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ORIGINAL PAPER

Mechanical soil disturbance as a determinant of arbuscular mycorrhizal fungal communities in semi-natural grassland

Tim Krone Schnoor · Ylva Lekberg · Søren Rosendahl · Pål Axel Olsson

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Abstract While the effect of disturbance on overall abundance and community composition of arbuscular mycorrhizal (AM) fungi has been researched in agricultural fields, less is known about the impact in semi-natural grasslands. We sampled two AM plant species, *Festuca brevipila* and *Plantago lanceolata*, from an ongoing grassland restoration experiment that contained replicated plowed and control plots. The AM fungal community in roots was determined using nested PCR and LSU rDNA primers. We identified 38 phylotypes within the Glomeromycota, of which 29 belonged to *Glomus* A, six to *Glomus* B, and three to Diversisporaceae. Only three phylotypes were closely related to known morphospecies. Soil disturbance significantly reduced phylotype richness and changed the AM fungal community composition. Most phylotypes, even closely related ones, showed little or no overlap in their distribution and occurred in either the control or disturbed plots. We found no evidence of host preference in this system, except for one phylotype that preferentially seemed to colonize *Festuca*. Our results show that disturbance imposed a stronger structuring force for AM fungal communities than did host plants in this semi-natural grassland.

T. K. Schnoor (✉) · P. A. Olsson
Plant Ecology and Systematics, Department of Biology,
Lund University,
The Ecology Building, Sölvegatan 47,
223 62 Lund, Sweden
e-mail: tim.krone_schnoor@ekol.lu.se

Y. Lekberg · S. Rosendahl
Terrestrial Ecology, Department of Biology,
University of Copenhagen,
Øster Farimagsgade 2D,
1353 København K, Denmark

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Introduction

Disturbance creates and alters ecosystems, and changes in disturbance intensity are hypothesized to influence species richness (Connell 1978; Huston 1979) and possibly ecosystem functions. Due to this, the impact of disturbance has been studied in many different ecosystems and groups of organisms, albeit with an emphasis on plants. Most land plants are colonized by arbuscular mycorrhizal (AM) fungi, where the fungi can provide the majority of plant required P (Smith et al. 2003) in return for up to 20% of the assimilated carbon (C; Jakobsen and Rosendahl 1990). Due to the ubiquitous nature and functional importance of AM, a better understanding of the effect of disturbance on plant communities will also require a consideration of the fungal partner of the symbiosis.

While little is known about the effect of disturbance on AM fungi in natural plant communities, more studies have been conducted in agricultural fields. Plowing and other forms of disturbances have been shown to reduce overall AM fungal abundance (Allison et al. 2005; Kabir 2005; Lekberg and Koide 2005), spore numbers (Galvez et al. 2001; Oehl et al. 2003), and species richness (Antunes et al. 2009). Other studies have indicated no change in overall richness, but a drastic shift in community composition (Hamel et al. 1994; Jansa et al. 2002; Jansa et al. 2003; Violi et al. 2008). These could be due to differences in life history strategies among fungal taxa generated by disparate growth patterns (Hart and Reader 2002) and infective propagules (Klironomos and Hart 2002). For example, *Glomus mosseae* and *Glomus caledonium* sporulate readily

and are common in disturbed agricultural soils, whereas natural undisturbed grasslands tend to host a greater proportion of unknown taxa that sporulate rarely and grow extensive mycelia (Rosendahl 2008; Rosendahl and Stukenbrock 2004). Because differences in growth and reproduction among AM fungi could have functional consequences, an increased knowledge regarding factors that drive fungal community composition is imperative.

Soil disturbance is an important component in some habitat restoration projects to counteract the loss of early colonizing plant species due to competitive exclusion (Dolman and Sutherland 1994). Similar comparisons are difficult in regards to AM fungi since we know so little about the distribution and abundance of different species. Furthermore, in situ studies of AM fungi have been limited by available methods for fungal identification, but recent advances in molecular analyses of AM fungi *in planta* have significantly increased our insights concerning their ecology. Here, we utilized molecular tools to test hypotheses regarding the effect of soil mechanical disturbance and degree of host preference in a replicated restoration field trial in calcareous semi-natural grassland in Sweden. We hypothesize that disturbance-sensitive, non-sporulating taxa will be lost, leading to an overall reduction in richness with disturbance. Furthermore, we speculate that the fungal communities will differ between disturbed and undisturbed areas due to variation in disturbance tolerance among AM fungal taxa (Antunes et al. 2009), and that disturbance will have a greater impact than host plants for structuring fungal communities.

