not match any fungal species. To avoid effects of imbalanced samples size on analyses, we used 9 randomly selected AMF clones per sample (total of 1296 clones) for all further analysis. To test uniformity of RFLP types, multiple clones (two to three) were sequenced from RFLP patterns representing >1% of all AMF clones. In total, 38 AMF clones were sequenced. The multiple sequenced RFLP patterns were highly similar (>99%, except clones A1/A2 and D1/D2 >97%) and formed distinct clades in the neighbour-joining phylogenetic tree (Fig. 1). Using the 27 AMF sequences, a total of 12 (95% similarity) and 17 (97% similarity) unique OTUs were found (Table 2). The different OTUs are evident in the neighbour-joining phylogenetic tree and showed AMF clades similar to OTUs defined at 97% and 95% sequence similarity (Fig. 1, Appendix). The OTUs as defined in Fig. 1 and Appendix were used for further analyses and discussion of the data.

Rarefaction curves (Mao Tau) of the pooled data showed a saturation of number of taxa found in our samples for both OTU groupings (95% and 97% similarity), but at the taxonomic level of 100% similarity the rarefaction curve did not level off completely, suggesting that more samples could have increased the number of RFLP types found in our maple roots (Fig. 2a). When observing taxon accumulation curves at the treatment by site level, no complete saturation was observed at the taxonomic level of 100% similarity. At the taxonomic level of 97% sequence similarity, it only showed saturation of the taxon accumulation curve for control plots at site C (Fig. 2b), which interestingly also had the highest Shannon diversity. At 95% sequence similarity, saturation was reached for control plots of site A and C and N-amended plots of site A and D. However, the estimated total taxon richness by functional extrapolation (using Michaelis-Menten function) was <3 RFLP types or 2 OTUs (97% and 95% similarity) greater than our observed number of taxa, indicating that we were very close to finding all taxa present within our system.

AMF Shannon's diversity was not significantly affected by treatment or site and varied from an average of 1.94 (100% similarity) to 1.64 (95% similarity) (Table 2). An average of 10.3 (±0.4), 9.0 (±0.3) and 7.3 (±0.2) OTUs were found overall at our study sites at the different taxonomic levels of 100%, 97% and 95% similarity, respectively. There was a significant site by treatment interaction at the taxonomic levels of 100% and 97% similarity (Table 2, Fig. 3). Site A showed a slight increase ($P = 0.11$) in taxon diversity with N-amendment, site B showed no difference ($P = 0.93$), and site C ($P = 0.03$) and D ($P = 0.21$) had a slight decrease (Fig. 3).

AMF community structure

Simulated increased N deposition led to a significant change in AMF community structure at all taxonomic

Fig. 1 Neighbour-joining phylogenetic tree of obtained arbuscular mycorrhizal fungi (AMF) sequences from this study (in bold), GenBank sequences of known Glomeromycota and of other GenBank AMF sequences of the 18S rDNA region highly similar to our sequences. Maximum parsimony phylogenetic trees had the same general topology. Tree is rooted to *Paraglomus occultum* (AJ276082). Bootstrap values of 70% (1000 replicates) and higher are shown, indicating well-supported branching. Operational taxonomic units as defined at 97% sequence similarity are displayed on the right side of the tree and family/group names of the Glomeromycota as defined in Schwarzott *et al.* (2001).

