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Seasonal influences on fungal community structure in unimproved and improved upland grassland soils

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Abstract

Seasonal and management influences on the fungal community structure of two upland grassland soils were investigated. An upland site containing both unimproved, floristically-diverse (U4a) and mesotrophic, improved (MG7b) grassland types was selected, and samples from both grassland types were taken at five times in one year. Soil fungal community structure was assessed using fungal automated ribosomal intergenic spacer analysis (ARISA), a DNA-profiling approach. Grassland management regime was found to strongly affect fungal community structure, with fungal ARISA profiles from unimproved and improved grassland soils differing significantly. The number of fungal ribotypes found was higher in unimproved than improved grassland soils, providing evidence that improvement may reduce the suitability of upland soil as a habitat for specific groups of fungi. Seasonal influences on fungal community structure were also noted, with samples taken in autumn (October) more correlated with change in ribotype profiles than samples from other seasons. However, seasonal variation did not obscure the measurement of differences in fungal community structure that were due to agricultural improvement, with canonical correspondence analysis (CCA) indicating grassland type had a stronger influence on fungal profiles than season.

Keywords: Upland grasslands; Fungal automated ribosomal intergenic spacer analysis; Seasonality; Improvement; Canonical correspondence analysis

Soil microbial communities play an important role in grassland ecosystems, regulating biogeochemical cycling and decomposition processes. Fungi in particular are key players in nutrient cycling processes such as proteolysis, phosphorus mobilization, and decomposition (Read and Perez-Moreno 2003; Wainwright 1988). In recent decades, many semi-natural, species-rich upland grasslands have been converted to species-poor but high-yielding mesotrophic plant communities (Rodwell 1992). This change in floristic diversity, known as improvement, is maintained through the application of lime and fertilizer, raising ecological and environmental concerns about changes in soil chemistry and loss of plant species (Stoate et al. 2001). The process of improvement has also been shown to result in changes to soil fungal and bacterial communities, with several studies finding unimproved grasslands often have higher fungal biomass and lower bacterial numbers than improved fields (Bardgett et al. 1999; Brodie et al. 2002, 2003; Grayston et al. 2001, 2004). In this study, soils from unimproved and improved grasslands were compared using ARISA, a recent advance in community profiling capable of providing a fuller characterization of fungal community structure than previously possible (Anderson and Cairney 2004).

The influence of temporal or seasonal changes upon grassland soil fungal communities has not been explored extensively, with most studies focusing on overall microbial biomass or bacterial communities. Measurements of microbial biomass-C and –N, as well as microbial phospholipid and nucleic acid profiles, have been observed to vary seasonally (Bardgett et al. 1999; Griffiths et al. 2003), but a consistent source of variation could not be found. A recent study of unimproved and improved grasslands concluded that seasonal variation in bacterial community profiles, although evident, did not obscure the effects of grassland management (Kennedy et al. 2005b). Although these studies indicate seasonal variation may be an important

factor influencing soil microbial populations, the effect of seasonality on fungal community structure remains largely unknown.

To assess both improvement and seasonal effects on fungal community structure, soil was collected from unimproved (corresponding to UK National Vegetational Classification U4a) and improved (MG7b) grassland sites at Long Hill, County Wicklow, Ireland (OS Coordinates O218 124) in February, May, July, October, and December 2001. Soil physico-chemical properties differed between the two grassland types, with MG7b soil having greater values for pH, phosphorus, and potassium, while U4a soil was higher in total % nitrogen (see Kennedy et al. (2005b) for full description of site vegetation, management, and soil chemistry). Soil samples were taken using a soil corer to remove three replicate cores (4 cm diameter, 10 cm depth) from five sampling points in each grassland type (U4a and MG7b). A reference sampling point was determined by measuring from a landmark to the centre of each grassland type, then measuring 2 m in each cardinal direction to designate five sampling points, which were used at each season. Soil temperatures were lowest in February and December, and highest in July (see Kennedy et al. (2005b) for full description).