Methods

Study area and sampling

The experimental site is located at the Rinkaby military training ground in eastern Scania, southern Sweden (55°58N 14°18E). The area has a mean annual precipitation of 500–550 mm, and a mean annual temperature of 7.5°C (based on data from 1956 to 2004 from the Swedish Metrological Institute, SMHI). The site lies on glacio-fluvial deposits within a belt of calcareous bedrock, where the pH often exceeds 7 and the soil is dominated by sand fractions with differing levels of silt. Due to the combination of previous disturbance and high soil pH, this 420 ha large area has hosted a sand steppe vegetation (Andersson 1950), or habitat type 6220 according to the Natura 2000 nomenclature, which includes many red-listed species such as *Koeleria glauca*, *Dianthus arenarius* ssp *arenarius*, *Phleum arenarium*, and *Alyssum alyssoides* (Olsson et al. 2009). Today, only small areas of this vegetation remain due to acidification and a discontinued mechanical soil disturbance.

In May 2006, large-scale restoration experiment was set up within a 0.5-ha section in Rinkaby in order to identify a mechanical soil disturbance that could restore the sand steppe and allow a reintroduction of red-listed plant species that belong in this habitat. Because these areas historically have been used for low-intensity agriculture, we used plowing as the source of disturbance, which overturned the soil to a depth of 30 cm and left no visible clumps of sod. While plowing is an unlikely form of disturbance in natural grasslands, it would be adequate as a means to restore habitats that has traditionally been used for extensive agriculture, such as the site studied here. Plowing was conducted in four 6×50-m areas, each located within a block that also contained four 6×50-m control areas without any disturbance. During the time of plowing, grasses such as *Festuca brevipila*, *Festuca rubra*, *Helictotríchon pubéscens,* and forbs such as *Medicago sativa* ssp. *falcata* and *Galium verum* had largely replaced the sand steppe vegetation within the experimental plots. Soil samples taken 1 week after the plowing showed that the disturbance did not significantly influence pH (H_2O) or available phosphorous (Bray1 and NaF+$NaSO_4$ extractable, data not shown).

In August 2008, about 2 years after the disturbance event, we destructively sampled one *F. brevipila* tussock and one *Plantago lanceolata* plant every 10 m along a 40-m transect within each plot. Thus, ten plants were collected within each plot, resulting in a total of 80 plants, of which 40 came from plowed plots and 40 came from control plots. *F. brevipila* and *P. lanceolata* were chosen because they were the most abundant AM hosts in both treatments and because they represent functionally different groups (grass and forb). Samples (containing shoots and whole root systems) were transported back to the laboratory and stored at 8°C until processed. Within a week, roots were washed clean from sand and stored in the freezer awaiting DNA extraction. AM colonization was assessed on pooled samples from within each species and plot using the gridline intersect method (Giovannetti and Mosse 1980) after staining with trypan blue (Brundrett et al. 1996).

DNA extraction and amplification

From each plant, ten root pieces (1 cm long for *Plantago* and 1.5 cm for *Festuca*) were collected randomly, and each root piece was transferred to 80 μL Tris EDTA pH 8 buffer and heated for 2 min at 95°C. Slightly longer root pieces were used for *Festuca* due to the lower AM colonization observed in these plants. The roots were ball-milled; 20 μl 20% Chelex-100 (BioRad) was added, and the homogenate was heated to 95°C for 2 min and centrifuged at 4,000 rpm for 15 min. The supernatant was diluted 50×, and 2 μl were used in the first polymerase chain reaction (PCR) using the universal primers 0061 and NDL22 (Kjøller and Rosendahl

2000). Amplicons were diluted 50× and used in a second PCR with the primers FLR3/FLR4 (Gollotte et al. 2004) that amplifies a region of the large subunit (LSU) rDNA of Glomalean fungi. This primer pair has shown to successfully amplify species within *Glomus* A and B group names refer to Da Silva et al. (2006), as well as members of Gigasporaceae and Acaulosporaceae (Gollotte et al. 2004; Mummey and Rillig 2007). Successful amplification was detected by gel electrophoresis, and positive samples were directly sequenced using FLR3 as sequencing primer by Macrogen (Seoul, South Korea). Samples showing mixed sequences were discarded from further analysis.

Alignment of sequences and phylotype definitions

The glomeromycotan origin of the sequences was verified by BLAST search and aligned manually with closely related BEG isolates if possible or with sequences from environmental samples (Altschul et al. 1997). A phylogenetic tree based on maximum parsimony implemented in MEGA version 3.1 (Kumar et al. 2004) was constructed. Phylogenetic groups were defined as terminal branches with more than 98% bootstrap support, as this level seems to separate morphologically defined species. The phylogenetic networks were inferred with the neighbor-net algorithm implemented in splits Tree 4.10 (Huson and Bryant 2006) based on uncorrected pair-wise distances.