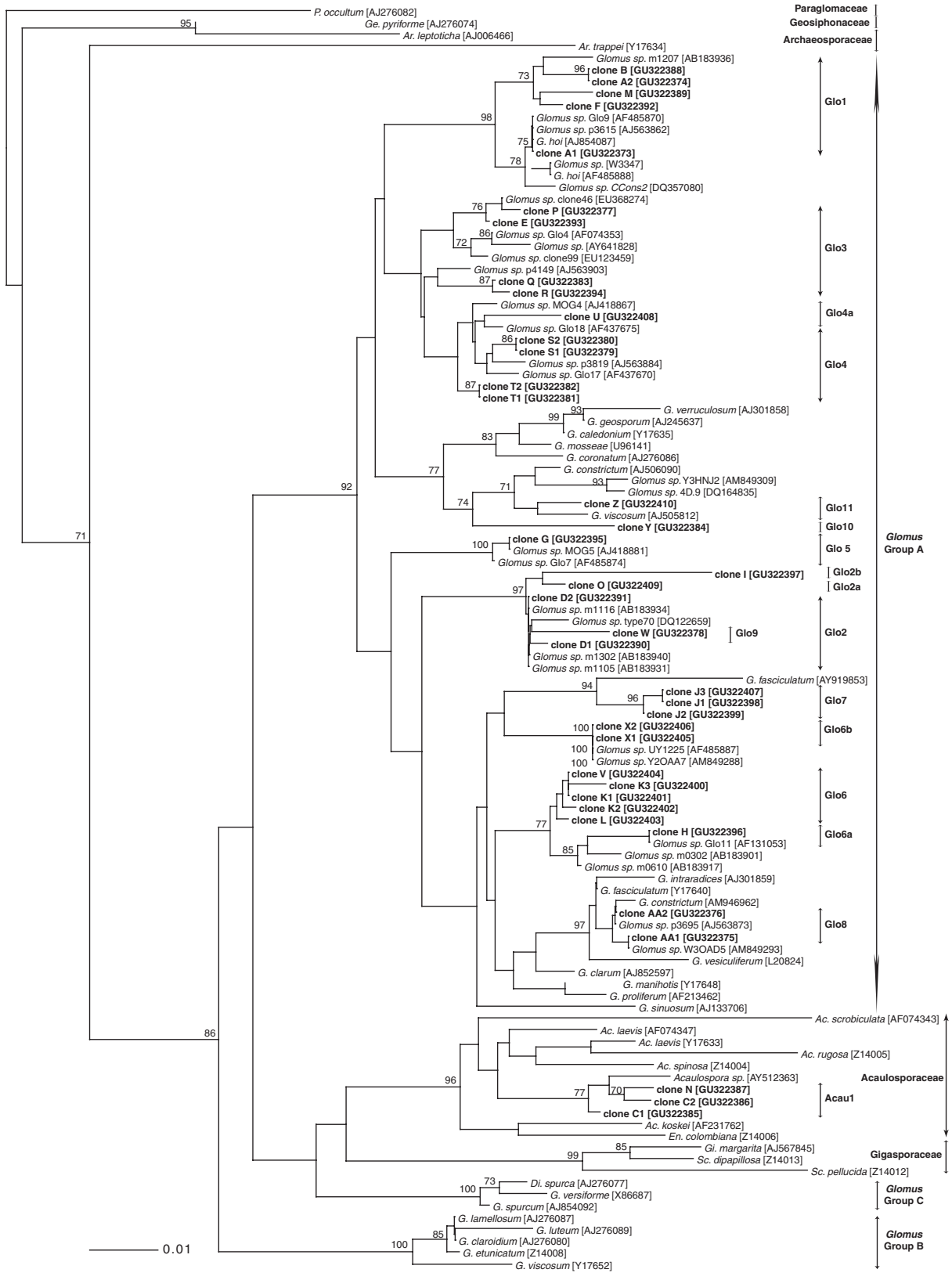

Table 2 Comparison of *P*-values of PERMANOVA of arbuscular mycorrhizal fungi community structure and ANOVA of Shannon diversity index for the 3 different taxonomic levels

Taxonomic level	No. OTUs	Community structure			Shannon diversity			Mean Shannon diversity
		Treatment	Site	Treatment * Site	Treatment	Site	Treatment * Site	
RFLP type	27	0.0315	0.0001	0.1637	0.908	0.172	0.035	1.94
97% similarity	17	0.0150	0.0001	0.1046	0.894	0.144	0.029	1.88
95% similarity	12	0.0132	0.0003	0.0998	0.416	0.441	0.810	1.64

OTUs, operational taxonomic units; RFLP, restriction fragment length polymorphism.

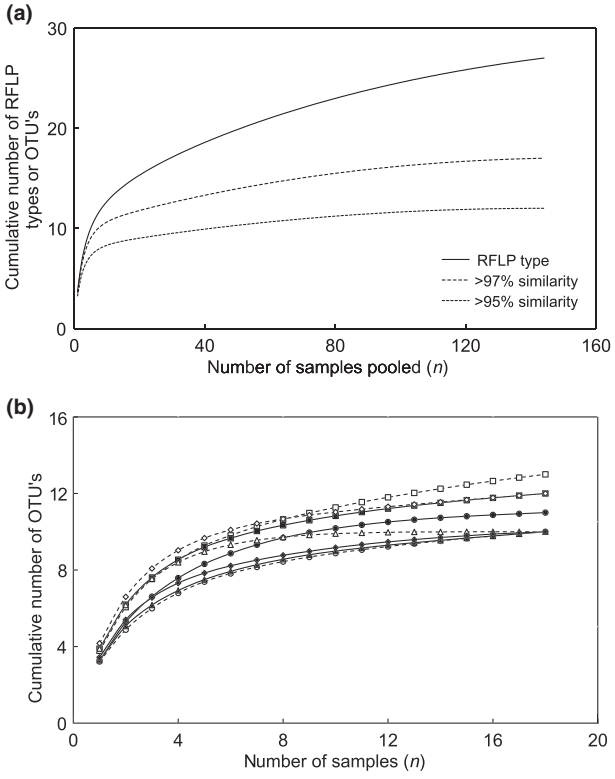

Fig. 2 Rarefaction curves with Mao Tau values estimating species accumulation at all taxonomic levels for number of arbuscular mycorrhizal fungi taxa of the pooled samples from all plots (a), and at treatment by site level at the taxonomic level of 97% similarity (b) (open symbols with dashed lines, control; closed symbols with solid lines, N-amended; circles, site A; squares, site B; triangles, site C; diamonds, site D).

