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454-sequencing reveals stochastic local reassembly and high disturbance tolerance within arbuscular mycorrhizal fungal communities

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Summary

1. Disturbance is assumed to be a major driver of plant community composition, but whether similar processes operate on associated soil microbial communities is less known. Based on the assumed trade-off between disturbance tolerance and competiveness, we hypothesize that a severe disturbance applied within a semi-natural grassland would shift the arbuscular mycorrhizal (AM) fungal community towards disturbance-tolerant fungi that are rare in undisturbed soils.

2. We used 454-sequencing of the large subunit rDNA region to characterize AM fungal communities in *Plantago lanceolata* roots grown in the field for 4 months and exposed either to no disturbance or to severe disturbance where fungi from undisturbed soil were either permitted or prevented from re-colonizing the disturbed area. This allowed for a distinction between AM fungi that survived the disturbance and those that quickly re-colonized after a disturbance. To identify AM fungi that could potentially colonize the experimental plants, we also analysed roots from adjacent, undisturbed vegetation.

3. We found 32 fungal operational taxonomic units (OTUs) distributed across five known AM fungal families. Contrary to our expectations, disturbance did not significantly alter the community composition and OTU richness. Instead, OTU abundances were positively correlated across treatments; i.e., common OTUs in undisturbed soil were also common after the severe disturbance. However, the distribution of OTUs within and between plots was largely unpredictable, with approximately 40% of all sequences within a sample belonging to a single OTU of varying identity. The distribution of two plant species that are often poorly colonized by AM fungi (*Dianthus deltoides* and *Carex arenaria*) correlated significantly with the OTU composition, which may indicate that host quality could be an additional driver of fungal communities.

4. *Synthesis*. Our results suggest that factors other than disturbance drive the relative abundance of OTUs in this grassland and question the long-held assumption that communities shift in a predictable manner after a disturbance event. The reassembly of this fungal community indicates a high community resilience, but substantial local stochasticity and dominance by single OTUs, which could be due to priority effects among abundant AM fungi possessing a similar – and high – degree of disturbance tolerance.

Key-words: arbuscular mycorrhizal fungi, community composition, disturbance, large ribosomal subunit, massively parallel pyrosequencing, plant–soil (below-ground) interactions, resilience, semi-natural grassland, spatial processes

Introduction

Understanding mechanisms that drive the relative abundance of species is a central goal of ecology. Disturbance – both natural and anthropogenic – is considered to be a major structuring force in communities and drives succession, promotes exotic invasions and influences overall species richness. For example, the intermediate disturbance hypothesis (IDH, Connell 1978) states that the highest richness is obtained at intermediate levels of disturbance, because it allows organisms that differ in

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disturbance tolerance and competitiveness to coexist. While the IDH has been supported in intertidal systems and some plant communities (see references and discussion in Roxburgh, Shea & Wilson 2004), relatively little is known about the role of disturbance for the richness and community composition of soil microbes that are often considered to be resistant, resilient and functionally redundant (Allison & Martiny 2008).

Approximately 80% of terrestrial plant species form a root symbiosis with arbuscular mycorrhizal (AM) fungi in the phylum Glomeromycota, where plants exchange carbon (C) for phosphorus (P) and possibly other services with the obligate biotrophic fungi (Smith & Read 2008). To date, about 200 morphospecies have been identified and a subset are in culture (Schüßler, Schwarzott & Walker 2001). Experiments and phylogenetic analyses with cultured fungi have shown that colonization strategies and subsequent growth patterns differ among AM fungal families (Hart & Reader 2002; Powell *et al.* 2009), and these differences could influence their response to disturbance. Indeed, whereas de Souza *et al.* (2005) argued that members of the Gigasporaceae harbour traits that may confer competitiveness but sensitivity to disturbance, some Glomeraceae species show a more disturbance-tolerant, opportunistic behaviour (Sykorova *et al.* 2007; Ijdo *et al.* 2010). For example, *Glomus mosseae* is common in agricultural fields and tends to have a fast growth rate and a high allocation to spores relative to mycelium (Jasper, Abbott & Robson 1991; Helgason *et al.* 1998). This species is also found in less-disturbed grasslands (Rosendahl & Stukenbrock 2004; Sykorova *et al.* 2007) but may depend on spatial and/or temporal disturbance events to persist.

Molecular analyses of AM fungi *in planta* have made it increasingly clear that the morphologically defined taxa drastically underestimate total AM fungal richness. For example, Vandenkoornhuyse *et al.* (2002) found 24 taxa, or operational taxonomic units (OTUs), in a temperate grassland and only one belonged to a morphologically defined species. Fitter (2005) even suggested that the fungal species richness may rival that of plant species richness within communities, and this was recently supported within a 100-m^2 boreal forest that hosted 47 fungal OTUs and an equivalent number of plant species (Öpik *et al.* 2009). Whether or not uncultured fungi display similar growth patterns and functions as their cultured relatives is currently unknown. Recent studies have suggested that this may not be the case, because several uncultured *Glomus* taxa grew extensive mycelia without any apparent spores (Rosendahl & Stukenbrock 2004) and occurred late in succession (Sykorova *et al.* 2007).