Total soil DNA was extracted as described previously (Brodie et al. 2002), using a modified CTAB buffer protocol followed by purification by phenol:chloroform:isoamylalcohol, lysozyme, and a High Pure PCR Product Clean Up Kit (Roche, Penzberg, Germany). The fungal intergenic spacer region containing the two internal transcribed spacers (ITS) and the 5.8S rRNA gene (ITS1-5.8S-ITS2) was amplified using primer set ITS1-F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns 1993) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). The forward primer ITS1-F was labeled with Beckman Coulter fluorescent dye D4 (Invitrogen, Paisley, Scotland). PCR reactions were

performed in 50 µl volumes containing 25 µl master mix (containing 50 units ml^{-1} Taq DNA polymerase in a proprietary reaction buffer (pH 8.5), 400 µM each dNTP, and 3 mM $MgCl_2$) (Promega, Southampton, UK), 15 pmol each primer, 25 µg BSA, and ~10 ng extracted total soil DNA. Thermocycling conditions were: a hot start at 95ºC for 4 min (1 cycle); 95ºC for 1 min, 53ºC for 30 s, 72ºC for 1 min (35 cycles); 72ºC for 7 min (1 cycle). PCR products were purified using a High Pure PCR product purification kit (Roche, Penzberg, Germany) and eluted in 50 µl of sterile water heated to 50ºC. The purified PCR products were visually quantified by comparison with a size standard on a 1.2% agarose gel before mixing aliquots (0.5 – 1 µl, equivalent to ~2 ng DNA) with 38.4 µl of deionized formamide, 0.2 µl of Beckman Coulter size standard 600 (dye D1), and 0.4 µl of custom-made marker (containing fragments ranging from 600-1000 bp in intervals of 20 bp, and 1000-1200 in intervals of 50 bp, all labeled with Beckman Coulter Dye D1) (BioVentures, Murfreesboro, TN, USA). Intergenic spacer lengths were determined by electrophoresis using a Beckman Coulter (CEQ8000) automated sequencer as described previously (Kennedy et al. 2005a). Analysis of ribotype profiles was performed using the Beckman Coulter fragment analysis package. Ribotypes with peak heights less than 1% of the total peak height for all ribotypes in a sample were regarded as background noise and excluded from analysis. Ribotypes that differed by less than 0.5 bp in different profiles were considered identical. The peak heights of individual ribotypes were relativized as a percentage of their abundance within a sample; this relativisation corrects for DNA quantity differences between replicate profiles while giving similar results to strictly quantitative data treatments (Yannarell and Triplett 2005).

Data for fungal ribotype number and relative abundances of individual fungal ribotypes were analyzed by one-way factorial analysis of variance (ANOVA) using Genstat v 6. The

significance level was set at $p < 0.05$. A randomization test was performed on fungal ARISA profiles using Genstat v 6 to test the null hypothesis that there were no significant differences between samples from different grassland types or different seasons, using a critical level of $p < 0.05$ (Kennedy et al. 2005a). This test compared variance between replicates to variance between treatment samples to determine if differences between treatments were significant. Fungal ARISA profiles were explored using canonical correspondence analysis (CCA) (Canoco for Windows, v 4.02) after initial analysis by detrended correspondence analysis (DCA) revealed that the data exhibited a unimodal, rather than linear, response to the environmental variables (grassland type and season). The resulting ordination biplot approximated the weighted average of each species (in this case, relative abundance of ribotypes) with respect to each of the environmental variables, which were represented as arrows. A Monte Carlo permutation test based on 199 random permutations was used to test the null hypothesis that fungal profiles were unrelated to environmental variables. As with all PCR-based fingerprinting approaches, caution must be used when interpreting ARISA profiles due to the potential for PCR bias (Dickie et al. 2002, von Wintzingerode et al. 1997). However, recent studies on bacterial community profiling found TRFLP, a similar method to ARISA, gave a quantitative view of the community not affected by PCR bias, particularly when non-degenerate primers (such as the ones in our study) were used (Lueders and Friedrich 2003). In addition, it must be noted that it may be possible for one fungal species to produce heterogeneous ITS regions; however, no evidence of such heterogeneity was detected in a similar survey of environmental fungi (Buchan et al. 2002).