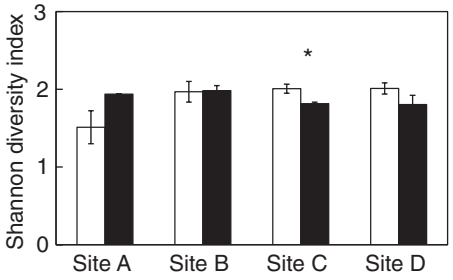

Fig. 3 Mean Shannon diversity index at the taxonomic level of 97% sequence similarity for the four study sites by treatment (open bars, control; closed bars, N-amended). Error bars indicate 1 SE of the mean. Overall treatment and site effect were not significant, but a significant treatment by site interaction was observed. *Mean of N treatments differs significantly at site ($P \leq 0.05$).

levels (Table 2, Fig. 4a, Appendix). The significance of the chronic simulated N deposition effect on AMF community structure increased slightly from 100% to 95% sequence similarity (Table 2). All taxonomic levels also showed a significant site effect (Table 2), and this site effect was stronger among control plots ($P = 0.001$) compared to N-amended plots ($P = 0.027$) when treatments were analysed separately. The first two axes of the CAP biplot with all plots explained 51%, 53% and 60% of the total variation in community structure among plots at the taxonomic level of 100%, 97% (Fig. 4a) and 95% sequence similarity, respectively.

All OTUs belonged to only two families of the Glomeromycota, with over 90% of all analysed clones from the genus *Glomus*, and the remainder (~6%) from the Acaulosporaceae (Acau1). Overall the most abundant OTUs were (in order of decreasing abundance) Glo2, Glo5, Glo6b, Glo8, Glo6, Glo1 [all belong to Glomus group A (Schwarzott *et al.* 2001)] and Acau1, which all formed distinct clades within the phylogenic analysis (Fig. 1) and represented on average more than 80% of all analysed clones for each treatment.

Of the most abundant OTUs, only Acau1 showed a significant N treatment main effect (Table 3) by increasing in abundance with simulated N deposition (Fig. 5g). Glo5 and Glo8 had a marginal N treatment effect ($P < 0.1$) (Table 3), with Glo5 increasing in abundance with N, whereas Glo8 decreased (Fig. 5b, d, respectively). For Glo2, a marginal negative N treatment effect was paralleled by a strong significant site effect (Table 3), with Glo2 decreasing in abundance

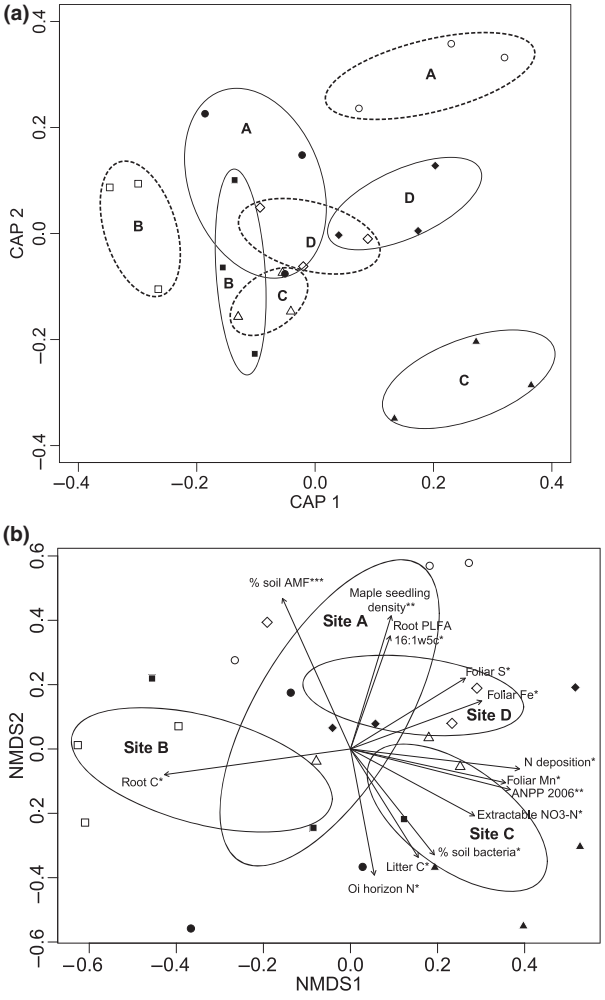

Fig. 4 Canonical Analysis of Principal Coordinates (CAP) biplots (a) and nonmetric multidimensional scaling (NMDS) plot (b) based on Bray–Curtis distance measure of arbuscular mycorrhizal fungi taxa structure of plots at treatment by site level with operational taxonomic units defined at 97% sequence similarity (open symbols, control; filled symbols, N-amended; circles, site A; squares, site B; triangles, site C; diamonds, site D). The first two axes of the CAP biplot explained 53% of the total variation. Solid circles in NMDS plot are 95% confidence ellipses of sites, and only significant environmental variables are shown, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ (see Table 4 for units).