The objectives with this study were to experimentally test whether co-occurring AM fungi in a seemingly undisturbed coastal grassland differ in resistance to – and recovery from – disturbance, and whether potential differences observed are phylogeny based. That is, will disturbance cause a shift in the identity of dominant taxa, and are those taxa going to be closely related? Previous studies in this grassland (Rosendahl & Stukenbrock 2004; Stukenbrock & Rosendahl 2005) have shown that the dominant AM fungi are uncultured and can grow extensive mycelia, but that more disturbance-tolerant taxa, such as *Glomus mosseae*, are present but rare (Rosendahl & Stukenbrock 2004). High disturbance tolerance and community resilience can result from taxa either surviving the disturbance or being able to rapidly re-colonize the disturbed area. To separate these traits, we applied two disturbance treatments in which AM fungi outside the disturbed area were either allowed or barred from re-colonizing. Treatment effects were evaluated based on comparisons of fungal communities in roots of *Plantago lanceolata* transplanted into either disturbed or undisturbed soil, and with those communities in mixed species roots collected from adjacent, undisturbed vegetation. We used recently developed second-generation 454-Sequencing™ (Margulies *et al.* 2005) to characterize fungal communities, which is a powerful alternative to cloning and Sanger sequencing (Öpik *et al.* 2009). Owing to the assumed trade-off between disturbance tolerance and competitive ability, we expect an inverse relationship in OTUs among treatments, such that disturbance-tolerant OTUs are rare in the un-disturbed treatments and *vice versa*. Furthermore, if traits associated with an ability to survive disturbance and to rapidly re-colonize are phylogenetically conserved, we hypothesize that treatments will cluster according to phylogenetic relatedness.

Materials and methods

FIELD SITE AND EXPERIMENTAL DESIGN

The study was conducted within a grassland on the north coast of Zeeland, Denmark (56°01′ N–11°59′ E), which hosts a diverse grass and forb community (Rosendahl & Stukenbrock 2004). On 2 July 2008, 11 plots (1.5 × 1.5 m) were selected within a 100 × 50 m area, and two disturbance treatments were applied within each plot; one that allowed re-colonization by surrounding AM fungi post disturbance (D), and another where a plastic barrier prevented re-colonization, and where colonization could only occur from fungi that survived the disturbance (DB). For both disturbance treatments, all shoots were cut off and removed from a 30-cm diameter area, and roots and soil were excavated with a trowel down to a depth of 20 cm. Roots were cut into 2-cm pieces, and the soil was passed through a 10-mm sieve; and in order not to drastically change the depth distribution of AM fungi, the upper 10 cm of roots and soil were kept separate from the lower 10 cm. In the DB treatment, a 25-cm-diameter plastic bucket was inserted into the hole before the soil was put back, which separated the disturbed area from the non-disturbed soil. The bottom of the bucket had been cut off at a depth of 20 cm to allow free drainage. This was considered to be sufficiently deep to prevent colonization from neighbouring, undisturbed mycelium, because the great majority of roots are in the upper 10 cm, and drastic differences in texture appeared at 20 cm in which the sand was replaced by large rocks. Furthermore, a separate experiment with in-growth tubes had shown that control plants grown in sterile soil with 1-μM screen windows and open bottoms at 20 cm had an AM colonization of < 5% after 1 year, while colonization was > 80% when grown in non-sterile soil. This colonization route is therefore very slow and can more or less be ruled out in this experiment where plants only grew for 4.5 months.

Two 1-month-old *Plantago lanceolata* seedlings were planted into each disturbed area immediately following the disturbance. Two

plants were also planted into native soil in each plot as controls (C), and care was taken to disturb the soil and surrounding vegetation as little as possible during planting. All three treatments were within 1 m of each other. *Plantago lanceolata* was chosen because it occurs naturally in the grassland, and the seedlings had been grown from seeds (kindly provided by Dr Pål Axel Olsson) in heat-sterilized field soil under greenhouse ambient conditions and watered as needed with tap water. The non-mycorrhizal status of three randomly selected seedlings was confirmed prior to outplanting by examining the roots under a dissecting microscope after staining with trypan blue (Brundrett *et al.* 1996). Owing to a persistent drought in July, the transplanted seedlings were watered twice weekly until 1 August, when the drought ceased. We surveyed the plant community non-destructively on 7 July by estimating the percentage coverage of individual plant species within an intact and undisturbed 1-m^2 area inside the plots (see Table S1 in Supporting Information). Except for some small, ruderal annuals that re-established from seeds, the transplanted *P. lanceolata* seedlings were the only plants inside the disturbed areas throughout the experimental period.

HARVEST

On 21 August, one plant was harvested per treatment. Efforts were made to minimize disturbance to the surrounding area. Plants and rhizosphere soil were placed in individual plastic bags and transported back to the laboratory, stored at + 4 °C and processed within 48 h. The rhizosphere soil from the C, D and DB treatments was passed through a 2-mm sieve and frozen prior to analysis. Shoots were separated from the roots and dried in 60 °C to constant weight, and dry weight was recorded. Roots were washed free of adhering soil, cut into 2-cm pieces and thoroughly mixed in water. A small subsample was taken and stained in trypan blue as above for analyses of AM colonization using the gridline intersect method (McGonigle *et al.* 1990). Coarse mycorrhizal hyphae were scored separately from fine endophytes (FE). Both types were aseptate and stained blue, but the FE hyphae were much finer, displayed an irregular and knobby growth and a fan-shaped growth pattern within the root. The remaining roots were freeze-dried and kept at −20 °C before DNA extraction.