Data analyses

Sampling effort curves were constructed in EstimateS (Colwell 2009) using each plant as a replicate and presence/absence of fungal phylotypes. The sampling effort curve for the disturbed and control treatments includes both plant species, and the sampling effort curve for the two plants includes both the disturbance and the control treatment. Phylotype richness was analyzed by a generalized linear model using Poisson error distribution, and AM colonization was analyzed by a generalized linear model using bionomial or quasibinomial error distribution (due to overdispersion) with plant species and disturbance as crossed factors, all in R 2.7.2 for Macintosh (R Developement Core Team 2008). We used multivariate analyses to determine if the AM fungal communities differed between the disturbed and control plots and whether or not differences were apparent between the two host plants. All analyses had samples as scaling focus, and all data were log-transformed. Canonical correspondence analysis (CCA) with manual forward selection, and 499 permutations were conducted with Canoco for Windows 4.54 (Ter Braak and Smilauer, Biometris Plant Research International, The Netherlands). Unimodal methods were justified by the length of the gradient in this data set. The proportion of variance in the AM fungal communities that could be explained based on treatment or host plant identity was calculated using variance partitioning through alternating the explanatory variables while using the other as a co-variable. Sequences only found once were excluded from the multivariate analyses but were included in the sampling effort curves and species richness analyses.

Results

AM colonization was significantly higher (t=4.93, p<0.001) in *P. lanceolata* (29±3 plowed, 41±1 control, mean percentage±se) than *F. brevipila* (16±4 plowed, 17±2 control), but there was no significant effect of plowing (t=−0.23, p=0.82) or significant interactions between species and disturbance (t=1.20, p=0.26). This low to moderate AM colonization could at least partly explain the low PCR success observed (21%). From the 800 root pieces extracted, we obtained 169 sequences, of which 33 were mixed and thus discarded from further analysis. Twelve of these sequences were from *P. lanceolata* in control plots (PC),

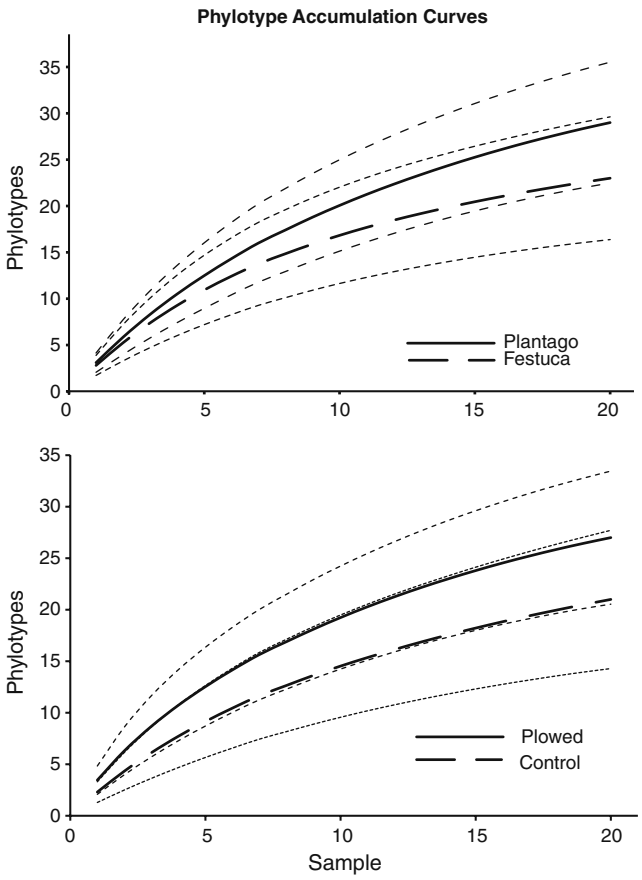

Fig. 1 Sampling effort curves for AM fungal phylotypes in **a** the two host species and **b** for the two treatments. *Dashed lines* represent 95% confidence interval

six from *P. lanceolata* in plowed plots (PP), ten from *F. brevipila* in control plots (FC), and five from *F. brevipila* in plowed plots (FP). This left us with 43 sequences from PC, 22 PP, 38 from FC, and 30 from FP. Within the restrictions of our extraction and amplification, sample effort curves indicated that the sampling intensity employed captured a large portion of the phylotype richness, both when considering host species and disturbance treatments (Fig. 1). The