going south (to higher ambient N sites) along the gradient (Fig. 5a). Glo6b and Glo6 (Fig. 5c, e, respectively) did not have a significant N treatment effect, but did have a significant site effect (Table 3), suggesting that their abundance was affected by the natural nitrogen gradient or by other site differences. The potential role of site differences for Glo6 is also observed in the significant treatment by site interaction, showing a significant increase at site A and a trend towards an increase at site B with N addition, while site C and D showed a trend towards a decrease with N addition (Fig. 5e).

Table 3 Comparison of *P*-values of ANOVA of dominant OTUs at 97% sequence similarity for the four study sites

OTU	Treatment	Site	Treatment * Site
Glo2	0.07	<0.001	0.16
Glo5	0.08	0.34	0.77
Glo6b	0.94	0.04	0.76
Glo8	0.09	0.16	0.73
Glo6	0.62	0.01	0.02
Glo1	0.40	0.71	0.77
Acau1	<0.001	<0.001	0.01

OTU, operational taxonomic unit, in order of decreasing overall mean abundance.

No treatment or site effects were found for Glo1 (Table 3, Fig. 5f) and OTUs that comprised the remainder of the clones (20%) found at our study sites. Glo1 was present in all the plots, while most of the remaining OTUs were only present in some of the plots (Appendix).

NMDS analysis with environmental variables had a significant treatment and site effect ($P = 0.033$ and $P = 0.002$, respectively, at 97% sequence similarity) and showed similar placement of plots as the CAP biplot (Fig. 4a,b). The 95%-confidence ellipses of the sites showed no overlap between sites B, C and D, indicating that these sites are significantly different in AMF community structure, while site A had some overlap in AMF community structure with all other sites (Fig. 4b, 95%-confidence ellipses of treatments are not shown for clarity of the figure, but did not overlap). The most significant environmental variables were AMF abundance in soil, fine root C, sugar maple seedling density, N in Oi horizon, total N deposition in 2005 and ANPP in 2006 and ($P \leq 0.01$, Table 4). Less significant variables were soil bacterial abundance, litter C, AMF abundance in fine roots, foliar Mn, S & Fe and extractable NO_3–N from soil ($P \leq 0.05$, Table 4). Other climatic factors such as temperature and precipitation, which describe the climatic gradient from north to south (site A–D), soil moisture & nutrients, and pH did not have a significant influence on the AMF community structure and did not explain the significant site differences we found.

The Unifrac test did not show a significant difference between treatments or sites at any taxonomic level, indicating that none of the branches in the phylogenetic tree of our sequences were unique to a treatment or site. This was confirmed by the PCoA of Unifrac metrics, which did not cluster the plots of similar treatments or sites together (data not presented). Furthermore, environmental cluster analysis using the Unifrac metrics did not show distinct clusters of treatments or