On 18 November, 4.5 months after planting, the remaining plants were harvested and processed as outlined above. In addition to the C, DB and D treatments, native *P. lanceolata* that were growing in, or in close proximity (< 3 m) to, the plots, were harvested to determine whether the AM fungal communities in experimental plants were similar to those of native plants. We found native *P. lanceolata* plants within, or adjacent to, four plots in our study, and these samples will hereafter be referred to as ‘N’ as in native. Furthermore, 10 mycorrhizal plants of various species that were common within each plot were harvested and pooled to identify the AM fungal OTUs that were present and could colonize the experimental plants. These samples will hereafter be referred to as ‘S’ for surrounding plants. We considered this approach appropriate given that no host preference has been documented in this site previously (Stukenbrock & Rosendahl 2005).

Soil samples (C, D, DB) from the second harvest were analysed for available P, NO_3 and NH_4 after extractions (1:10, w:v) in 0.01 M $CaCl_2$ (Houba *et al.* 2000). Soil pH and electrical conductivity (EC) were measured in water (1:1, v:v), and soil organic matter (SOM) was determined based on loss of ignition. Soil samples from C and D treatments within four randomly selected plots were also analysed from the first harvest for comparisons.

DNA EXTRACTIONS AND MOLECULAR ANALYSES

Owing to the low AM colonization (< 10%) observed in the transplanted seedlings at the first harvest in all treatments, DNA was only extracted from roots from the second harvest. DNA was extracted from 25 mg of freeze-dried, milled roots using chloroform and isopropanol according to Gardes & Bruns (1993) and stored in TE buffer at −20 °C. Non-mycorrhizal pea roots were included as a negative control to ensure that no contamination occurred during the extraction. DNA was extracted from a total of 48 samples, including 11 D, DB, C, S and 4 N. Samples were prepared for 454-sequencing in a two-step PCR procedure. In PCR1, the 3′ end of the large ribosomal subunit (LSU) was amplified with eukaryotic primers 0061 (Van Tuinen *et al.* 1998) and LR5 (Vilgalys & Hester 1990). We used a regular Taq polymerase (Ampliqon, Skovlunde, Denmark) and the following thermocycling parameters: 1 min 95 °C, 25 cycles of 30 s at 95 °C, 30 s of 54 °C and 1 min at 72 °C (+ 4 s per cycle) with a final extension phase of 5 min at 72 °C. The 900-bp PCR products were purified by membrane filtration (Macherey-Nagel, Düren, Germany) and diluted 50× prior to PCR2, which was run for 10 cycles under the same conditions as PCR 1, except for a 56 °C annealing temperature and no increase in extension time. PCR2 products were about 350 bp long, and bands were gel-purified using the QIAEX II kit (Qiagen, Hilden, Germany). Target DNA was amplified with the novel forward primer glo454 (3′-TGAAAGGGAAACGATTGAAGT-5′) in combination with NDL22 (Van Tuinen *et al.* 1998) in PCR 2, which targets the nLSU-D2 region that was recently suggested to be suitable for high-throughput sequencing (Stockinger, Kruger & Schüßler 2010). This primer combination amplifies all known AM fungal clades, including the deep-branching *Paraglomus*, but may also amplify some non-AM fungi, especially basidiomycetes. A preliminary cloning and sequencing of glo454-NDL22 products from the extracted root samples showed that sequences of AM fungal origin always dominated the amplicon pools (results not shown). The glo454 primer sequence included the 454 linker A, while NDL22 included linker B (Margulies *et al.* 2005), i.e. amplicons were sequenced from the glo454 primer end. Additionally, three different 10-base tags were included between linker A and the glo454 primer (Dowd *et al.* 2008), allowing all 48 samples to be distributed among sixteen 454-sectors with three samples in each sector. The three-tag sequences used were tag1: TGTACCGATG, tag2: CTCACTTAGG and tag3: CGCCGTTATA. The amplified fragments with adapters and tags were quantified using a Qubit™ fluorometer (Invitrogen, Naerum, Denmark) and qPCR (Mx-3000, Stratagene) as previously described (Larsen *et al.* 2010). The sample amplicons were mixed in equal amounts (4×10^5 copies μL^{-1}) to ensure equal representation of each sample. A 16-region 454-sequencing run was performed on a 70_75 GS PicoTiterPlate (PTP) using a GS FLX pyrosequencing system according to manufacturer instructions (Roche Hvidovre, Denmark). The sequencing run including metadata has been stored in the Short Read Archive at NCBI with accession number SRA029261. Sorting and trimming of sequences based on size (> 150 bp) and quality (> 20) was performed by the Pipeline Initial Process at the RDP’s Pyrosequencing Pipeline (http://rdp.cme.msu.edu/; Cole *et al.* 2009).

SEQUENCE ANALYSIS AND DESIGNATION OF OTUS

Sequences from each of the 48 root samples were aligned using MAFFT (Katoh *et al.* 2002) with output order set to ‘aligned’. Singletons were removed from the alignments, and each alignment was then roughly divided into preliminary OTUs using a 97% similarity

criterion. Representatives from these preliminary OTUs were blasted against NCBI GenBank to identify non-AM fungal sequences that were subsequently removed from the data sets. Non-AM fungal sequences were defined either as having their closest match with Basidiomycota, or as having no known match. Identical sequences were then counted and removed to create 48 alignments with no duplicate sequences. These files were combined and aligned with MAFFT, and a Neighbor Net was constructed using SplitsTree4 v. 4.10 (Huson & Bryant 2006). OTUs were identified as clades with greater than 97% bootstrap support. Representative sequences from each clade were blasted against NCBI GenBank to obtain the most similar sequences from other studies. Only sequences with 98–100% coverage resulting in *E*-values close to zero were considered. Accession numbers, origin and identity of these sequences are listed in Table S2.