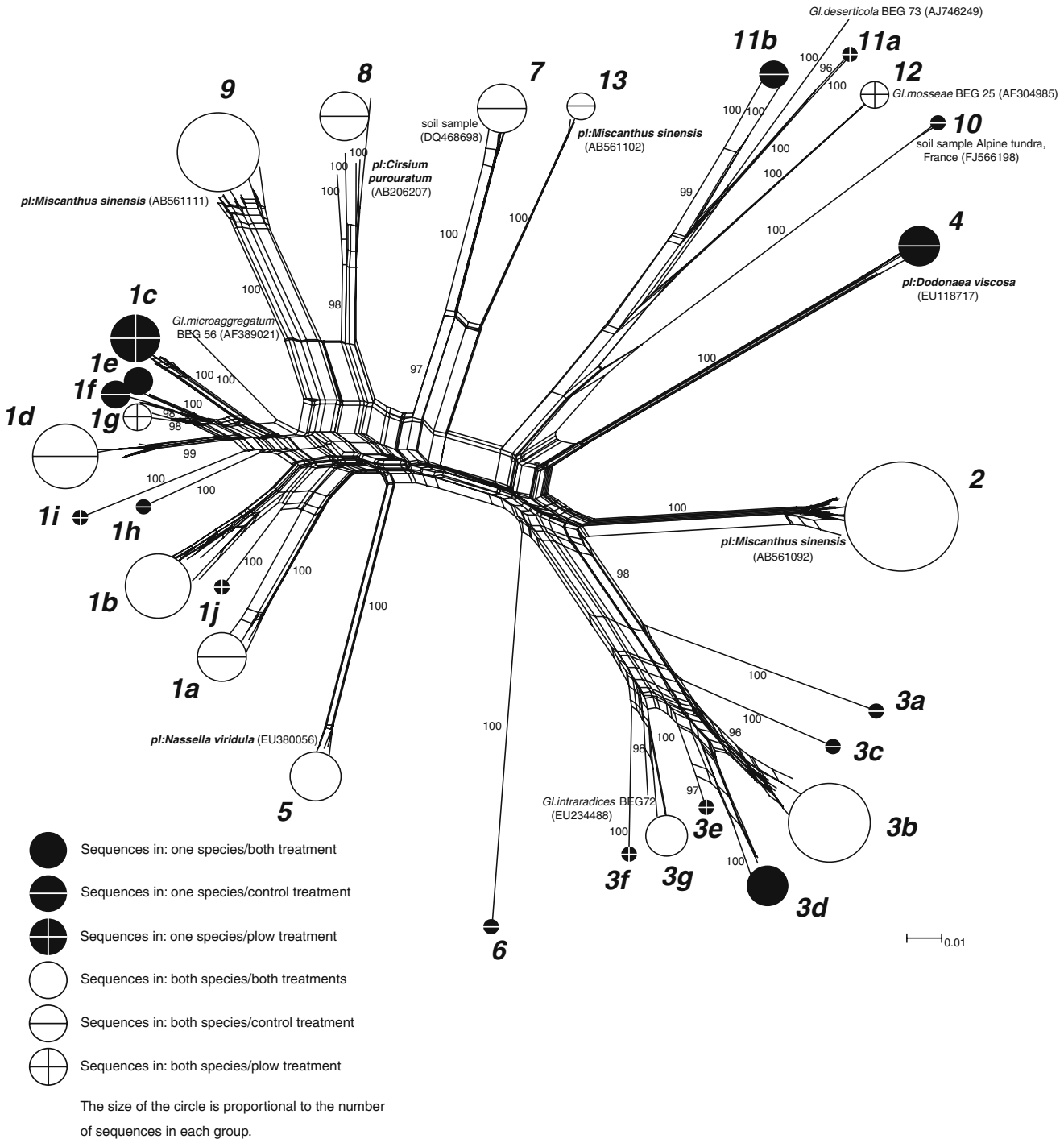

Fig. 2 Phylogenetic network of the LSU rDNA sequences belonging to *Glomus* group A extracted from *F. brevipila* and *P. lanceolata* roots in control and plowed plots. *Numbers* denote bootstrap support. A detailed description of sequences belonging to each group and sequence accession numbers is found in Table 1. The letters "*pl:*" in reference sequences denotes that the sequence is derived from the plant species given after the abbreviation