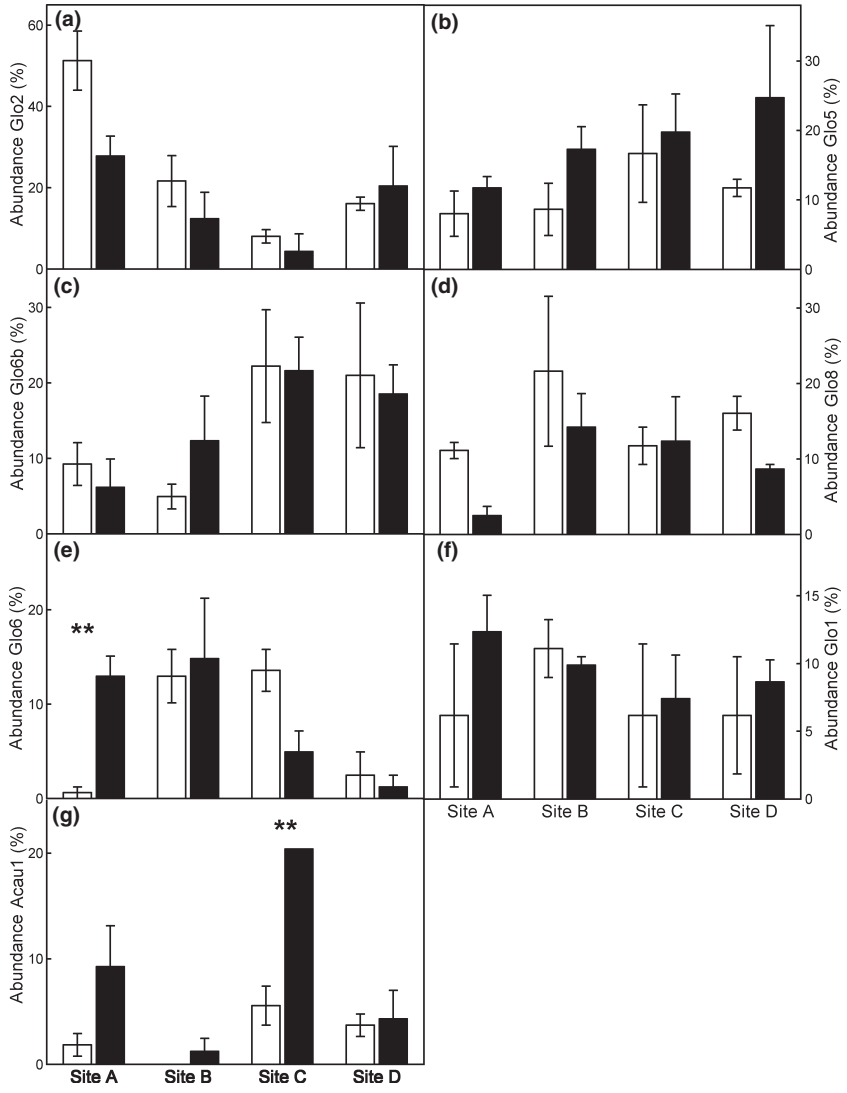

Fig. 5 Mean percentage abundance of dominant operational taxonomic units in order of decreasing overall dominance (a–g) for the four study sites by treatment (open bars, control; closed bars, N-amended). Abundance is based on number of clones, and error bars indicate 1 SE of the mean. See Table 3 for *P*-values of treatment and site effects. Means of N treatments differ significantly at site (**$P \leq 0.01$).

sites with high statistical confidence using Jackknife support (data not presented).

Discussion

Our finding that AMF community structure was significantly affected by N-amendment and site lends strong support to our hypothesis. This is the first study that tested for N-mediated community change in temperate forest AMF communities. Studies in grasslands have found similar results, showing AMF community shifts under N-amendment (Jumpponen *et al.* 2005; Egerton-Warburton *et al.* 2007; Porras-Alfaro *et al.* 2007). The higher significance found for differences in AMF community structure among control plots compared to N-amended plots could possibly indicate site homogenization because of N-amendment. This is supported by the greater similarity in N-amended communities than control communities for sites A, B and D, but not for community C, which diverged from the others after N-amendment (Fig. 4a). Egerton-Warburton *et al.* (2007) found a similar homogenization of AMF community following fertilization in five different grassland ecosystems, which persisted long after fertilization (>20 years) had stopped.

By fitting environmental variables measured at our sites, we were able to explain part of the site differences in AMF community structure. However, most of the significant variables were indirect effects caused by increased N deposition (e.g. ANPP, soil extractable

Table 4 Comparison of *P*-values and R^2 of significant environmental variables fitted in NMDS plot (Fig. 4b) for the four study sites. Variables are ranked based on R^2

Variable	Unit	R^2	*P*-value
AMF abundance in soil (2006)*	% PLFA 16:1ω5c of total PLFA	0.55	0.001
Fine root C (2006)*	%	0.43	0.007
Sugar maple seedling density	Stems/m^2	0.41	0.006
N in Oi horizon	mg/g	0.36	0.011
Total N deposition (2005)	kg/ha	0.35	0.011
ANPP (2006)	g/m^2	0.34	0.010
Bacterial abundance in soil (2006)*	% bacterial PLFA of total PLFA	0.33	0.013
Litter C (2006)	%	0.32	0.024
Foliar manganese (2006)	mg/kg	0.31	0.017
AMF abundance fine roots (2006)*	nmol PLFA16:1ω5c/mg fine root	0.30	0.026
Extractable NO_3–N from soil (2004)	μg/g	0.28	0.028
Foliar sulphur (2006)	mg/kg	0.27	0.035
Foliar iron (2006)	mg/kg	0.26	0.037

*Analyses were carried out on same samples as used in this study.
PLFA, phospholipid fatty acid; AMF, arbuscular mycorrhizal fungi.