STATISTICAL ANALYSES

Treatment and plot effects on shoot dry weight, AM colonization and soil characteristics were analysed using a two-factor anova in Minitab (Minitab Inc., State College, PA, USA), with plot as a random factor. Residuals were plotted and transformed when necessary to improve normality and homoscedacity of the data, and mean separations were performed using the least significant difference method with $\alpha = 0.05$.

The fungal community composition was analysed using multivariate analyses in canoco 4.54 (ter Braak & Šmilauer 2002), where the sequence number of each OTU was used as a measure of abundance. All analyses had samples as scaling focus, and all species data were Hellinger-transformed using the program PrCoord 1.0 (Legendre & Gallagher 2001; ter Braak & Šmilauer 2002). Detrended correspondence analysis (DCA) of the transformed OTU abundance data showed axes lengths < 3.0, suggesting a linear treatment of the data (Ramette 2007). To assess the influence of the spatial distribution of the sample locations on AM fungal species composition, plot locations were assigned two-dimensional (x and y) coordinates, which were used to create spatial polynomials for inclusion in multivariate analyses (Borcard, Legendre & Drapeau 1992). Redundancy analysis (RDA) with manual forward selection and 499 permutations was used to evaluate effects of environmental variables (treatment, spatial distribution, soil chemical parameters and vegetation) on the fungal community composition. An indirect gradient analysis (PCA) was used to plot the distribution of samples in ordination space, with important environmental variables (as indicated by forward selection) overlaid as supplementary data. Vegetation cover data for each sample site were also analysed as response variables in canoco. A DCA of vegetation cover data showed gradients lengths below 3.0, so an RDA was performed with forward selection (499 permutations) to test for covariance with explanatory variables. Finally, we used the 'Betadisper' command in the Vegan package (Oksanen *et al.* 2011) for R 2.11.1 (R Development Core Team, 2010) to test for differences in sample dissimilarities in OTU composition among treatments (Anderson, Ellingsen & McArdle 2006).

Results

AM FUNGAL RICHNESS, DIVERSITY AND OTU COMPOSITION

Overall, 213 323 sequences with an average length of 210 bp passed the initial quality assessment (see above). Of those, 88 351 were identical to sequences previously obtained from this grassland or matched with Glomeromycota sequences in GenBank. The remaining sequences were either of non-Glomeromycotan origin (predominately basidiomycetes) or of poor quality (fewer than 100 sequences). There were no significant differences in the number of AM fungal sequences among treatments ($F_{3,30} = 1.62$, $P = 0.21$) or plots ($F_{10,30} = 1.31$, $P = 0.27$). In all, we identified 32 OTUs in this grassland (Figs 1 and 2), and their similarities to previously published

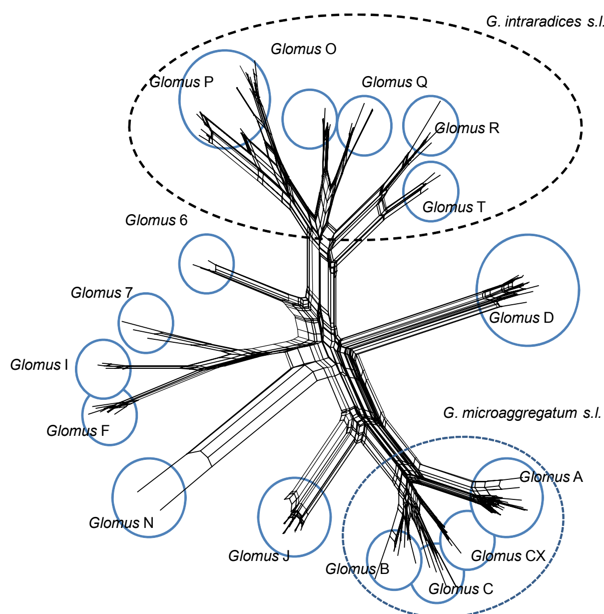

Fig. 1. NeighborNet split network based on least-square distances of *Glomus* group A LSU rDNA sequences. The circles indicate operational taxonomic units (OTUs) defined as lineages with more than 97% bootstrap. *Glomus* O, P, Q, R and T showed high similarity to published *G. intraradices* sequences, and A, B and C to *G. microaggregatum*. None of the other OTUs showed similarity to described species.

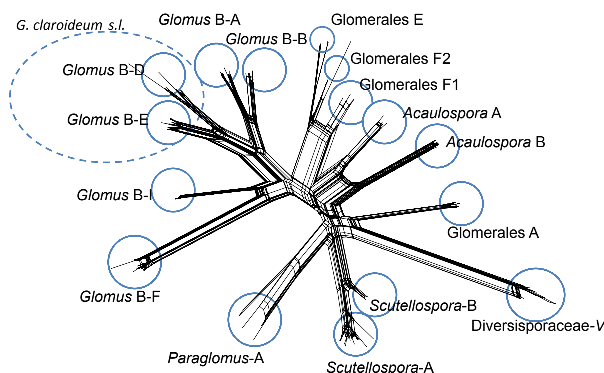

Fig. 2. NeighborNet split network based on least-square distances of *Glomus* group B, *Paraglomus*, Gigasporaceae and Diversisporaceae LSU rDNA sequences. The circles indicate operational taxonomic units (OTUs) defined as lineages with more than 97% bootstrap. *Scutellospora* A and B showed high similarity to *S. calospora* and *S. gilmorei*, respectively, *Acaulospora* A and B to *A. lacunosa* and *A. paulinae* and *Glomus* B-D and *Glomus* B-E showed high similarity to sequences from *G. claroideum*.