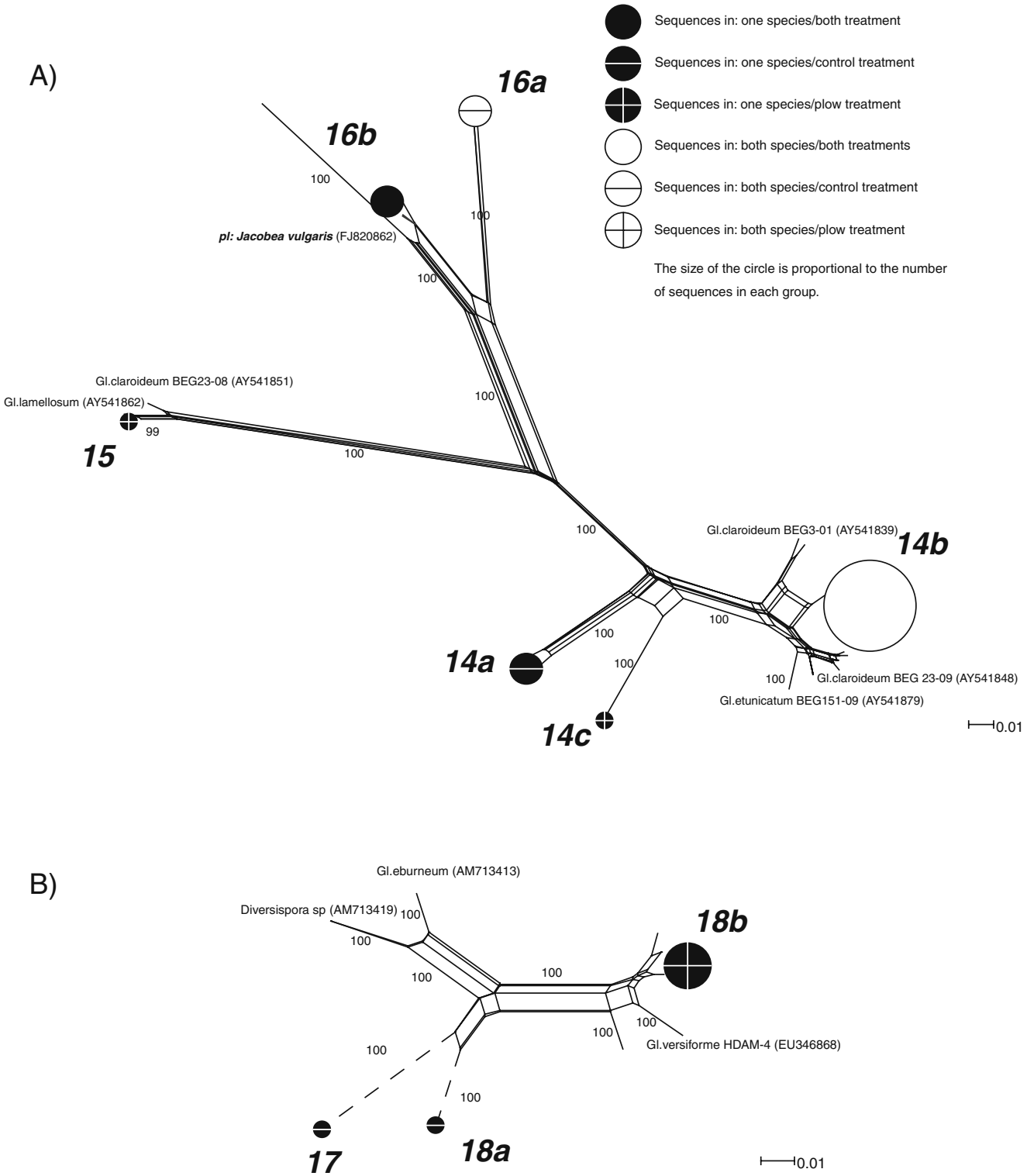

Fig. 3 Phylogenetic network of the LSU rDNA sequences belonging to **a** *Glomus* group B **b** *Glomus* group C/Diversisporaceae, extracted from *F. brevipila* and *P. lanceolata* roots in control and plowed plots. *Numbers* denote bootstrap support. A detailed description of sequences belonging to each group and sequence accession numbers is found in Table 1. *Dashed lines* were reduced to 20% of its original length to increase readability. The letters "*pl:*" in reference sequences denotes that the sequence is derived from the plant species given after the abbreviation

estimated total richness (mean Chao1 and Chao2, EstimateSMac800, Colwell 2009) suggest that we captured between 74% and 80% of the richness in our plots.

In all, we found 38 phylotypes of which 29 belonged to *Glomus* A (Fig. 2), six to *Glomus* B (Fig. 3a), and six to Diversisporaceae (Fig. 3b). The 29 phylotypes within *Glomus* A were divided into 17 clades, in which closely related phylotypes were placed. For example, clade 3 in Fig. 2 is divided into 3a–3g, denoting seven related groups. *G. mosseae*, *Glomus intraradices*, and *Glomus microaggregatum* were the only species with closely related phylotypes within *Glomus* A in our study. The phylotypes named *Glomus* D/F/G are related to sequences found by Rosendahl and Stukenbrock (2004) in a similarly dry, sandy grassland in Denmark. The remaining phylotypes showed little or no association to previously published sequences. Phylotypes belonging to *Glomus* B appeared to be closely related to *Glomus claroideum* and *Glomus etunicatum*, whereas phylotypes within the Diversisporaceae were related to *Glomus versiforme*, except for two sequences that showed little or no relationship with known morphospecies or environmental samples. Overall, disturbance significantly decreased phylotype richness ($z=-2.532$, $p=0.01$), but there was no difference in richness between the two host species ($z=0.36$, $p=0.72$). This was true regardless if singletons (phylogenetic groups with only one sequence) were included in the analysis or not (Fig. 4). Table 1 lists the distribution and abundance of sequence types in the two treatments and on the two plant species.