NO_3–N, abundance of AMF in soil and roots, sugar maple seedling density), with N deposition being a combination of simulated N deposition and ambient N deposition along the gradient. Direct effects of site differences such as the climatic factors temperature and precipitation, non-N soil nutrient chemistry, overstory tree species diversity or abundance did not explain the differences in AMF community structure. Significant relationships with foliar S, Fe and Mn patterns suggest that part of the community response might be related to sulphur deposition or acidification, but there is no indication of a significant soil pH effect on the fungal community, and N additions have not significantly altered soil pH (D.R. Zak, unpublished data). Site D had a higher foliar S and Fe, and lower root C concentration (L.T.A. van Diepen, unpublished data), and was also the site that had the least response to simulated N deposition, both in AMF abundance (Van Diepen *et al.* 2007, 2010) and in community structure. Sites with older soils are hypothesized to have greater iron oxide and as a result greater sulphate adsorption capacity than other soils (Johnson *et al.* 1986). All soils in the present study are of recent glacial origin, so extreme differences in soil age are unlikely. Whether the differences in the present study arise from soil age, and whether the relationships with AMF community are spurious or causal awaits further investigation.

AMF play an important role in uptake of phosphorus (P), especially in P-limited systems (Smith & Read 2008), and experimental P limitation has been found to increase allocation to mycorrhizal fungi (e.g. Treseder & Allen 2002). We did not observe a shortage of P in our foliage, N treatment effect on foliar P ($P = 0.82$), or significant influence of foliar P on AMF community structure ($P = 0.11$). However, foliar P concentration did differ significantly among sites ($P < 0.001$). Sites C and A were the main drivers behind this site difference in foliar P concentration, because of the significantly higher foliar P concentration compared to the other two sites [site C: 1124 ± 40 mg/kg, site A: 984 ± 24 mg/kg, site B & D: 890 ± 17 mg/kg (mean ± SE)]. AMF species vary in their efficiency of P uptake and transfer to host plant, and Jakobsen *et al.* (1992) found that *Acaulospora* species were more effective in P uptake and transfer to the host plant compared to *Glomus* species. Consistent with this, foliar P concentration was significantly positively correlated with Acau1 abundance at our study sites ($R^2 = 0.26$, $P = 0.01$), while none of the other OTUs had a significant relationship with foliar P, which suggested a potential important role of Acau1 in P uptake and transfer to maple trees at our sites.

AMF community composition has also been found to vary seasonally and annually and with host plant (e.g. Helgason *et al.* 1998; Egerton-Warburton *et al.* 2007). Our samples were taken in fall of 1 year from only one tree species, so caution must be used in extrapolating our results to other forest types and seasons.

AMF community response was reflected in positive response to N addition for several taxa. Of our most abundant OTUs, Glo5 showed a positive response to N-amendment similar to a closely related environmental isolate found by Jumpponen *et al.* (2005). We also observed a positive response to N-addition for OTU Acau1, which suggested that our *Acaulospora* species was more tolerant of high nitrogen levels than some of the *Glomus* taxa found at our sites, or better at competing with species from the *Glomus* genus at higher levels of nitrogen.

Negative effects of N-amendment found for Glo8 parallel effects seen on close relatives of Glo8 found by Porras-Alfaro *et al.* (2007) in a semi-arid grassland, but

contrasted with increased abundance with N-amendment for a closely related isolate found by Jumpponen *et al.* (2005) in a tallgrass prairie. Glo8, closely related to the *Glomus intraradices/fasciculatum* group (Fig. 1), was a dominant OTU in grasslands and wetlands (Öpik *et al.* 2006). This suggests either context-dependence of response to N, or functional diversity within this clade.

The changes in AMF community structure found at our sites together with an overall decrease in intraradical and extraradical AMF abundance with N-amendment (Van Diepen *et al.* 2007, 2010) could have implications for the functioning of this type of ecosystem. Johnson (1993) found that N-amendment may select less mutualistic AMF taxa, which is reflected through decreased nutrient uptake efficiency or an increased carbon cost for the host plant. AMF taxa that are abundant at high N-levels have been found to be less beneficial or even detrimental to the host plant when compared with those from low N environments (Corkidi *et al.* 2002). Our observed change in AMF community structure thus has the potential to substantially change both nutrient and carbon cycling within northern hardwood forests. Although no dominant taxa were lost from sites as a result of simulated N deposition for 12 years, there was a substantial decline in abundance of Glo2 in three of four sites. Interestingly, these were the same three sites that also exhibited declines in intraradical and extraradical AMF biomass (Van Diepen *et al.* 2007, 2010). We hypothesize that functional differences between Glo2 and the increasing taxa at those sites (i.e. Acau1) will reveal fundamental differences in growth and nutrient supply to seedlings indicative of a less beneficial community under high N conditions. This hypothesis is supported by the significant positive relationship of Glo2 abundance and sugar maple seedling density (D.R. Zak, unpublished data) at our sites ($R^2 = 0.53$, $P < 0.0001$) together with a significant relation of sugar maple seedling density with AMF community structure (Fig. 4b, Table 4). In addition, none of the other OTUs had a significant relationship with sugar maple seedling density. Given the various stressors and declines associated with sugar maple forests, and in which pollutant deposition has been implicated (e.g. Minorsky 2003; Lovett & Mitchell 2004), these community changes suggest that changes in mycorrhizal fungal communities in response to nitrogen deposition could be one of the contributors to sugar maple decline.