sequences are in Table S2, and their observed sequence numbers across treatments in the 11 plots are in Table S3. The great majority of sequences belonged to *Glomus* group A (16 OTUs), and the remaining belonged to *Glomus* group B (6), *Scutellospora* (2), *Acaulospora* (2), *Paraglomus* (1), *Diversispora* (1) and four unknown OTUs within the Glomeromycota that show limited similarity with known AM fungal clades. A rarefaction analysis indicated that our sampling intensity was sufficient to identify the great majority of AM fungal OTUs in the C, D, DB and S samples within this grassland, although even with this deep sequencing, none of the curves had reached saturation (Fig. S1). Because we only had four native *P. lanceolata* plants, sampling effort was not estimated for these samples. The OTU richness per sample did not differ significantly among treatments ($F_{3,40}$ = 0.10; P = 0.96), and each sample was colonized by an average of 11 OTUs. Furthermore, OTU dominance (sequence number of the most abundant OTU to the total sequence number in each sample) did not differ significantly across treatments ($F_{3,40}$ = 1.79; P = 0.16), and an average of 44% of all sequences in a sample belonged to one OTU, but the identity of this OTU differed among samples.

Contrary to our expectations, disturbance did not alter OTU composition, and samples appeared to be almost randomly distributed in ordination space (Fig. 3). The dissimilarity analyses showed that the variance among samples within treatments did not differ significantly from each other, indicating no increased compositional variation because of disturbance treatments. To our great surprise, we found that OTUs that were abundant in control samples also tended to be abundant in disturbed samples where re-colonization from adjacent undisturbed areas was prevented (Table 1), which suggests that contact with the surrounding soil did not alter composition and that the disturbed soil contained sufficient propagules to allow for community recovery. This was supported by significant and positive (P < 0.003, r > 0.53) pairwise correlations of OTU abundances across all treatments (C vs. D, C vs. DB, D vs. DB, S vs. C, S vs. D and S vs. DB).

The coverage of the plants *Dianthus deltoides* and *Carex arenaria* explained a significant (P < 0.01, RDA) proportion (16.4%) of the variation in OTU composition, whereas spatial variables did not correlate significantly with OTU abundance. None of the measured soil variables covaried with the fungal community composition, but a separate constrained ordination (RDA) showed that the plant community composition covaried significantly with NO_3 (P = 0.002) and pH (P = 0.024). Thus, while certain soil parameters can be predictive of the plant community composition, this does not appear to extend to the fungal community. As with fungal community structure, plant community composition did not significantly covary with spatial variables.

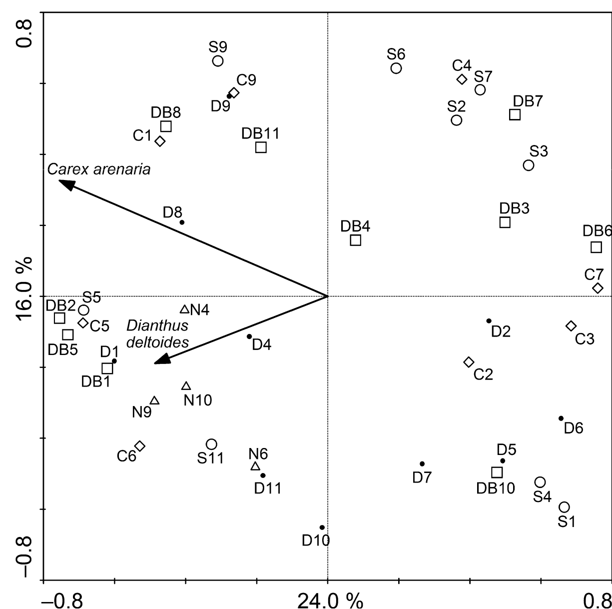

Fig. 3. Principal component analysis plot of operational taxonomic unit (OTU) composition in *Plantago lanceolata* roots exposed to either minimal disturbance during planting (C), or to extensive disturbance where AM fungi surviving the disturbance and from outside the disturbed area could re-colonize (D), or where only AM fungi surviving the disturbance could colonize the roots (DB). OTU composition in these three treatments was compared to that in surrounding (S) plants found inside the eleven experimental plots and to native *P. lanceolata* found in four plots. The arrows indicate the plant species that explained a significant proportion of the OTU composition (using forward selection under a constrained – RDA – analysis). The N samples and 11S were excluded from the RDA because we lacked soil chemical data for those samples. Distance among samples approximates their OTU dissimilarity. PCA axis 1 accounts for 24.0% of the species variance, and PCA axis 2 accounts for 16.0% of the variance.

SHOOT DRY WEIGHT, SOIL CHEMICAL PROPERTIES AND AM COLONIZATION

Disturbance treatments significantly affected shoot dry weight at the second harvest ($F_{2,20}$ = 17.9; P < 0.001) and were highest in DB treatment (1.18^{a} ± 0.11, mean ± SE), followed by the D (0.83^{b} ± 0.10) and C (0.47^{c} ± 0.06). The larger plants in the disturbed treatments could be the result of reduced competition, or an increased availability of nutrients relative to the control. The nutrient concentrations in the disturbed treatments were 40–130% higher compared to the control at the first harvest (data not shown), but these differences had disappeared at the second harvest. Based on the non-significant treatment effects on all measured soil variables at the final harvest, their overall means are given in Table S4. There were plot differences, however, in NO_3 concentrations ($F_{10,18}$ = 9.39; P < 0.001), and close to significant differences in NH_4 (P = 0.08), as well as available P (P = 0.06), suggesting that nutrients differ spatially in this system.