Disturbance significantly altered the phylotype composition ($F=2.39$, $p=0.002$) as revealed by CCA with Monte Carlo permutations of sequences (excluding singletons). There was no significant difference, however, between the two plant species (Fig. 5). Variance partitioning showed that disturbance explained 14.8% of the total variance in phylotype distribution, whereas plant species explained 7.0%. There was virtually no overlap in the variation explained by the two variables (0.1%), suggesting little or no interaction. A more careful examination of the CA ordination plot (Fig. 5) indicated that the variation in phylotype composition (i.e., the distance among individual plots in ordination space) was greater in disturbed plots compared with control plots, which suggests that the disturbed plots were less uniform. Also, we found four sequences of Glom A1c in *Festuca* roots in three different disturbed plots (Table 1), suggesting that this particular phylotype may, apart from preferring disturbed habitats, display some degree of host preference. Other phylotypes, such as Glom A3d, 1f, 4, 11b, 14a, and 18b were also found in either *Plantago* or *Festuca*, but due to their low sequence number (≤3), this should be considered with caution.

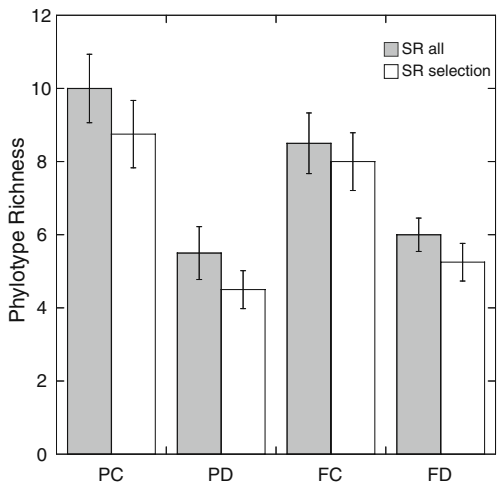

Fig. 4 Number of phylotype groups per treatment, both including (SR all) and excluding singletons (SR selection). *Error bars* show SE. *PC=Plantago* control, *PD=Plantago* disturbed, *FC=Festuca* control, and *FD=Festuca* disturbed

Discussion

Our results show that soil disturbance had a large impact on the AM fungal community. In agreement with our first hypothesis, we observed reduced phylotype richness in disturbed plots, most likely due to a loss of disturbance sensitive phylotypes that was not fully compensated for by an increase in the number of disturbance-tolerant phylotypes. Plant species richness, on the other hand, increased in disturbed plots (Schnoor and Olsson, unpublished data), and this differential response by plants and AM fungi is interesting as it suggests that the positive correlation between plant and AM fungal species richness observed in controlled experiments (van der Heijden et al. 1998) may depend on the environment. One can speculate about potential differences in structuring forces for plants and AM fungi during secondary succession. For example, whereas plowing may reduce overall plant density that could lower interplant competition and allow inferior competitors to persist, fungal competition may actually *increase* as a result of the lower abundance of roots to colonize, especially since many early successional plants are non-mycorrhizal or facultative mycotrophs. This raises the possibility that early successional AM fungi must not only be disturbance-tolerant, but also competitive. Our results also suggest that the level of disturbance required to optimize richness in this habitat differ among plants and fungi. Given the intimate relation between plant and AM fungi, this should be considered in restoration projects.

Table 1 List of phylogenetic types as defined by the network analysis (≥98% similarity)