Our finding that chronic N-addition had different effects on AMF taxon diversity per site, indicated by a significant treatment by site interaction, might reflect the conditional effect of treatments as a function of background N deposition levels. Diversity at our lowest N deposition site (A) had a trend towards a positive response to N-amendment, while the N-amended plots at our highest deposition sites (C and D) showed a trend towards a negative response by decreasing in taxon diversity. The switch in the sign of the diversity response to N fertilization as N deposition increases could reflect a response to the underlying N deposition gradient, suggesting that diversity in these communities might be highest in sites with intermediate N availability. Consistent with this hypothesis, Porras-Alfaro *et al.* (2007) also found higher taxon diversity with N-amendment in a semi-arid grassland, which had comparable ambient N deposition (2 kg N/ha per year) to our site A (3 kg N/ha per year).

Shannon diversity index at our sites for a single tree species was in the same range ($H = 1.64$–1.94, Table 2) as values for a grassland system for two plant species ($H = 1.71$, Vandenkoornhuyse *et al.* 2002), lower than in a tropical forest for two plant species ($H = 2.33$, Husband *et al.* 2002) but much higher than an arable site ($H = 0.39$, Helgason *et al.* 1998). Although it is tempting to speculate that lower taxon diversity has led to lower functional diversity, lower taxon diversity does not necessarily indicate lower functional diversity. For example, Munkvold *et al.* (2004) found a substantial functional heterogeneity with low AMF species diversity by looking at species isolate characteristics and their benefits to the plant. This intraspecific functional variation raises another challenging aspect of interpreting AMF community structure and diversity and their related function in ecosystem processes (Sanders 2004; Van der Heijden & Scheublin 2007). Conclusive understanding of the functional consequences of these changes awaits studies directly addressing this topic.

Although sequence matches of 97–100% similarity with AMF taxa in GenBank were found for all our AMF sequences, most matches were to unidentified environmental isolates, with only a few sequences similar to known AMF species within the GenBank database. This could indicate that our forests contain a large number of species that have not been characterized, or that the GenBank database is insufficient in coverage of known species. Natural forest ecosystems of North America have been much less sampled than other ecosystems types such as agroecosystems and grasslands, so it is entirely possible that these lineages are not well characterized, and future efforts should focus on isolation, morphological and taxonomic description and physiological characterization of these taxa.

Phylogenetic analysis confirms that most of our sequences were from one subclade of the genus *Glomus* (Glomus group A, Schwarzott *et al.* 2001) and a few from one subclade of the genus *Acaulospora*. Phylogenetic analysis of the AMF community structure using Unifrac resulted in no significant effect, indicating that the taxa present at our plots were not clustered in

specific lineages or branches by treatment or site. A possible cause for this lack in significant clustering could be the low variety in genera and subclades within the *Glomus* genus found at our sites. The primer pair we used in this study, NS31 and AM1, adequately amplifies the families Glomeraceae, Gigasporaceae and Acaulosporaceae (Helgason *et al*. 1998), but does not properly target the Archaeosporaceae and Paraglomeraceae (Redecker *et al*. 2000). However, AMF spore studies carried out in sugar maple forests have not found spores of the Archaeosporaceae or Paraglomeraceae (e.g. Moutoglis & Widden 1995; Coughlan *et al*. 2000; Lansing 2003; Lerat 2003).

In summary, we found a significant effect of simulated and increased ambient nitrogen deposition on AMF community structure, and the responses of some AMF taxa to N-amendment within our study paralleled responses found for those AMF taxa in other studies. Finding this repeated pattern is a prerequisite for determining universal patterns and rules for responses of AMF taxa to environmental change (Van der Heijden & Scheublin 2007). This study has contributed to our understanding of responses of some AMF taxa in sugar maple–dominated forests, which form extensive stands in northern temperate biomes, and indicates the need for characterization of the functional consequences of these community shifts.