Colonization of coarse mycorrhizal hyphae differed significantly among treatments ($F_{4,32}$ = 8.11; P < 0.001) and was highest in the DB treatment and in roots collected from the surrounding vegetation (Table 2). There were also significant differences in overall colonization among plots ($F_{10,32}$ = 2.61; P = 0.02), suggesting that fungal abundances differ spatially. Arbuscules and vesicles were present, but not specifically recorded. Colonization by fine endophytes also differed significantly among treatments

Table 1. Abundance of operational taxonomic units (OTUs) based on number of sequences on *Plantago lanceolata* seedlings exposed to minimal disturbance (C) or a disturbance where surrounding AM fungi were allowed to re-colonize the soil (D) or not (DB), or on surrounding plants within each plot (S), $n = 11$. Native *P. lanceolata* OTU composition is not shown as those plants were only found in and around four plots

OTU name	C	D	DB	S	Total
Glomus P	3021	2136	1479	5945	12 581
Glomus D	892	1284	3550	4399	10 125
Glomus F	2268	1088	3546	3712	10 614
Glomus B-D	605	2919	3106	112	6742
Paraglomus A	1071	1634	522	1658	4885
Glomerales E	793	636	272	2586	4287
Glomerales A	181	568	1549	2653	4951
Acaulospora B	25	814	1172	2566	4577
Glomus O	1014	585	869	1624	4092
Glomus B-B	1368	62	665	1128	3223
Glomus C	796	1587	523	2	2908
Glomus I	494	235	812	696	2237
Glomus A	470	491	293	573	1827
Glomus B-A	982	20	387	471	1860
Glomus B	350	520	61	697	1628
Glomus Q	404	532	333	337	1606
Scutellospora-A	322	507	377	232	1438
Glomus B-E	793	17	182	129	1121
Glomus T	115	169	78	156	518
Glomus J	12	50	0	22	84
Glomus R	71	114	58	74	317
Glomus B-F	119	26	18	29	192
Glomerales F2	13	168	0	0	181
Glomerales F1	0	100	12	58	170
Acaulospora A	0	109	1	0	110
Glomus CX	21	64	0	0	85
Diversisporales V	0	0	0	61	61
Glomus 7	0	0	10	39	49
Glomus B-I	12	0	4	6	22
Glomus 6	0	0	0	8	8
Glomus N	0	4	0	2	6
Scutellospora-B	0	0	6	0	6
Total	16 212	16 439	19 885	29 975	82 511

Table 2. Means (SE) of arbuscular mycorrhizal (AM) colonization by coarse AM fungi as well as blue-staining, aseptate fine endophytes (FE) on *Plantago lanceolata* seedlings exposed to minimal disturbance (C) or a disturbance where surrounding AM fungi were allowed to re-colonize the soil (D) or not (DB). Native *P. lanceolata* plants (N), where available, were also harvested from within each plot, as well as other mycorrhizal plants growing in each plot (S), $n = 11$ except for $n(N) = 4$. Different superscripts indicate a significant ($p \leq 0.05$) difference within column means

Treatment	AM (coarse) (%)	FE (%)
C	33.1 (4.88)^{b}	4.71 (0.75)^{c}
D	34.1 (5.36)^{b}	17.6 (3.19)^{ab}
DB	54.2 (5.29)^{a}	22.1 (4.66)^{a}
N	39.8 (9.09)^{ab}	14.6 (8.44)^{abc}
S	58.9 (4.07)^{a}	10.0 (3.20)^{bc}

($F_{4,26} = 6.04$; $P = 0.001$) and tended to be higher in the disturbed treatments (Table 2), but showed no significant differences among plots.

Discussion

DISTURBANCE DID NOT INFLUENCE OTU COMPOSITION

This study was driven by the general assumption that there is a trade-off between competitive ability and disturbance tolerance and that disturbance increases the abundance of OTUs that are rare under undisturbed conditions. Disturbance has previously caused successional shifts in AM fungal communities in agricultural systems (Jansa *et al.* 2002, 2003; Rosendahl & Matzen 2008) as well as in natural plant communities (Husband *et al.* 2002; Sykorova *et al.* 2007; Fitzsimons, Miller & Jastrow 2008), but had no significant effect here. The fact that the fungal community in the DB treatment did not differ from the one observed in the undisturbed, surrounding vegetation suggests that abundant OTUs in this grassland possess a similar – and high – degree of disturbance tolerance. This was unexpected given the apparent lack of any severe disturbance in this grassland. It is possible that less-obvious forces, such as frost heave, wind and trampling from humans and animals are sufficient to select for disturbance tolerance, although these disturbances – if present – are considerably less intense than what was employed in this study. Furthermore, a tolerance to other stresses, such as recurrent droughts (Hawkes *et al.* 2011), changing environmental conditions, and a temporal and spatial flux in host carbon may select for resilient traits, which makes it less likely to observe treatment effects. Regardless, we find the lack of treatment effects remarkable given that only 4.5 months had passed after the severe disturbance was applied. This high disturbance tolerance could arise from the ability to colonize seedlings from pieces of external mycelia and colonized roots rather than spores, because the AM colonization in the DB treatment was high (Table 2) even though spore numbers are consistently low (Y. Lekberg & S. Rosendahl, pers. observ.). Because fungal communities did not differ between the two disturbance treatments, there does not appear to be a great difference among AM fungi in their ability to resist and recover from disturbance. Based on the overall lack of treatment effects, we reject our first hypothesis, which predicted an inverse relationship between OTU abundances and disturbance in this grassland. Further experiments are required to verify the commonality of these findings in other systems. Our second hypothesis regarding phylogenetic trait conservatism is difficult to address because of the dominance of *Glomus* group A fungi, but it is noteworthy that the one *Scutellospora* with reasonable abundance was not reduced in the DB treatment (Table 1), which would be expected based on previous studies (Klironomos & Hart 2002). This suggests that caution may be warranted before extrapolating traits observed in cultured fungi to their uncultured relatives.