Phylotype	Hits	Sequences found in each treatment		Closest reference sequence	Accession no.
		Control	Plowed		
Glom A					
1a	3	P2, P3, F3	–	*G. microaggregatum*	GU108353
1b	8	P3	2 P1, P2, F1, 2 F2, F3	″	GU108366
1c	4	–	F2, F3, 2 F4	″	GU108347
1d	7	P1, P3, P4, F1, F2, F3, F4	–	″	GU108336
1e	2	P2	P4	″	GU108370
1f	2	F1, F4	–	″	GU108337
1g	2	–	P4, F3	″	GU108345
1h	1	P3	–	″	GU108358
1i	1	–	P2	″	GU108368
1j	1	–	F4	″	GU108348
2	13	3 P1, P2, P4, 2 F1, 2 F2, 2 F4	P3, F1	ND[a]	GU108359
3a	1	F4	–	*G. intraradices*	GU108342
3b	11	2 P2, P3, F3, 2 F4	P2, P4, F1, 2 F3	″	GU108340
3c	1	F2	–	″	GU108338
3d	3	P3	P1, P2	″	GU108373
3e	1	–	F1	″	GU108343
3f	1	–	P4	″	GU108372
3g	4	P2, F1, F4	P2	″	GU108350
4	2	P2, P4	–	ND	GU108351
5	4	P1, P2	F1, F2	″	GU108362
6	1	P4	–	″	GU108360
7	4	P1, P2, F3, F4	–	″	GU108341
8	4	P1, P3, F1, F4	–	″	GU108352
9	10	P1, 2 P2, P3, 2 F1, F4	P3, F1, F2	″	GU108356
10	1	P2	–	″[b]	GU108349
11a	1	–	F3	″	GU108346
11b	2	F1, F3	–	″	GU108339
12	2	–	P2, F2	*G. mosseae*	GU108367
13	2	P3, F3	–	ND[c]	GU108357
Glom B					
14a	2	P2, P4	–		GU108361
14b	8	P3, F2, 2 F4	P1, 2 P2, P4	*G. claroideum*	GU108354
14c	1	–	P2	ND	GU108365
15	1	–	P4	″	GU108371
16a	2	P1, F2	–	″	GU108363
16b	2	P2	P1	″	GU108369
Diversisporae					
17	1	P3	–	″	GU108355
18a	1	P1	–	″	GU108364
18b	3	–	2 F1, F2	*G. versiformae*	GU108344

Number of hits for each phylotype is shown as well as their distribution among treatments, their closest reference sequences in GenBank, and the accession number of submitted sequences for each phylotype. *P* denotes sequences found in *P. lanceolata* and *F* in *F. brevipila* and the *numbers following letters* denotes plot number and the *numbers before letters* denote the number of sequences found in that plot; no number means one sequence; ″ same as above

[a] *ND* none described. Refers to when sequences are most similar to sequences that have only been found in environmental samples with no species name

[b] Similar to type F and G in Rosendahl and Stukenbrock (2004)

[c] Similar to type D in Rosendahl and Stukenbrock (2004)

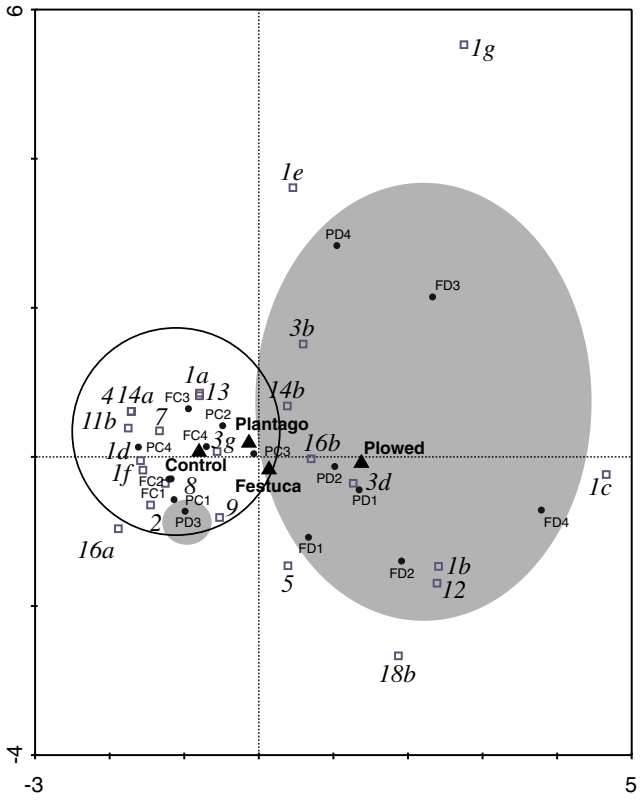

Fig. 5 Correspondence analysis of the phylogenetic groups and their association with treatments and host species superimposed in the figure. *Circles* denote plots and *squares* phylogenetic groups. *Numbers* of the groups refer to numbers in Table 1. The *ring* encircles all control plots, and the *shaded ovals* cover all plowed plots. Eigenvalue for axis 1 is 0.61 and for axis 2 0.45. Total inertia is 3.19