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Appendix

Abundance of RFLP types (percentage of 54 clones) across a nitrogen deposition gradient in Michigan, USA [mean and standard error (n = 3)]

RFLP/ clone type	Site A		Site B		Site C		Site D		RFLP types > 97% similarity	RFLP types > 95% similarity	OTU name
	C	N	C	N	C	N	C	N			
A	5.6 (4.7)	11.1 (2.8)	11.1 (2.1)	9.3 (1.1)	6.2 (5.3)	7.4 (3.2)	6.2 (4.3)	8.6 (1.6)	A,B,F,M	A,B,F,M	Glo 1
B	0.6 (0.6)	–	–	–	–	–	–	–	A,B,F,M	A,B,F,M	Glo 1
F	–	1.2 (0.6)	–	–	–	–	–	–	A,B,F,M	A,B,F,M	Glo 1
M	–	–	–	0.6 (0.6)	–	–	–	–	A,B,F,M	A,B,F,M	Glo 1
C	1.9 (1.1)	9.3 (3.9)	–	1.2 (1.2)	5.6 (1.9)	19.8 (0.6)	3.7 (1.1)	4.3 (2.7)	C,N	C,N	Acau 1
N	–	–	–	–	–	0.6 (0.6)	–	–	C,N	C,N	Acau 1
D	51.2 (7.3)	27.8 (4.9)	21.6 (6.3)	12.3 (6.5)	8.0 (1.6)	4.3 (4.3)	16.0 (1.6)	20.4 (9.8)	D	D,O,I	Glo 2
O	1.2 (1.2)	0.6 (0.6)	–	–	–	–	–	–	O	D,O,I	Glo 2a
I	–	–	–	–	–	–	0.6 (0.6)	–	I	D,O,I	Glo 2b
E	5.6 (2.1)	3.1 (1.2)	3.7 (1.1)	4.9 (4.0)	4.3 (1.6)	–	1.9 (1.1)	4.3 (1.2)	E,P,Q,R	E,P,Q,R	Glo 3
P	0.6 (0.6)	0.6 (0.6)	-	0.6 (0.6)	1.2 (0.6)	–	3.1 (1.2)	0.6 (0.6)	E,P,Q,R	E,P,Q,R	Glo 3
Q	–	–	0.6 (0.6)	–	–	–	–	–	E,P,Q,R	E,P,Q,R	Glo 3
R	0.6 (0.6)	–	–	–	0.6 (0.6)	–	0.6 (0.6)	–	E,P,Q,R	E,P,Q,R	Glo 3
S	–	–	5.6 (2.1)	3.7 (1.9)	1.2 (1.2)	3.1 (3.1)	2.5 (1.2)	0.6 (0.6)	S,T	S,T,U	Glo 4
T	–	–	3.7 (1.9)	1.9 (1.9)	3.1 (1.2)	2.5 (1.6)	2.5 (0.6)	1.2 (0.6)	S,T	S,T,U	Glo 4
U	–	–	1.2 (0.6)	0.6 (0.6)	–	–	–	–	U	S,T,U	Glo 4a
G	8.0 (3.3)	11.7 (1.6)	8.6 (3.8)	17.3 (3.3)	16.7 (7.0)	19.8 (5.5)	11.7 (1.2)	24.7 (10.4)	G	G	Glo 5
K	0.6 (0.6)	11.1 (3.9)	13.0 (2.8)	13.6 (5.9)	12.3 (2.2)	4.3 (1.6)	1.9 (1.9)	1.2 (1.2)	K,L,V	H,K,L,V,X	Glo 6
L	–	1.9 (1.9)	–	1.2 (0.6)	1.2 (1.2)	0.6 (0.6)	–	–	K,L,V	H,K,L,V,X	Glo 6
V	–	–	–	–	–	–	0.6 (0.6)	–	K,L,V	H,K,L,V,X	Glo 6
H	3.7 (2.1)	8.6 (5.9)	1.9 (1.1)	5.6 (1.1)	5.6 (2.1)	2.5 (1.6)	11.1 (2.8)	6.8 (5.1)	H	H,K,L,V,X	Glo 6a
X	9.3 (2.8)	6.2 (3.8)	4.9 (1.6)	12.3 (5.9)	22.2 (7.5)	21.6 (4.5)	21.0 (9.6)	18.5 (3.9)	X	H,K,L,V,X	Glo 6b
J	–	4.3 (4.3)	–	–	–	–	–	–	J	J	Glo 7
AA	11.1 (1.1)	2.5 (1.2)	21.6 (9.9)	14.2 (4.5)	11.7 (2.5)	12.3 (5.9)	16.0 (2.2)	8.6 (0.6)	AA	AA	Glo 8
W	–	–	0.6 (0.6)	–	–	1.2 (1.2)	–	–	W	W	Glo 9
Y	–	–	1.2 (1.2)	–	–	–	0.6 (0.6)	–	Y	Y	Glo 10
Z	–	–	0.6 (0.6)	0.6 (0.6)	–	–	–	–	Z	Z	Glo 11