We cannot exclude the possibility that the lack of treatment effects was an artefact of our experimental protocol, but for reasons outlined below, we find this to be unlikely. For example, if hyphae were able to colonize DB plants from the bottom of the barrier (something we find improbable for reasons stated earlier), differences between the D and DB treatments would

be reduced. Even if that were the case, it would not influence our overall finding that this AM fungal community is highly resilient. One can also argue that the immediate planting of *P. lanceolata* into the disturbed soil may have facilitated a rapid recovery by AM fungi by providing an immediate host plant. However, significant differences *were* observed among AM fungi in their ability to colonize plants from spores, hyphal and root fragments using a similar approach in a previous study (Klironomos & Hart 2002). Furthermore, opportunistic, early colonizing fungi may have been missed because we chose not to analyse samples from the first harvest. However, roots in *all* treatments were poorly colonized (< 10%), which suggests that even if this group of fungi was present, they constituted a relatively small proportion of the community that was rapidly replaced. Including a third sampling of older roots is also unlikely to have affected the results, because AM colonization and OTU composition did not differ among our treatments and undisturbed surrounding vegetation 4.5 months after a severe disturbance event, which indicates that the fungal community had reached some sort of equilibrium.

Similar to other community studies, we found some OTUs to be common and others to be rare, but disturbance did not influence this relationship because abundance numbers were positively correlated across treatments. That is, OTUs that were common in the control samples were also common after severe disturbances. What factors may cause this cannot be deduced from this study, but clearly some abiotic and/or biotic conditions favour certain OTUs. It is interesting to note that many of the OTUs found here are also abundant in a similar coastal grassland in Sweden (Schnoor *et al.* 2011), indicating that significant landscape level or vegetation type structuring of AM fungal communities occurs. It is also noteworthy that large-scale perturbation *did* influence the AM fungal community composition in the Swedish grassland (Schnoor *et al.* 2011), suggesting that treatment effects may be scale-dependent, especially if there are concomitant changes in the plant community composition.

The relative importance of host plant identity for OTU composition has been debated, and correlations between plant and fungal communities have been found in some studies (Husband *et al.* 2002), but not others (Santos, Finlay & Tehler 2006). OTU abundances correlated significantly with the coverage of *Carex arenaria* and *Dianthus deltoides* in our study, which is intriguing because these two plant species belong to families (Cyperaceae and Caryophyllaceae) that are either considered to be non-mycorrhizal or poor hosts (which we define here as being poorly colonized and therefore allocating relatively little C to their fungal symbiont) to AM fungi (e.g. Koide & Schreiner 1992; Miller *et al.* 1999). AM hyphae and vesicles have been observed in both plants at our field site, but arbuscules are rare or absent (Y. Lekberg, *pers. observ.*). This is in agreement with previous studies of plants in non-mycorrhizal families (Hirrel, Mehravaran & Gerdeman 1978) and may indicate that this symbiosis is non-functional. AM fungi may use poor host plants to store C acquired from better hosts (Lekberg, Hammer & Olsson 2010), but this strategy may not extend to all AM fungal taxa and could be one possible explanation for the correlation between certain OTUs and *C. arenaria* and *D. deltoides* observed here. We do not believe that our results indicate host preference, because there was no difference in OTU composition between the *P. lanceolata* (planted and native) and surrounding plants. This agrees with previous findings from this grassland (Stukenbrock & Rosendahl 2005). Instead, one may speculate that host quality, rather than host identity, could be an important factor for structuring fungal communities.

While the plant community explained a significant portion of the variation in the OTU composition, the great majority remains unexplained, and the OTU distribution among individual samples was largely unpredictable. For example, while *Glomus* P was absent in the surrounding plants but present – and even highly abundant – in the C, D and DB treatments in plots 1 and 4, the opposite was found in plots 2 and 3 (Table S3). To obtain patterns like these, OTUs must not only harbour a similar disturbance tolerance, but also differ greatly on small spatial scales, because treatments within plots were within 1 m of each other. Small-scale patterns in AM fungal communities have been documented previously within natural plant communities (Wolfe *et al.* 2007; Mummey & Rillig 2008; Van der Voorde *et al.* 2010), and a recent survey in this grassland showed that plants less than 1 m apart were sometimes colonized by different AM fungal taxa (I. Galløe & Y. Lekberg, unpubl. data). Clearly, if spatial patterns such as these are common, they need to be considered during the planning, analysis and interpretation of field experiments involving AM fungi.