In accordance with our second hypothesis, disturbance caused a shift in fungal communities. We are not the first to report this (Jansa et al. 2003; Violi et al. 2008), but our results show that responses may be similar between semi-natural grasslands and agricultural soils. This is not surprising if one assumes that co-occurring species possess different life history strategies (Hart and Reader 2002; Helgason et al. 2007), where ruderal, disturbance-tolerant phylotypes are expected to proliferate after plowing, whereas more competitive phylotypes dominate in undisturbed grassland. Indeed, the majority of phylotypes found here showed little or no overlap between disturbed or control plots. For example, Glom A1c, Glom A12, and Div 18b were only found in disturbed plots. These three phylotypes are closely related to *G. microaggregatum*, *G. mosseae*, and *G. versiforme*, which have been shown previously to occur preferentially in disturbed environments (Helgason et al. 1998; Sykorova et al. 2007). On the contrary, Glom A1d, Glom A7, and Glom A8 were only found in control plots (Table 1). Glom A7 is closely related to *Glomus* type D in the study by Rosendahl and Stukenbrock (2004), which has been hypothesized to spread by hyphal growth instead of spores. These results suggest that while plants had re-colonized the plowed plots and showed a higher richness than control plots after 2 years (Schnoor and Olsson, unpublished data), this time may have been insufficient for the re-colonization by fungal phylotypes that spread by hyphae rather than spores. It is interesting to note, however, that phylotypes closely related to *G. microaggregatum* showed a disparate distribution and occurred in either control or disturbed plots. Wu et al. (2007) also found two clades related to *G. microaggregatum* along an altitudinal gradient within a volcanic desert of Mount Fuji where one was a generalist and the other was more common in higher altitudes and restricted to two out of four host species. These disparate distribution patterns by closely related phylotypes challenges the assumption that closely related species are functionally more similar than more distantly related species due to niche conservatism (e.g., Maherali and Klironomos 2007), as this may not necessarily be true for AM fungi.

While most AM fungi showed a preferential allocation and occurred in either disturbed or control plots, others, such as Glom A3b, Glom A9, and Glom B14b were more evenly distributed between the two treatments. *Glomus intraradices* is often considered a generalist species (Helgason et al. 2007; Sykorova et al. 2007), and this was supported here because sequences most similar to this species were present in both treatments and host species (Glom A3a–g; Table 1). Many of the phylotypes within this clade were singletons, which is in accordance with the previously documented high genetic variability within *G. intraradices* (Boerstler et al. 2008). Due to this high variability, it is hard to determine whether the rare phylotypes in this clade show a similar non-specific behavior as the more abundant types, and the generalist behavior of this whole group should therefore be treated with caution.

Supporting our third hypothesis, disturbance appeared to be a stronger structuring force than host preference because no general pattern of host preference could be found even though there were indications that some phylotypes occurred more frequently in one of the two plant species. Our finding of no community level host preference is therefore in line with the idea that AM fungi are more or less generalist (Smith and Read 2008), even though several studies have indicated some degree of host preference (Helgason et al. 2002; Vandenkoornhuyse et al. 2002). However, the number of sequences per phylotype and plant species was low in our study, and before the degree of host preference in our system could be determined with certainty, substantially more sequences are required.

Not only did the disturbance change the community composition and reduce richness, it also increased the

variation in phylotype distribution among plots. That is, the community composition in disturbed plots differed more from each other than did control plots. We propose that this is caused by spurious associations between proximate ruderal phylotypes and plant individuals establishing after plowing, which results in the documented higher influence of stochastic processes during early successional stages (Christensen and Peet 1984; Peet and Christensen 1980). As succession proceeds, these ruderal fungi will subsequently be replaced by competitively strong AM fungal species, leading to community convergence (del Moral 2009) and the lower variation seen in the control plots.

Contrary to many molecular studies that have used cloning, we extracted small root fragments that were directly sequenced. This method has been used previously (e.g., Rosendahl and Stukenbrock 2004) and has the advantage of being quantitative, less costly, and fast, which means that significantly more samples can be processed compared with cloning studies. Also, this method can favorably be used for investigations of small-scale spatial patterns of AM fungi. The disadvantage with the direct sequencing approach is the mixed sequences, where potentially valuable information has to be omitted. Regrettably, we had to discard 20% of our sequences, but it is unlikely that an inclusion of these would have drastically altered our results since we found only slightly more mixed sequences in control plots compared with plowed plots, and control plots had more clean sequences (81) compared with plowed plots (52). It is possible that these mixed sequences represents intra-species sequence divergence rather than a mixture of two phylotypes, but this is less likely because divergence is relatively low in the LSU region. Overall, the number of sequences was relatively low in our study based on the number of extractions, but this is not too surprising given the low to moderate AM colonization observed. Extracting more root samples and obtaining more sequences would unlikely have altered the significant disturbance effect, but could have allowed us to discuss the degree of host preference with more certainty. Based on this, we want to re-emphasize that the observed lack of host preference within this grassland should be considered with caution until further studies are conducted.

In conclusion, we showed that soil disturbance—but not host species identity—was an important structuring force for AM fungal communities in this disturbance-dependent semi-natural grassland. These findings could have implications for plant and fungal restoration success and need to be considered.

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