The observed difference in OTU composition among samples could have been amplified through priority effects, in which the establishment of one OTU influences subsequent colonization by others. Pre-inoculation with certain taxa has been shown to influence subsequent communities for both AM and ectomycorrhizal fungi (Hepper *et al.* 1988; Kennedy *et al.* 2009; Mummey, Antunes & Rillig 2009). Dumbrell *et al.* (2010) proposed that priority effects and subsequent positive feedback could help explain the overdominance observed in AM fungal communities, in which the most abundant taxon constitutes about 40% of the total abundance. Because the dominant OTU differed among sites, Dumbrell *et al.* (2010) also argued that stochastic processes may play an important – yet underappreciated – role in shaping AM fungal communities. Our results are in agreement with those presented by Dumbrell *et al.* (2010), because the AM fungal community assembly in the *P. lanceolata* roots was largely stochastic, and the OTU dominance was almost identical to the one calculated by Dumbrell *et al.* (2010). This suggests that the mechanisms proposed by Dumbrell *et al.* (2010) to explain differences among communities also operate on smaller scales. Furthermore, because overdominance and stochasticity were present in the undisturbed surrounding plants in our study, these patterns appear to persist over time. The AM colonization in our experiment ranged between 35% and 60% and may not be considered sufficiently high for competition among OTUs to occur, but AM fungi may not only compete for root space but also for carbohydrates (Pearson, Abbott & Jasper 1994). The

occurrence and severity of competition is not only of interest for AM fungal community ecology, but could also have functional consequences as more competitive AM fungi may be poorer mutualists to plants (Bennett & Bever 2009).

THE FUNGAL COMMUNITY AS REVEALED BY 454-SEQUENCING

Thorough molecular characterization of fungal communities has been limited because of time and cost constraint related to cloning and Sanger sequencing. 454 and other next-generation sequencing techniques hold great promise for thorough community analyses as they eliminate the need for cloning and appear to generate sequences in a quantitative manner (Thomas *et al.* 2006) as long as care is taken to avoid biological and technical artefacts (Tedersoo *et al.* 2010). For example, Öpik *et al.* (2009) compared results from cloning and Sanger sequencing with those from 454-sequencing and found 33% more OTUs with the 454-sequencing using the same PCR protocol and primers. Because we designed new primers for this study, we are unable to compare our richness estimates with those found previously (Rosendahl & Stukenbrock 2004; Stukenbrock & Rosendahl 2005) also targeting the LSU. Nonetheless, abundant OTUs in earlier studies, such as *Glomus* D and F, were also abundant in this study. The exception was *Glomus mosseae*, which has been documented previously (Rosendahl & Stukenbrock 2004; Stukenbrock & Rosendahl 2005) but was conspicuously absent here. It is also interesting to note that even though comparisons to other studies are difficult because of differences in OTU delineation and target gene sequences, the 32 OTUs found here are within the range found in other studies (Vandenkoornhuyse *et al.* 2002; Öpik *et al.* 2009). It is possible that our reported richness underestimates the actual AM fungal richness, because we excluded some OTUs with no match to know fungal groups. Paraglomeraceae is the most basal group in the Glomeromycota, and there could potentially be more basal groups of non-sporulating – yet unidentified – AM fungi. While these excluded OTUs were rare at our study site, they could be abundant in other vegetation systems. Overall, this surprising fungal diversity may be important, because the long-held assumption that AM fungi are functionally equivalent has been refuted by research showing that fungal species richness influences plant diversity and productivity (van der Heijden *et al.* 1998) as well as ecosystem properties such as soil aggregation and carbon and nutrient cycling (as discussed in Rillig 2004). Because a majority of functional studies have focused on culturable taxa and been conducted under controlled greenhouse conditions, the function of the majority of AM fungi remains unknown, and field studies assessing mycorrhizal function are sorely needed. For example, two of our three most abundant OTUs have no close match to known taxa in GenBank and may constitute locally adapted taxa with unknown traits. This is also true for many of the fine endophytes whose identity and function are uncertain, and it is unclear to what extent our primers were able to amplify this group of fungi.

As the number of next-generation sequencing studies increases, it becomes imperative that some standards are put in place to allow for comparisons among studies. An initial proposal is put forward by Nilsson *et al.* (2011), in which guidelines for how data and results should be reported and made available to the scientific community are suggested. Other areas that complicate comparisons include OTU delineation and abundance data, but these potential sources of bias are not necessarily unique to next-generation sequencing techniques. For example, OTUs are often defined based on a 97% sequence similarity in AM fungal community studies (Buée *et al.* 2009). Such criteria are operational, but may suffer from the problem that the genetic diversity within species may vary considerably, even between related species. The neighbour net analysis (Figs 1 and 2) shows that some OTUs (e.g. *Glomus* F) belong to well-resolved lineages, whereas others (e.g. *Glomus* P) show considerable genetic variation and belong to less-resolved lineages. This phenomenon is not unique for AM fungi and is indicative of differences in the speciation process among lineages (Taylor *et al.* 2000). With the increasing number of sequences available with 454-sequencing, these differences can be more categorically assessed.

Conclusions

This study questions the long-held assumption that disturbance is a strong structuring force in communities and that communities shift in a predictable manner after a disturbance event. Instead, the reassembly of this fungal community indicates a high community resilience and substantial local stochasticity, which could be due to priority effects among abundant and disturbance-tolerant AM fungi.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Vegetation survey of the 11 plots.

Table S2. Operational taxonomic unit (OTU) names and their matches with Genbank sequences.

Table S3. Sequence numbers of OTUs in the 11 plots.

Table S4. Overall mean (min–max) of measured soil variables.

Figure S1. Sampling effort curves of C, D, DB and S samples.

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