Fungal ribotype number was significantly affected only by grassland type, with U4a soil having significantly more fungal ribotypes than MG7b soil, while season and season-grassland type interaction had no significant effect on fungal ribotype number (Table 1). Fungal ARISA

profiles were analyzed using a randomization test to determine if changes in season and grassland type caused statistically significant alterations to fungal community structure (data not shown). Fungal community profiles from U4a and MG7b soil were significantly different at every season except October, indicating a large impact of grassland type on fungal community structure. In contrast, season appeared to have a smaller effect, with profiles of U4a soil taken in July different from those taken in May and October, and no significant differences found in fungal profiles from MG7b soil taken during different seasons.

The top 20 most abundant fungal ribotypes, accounting for 48% of overall abundance, were analyzed by CCA (Figure 1). Axis 1 (eigenvalue 0.842) and Axis 2 (eigenvalue 0.542) were found to explain 15% of the total variation within the fungal ribotype profiles, and 66% of the total variation that could be related to season and grassland type. Monte-Carlo significance tests revealed that Axis 1 alone, and all axes together, explained a significant amount of the variation within fungal community profiles. Species-environment correlations for both axes were high (0.969 and 0.824, respectively), indicating that there was good correlation between fungal ribotype profiles and environmental variables (season and grassland type).

The plot presented in Figure 1 may be interpreted qualitatively by noting the length of the arrows representing environmental variables, with longer arrows indicating higher correlation with variation in fungal community profiles (Jongman et al. 1995). Variables for U4a, MG7b, and October appeared dominant on the CCA plot. Values for intra-set correlations confirmed this, and indicated that Axis 1 was most highly correlated with U4a and MG7b (grassland type), while Axis 2 was most closely correlated with a particular season, October. The large impact of grassland type on fungal community structure was evident from both the long length of the arrows for U4a and MG7, and by noting that most fungal ribotypes (represented by dots)

appeared on either end of the axis formed by their arrows. Correlations between grassland type and fungal ribotypes were confirmed by ANOVA of individual ribotypes (Table 2), which found grassland type significantly affected the abundances of the top four most abundant fungal ribotypes.

Overall, fungal community profiling results indicated that grassland type had a more consistent effect upon fungal communities than did seasonal influences. This finding that the soil fungal community was strongly affected by grassland type correlated well with other studies of upland grassland management. Several studies have found higher fungal:bacterial phospholipid fatty acid ratios in unimproved than improved areas (Bardgett et al. 1999; Grayston et al. 2001, 2004), corresponding to the higher numbers of fungal ribotypes, and lower numbers of bacterial ribotypes (Kennedy et al. 2005b) found in unimproved versus improved grasslands at our field site. The effect of grassland management on soil fungi at our site was also investigated by Brodie et al. (2003), who discovered improvement resulted in a decrease in fungal biomass, and a shift in the evenness of fungal community DNA profiles; however, as the results were from a single sampling, it was unclear if this alteration in community composition was influenced by season.

The factors driving shifts in fungal community structure due to improvement have not been precisely identified. Unimproved and improved grasslands differ in plant species composition and soil chemistry, both of which may be controllers of microbial community structure. Microcosm experiments have indicated that addition of nitrogen can strongly impact fungal populations in grassland soils (Kennedy et al. 2005a). Changes in diversity and composition of plant species and soil chemistry due to improvement may also cause differences in substrate availability between grasslands; it has been noted that the higher organic matter content of unimproved soils may provide substrates more suitable for fungi than bacteria (Bardgett et al.

1999; Grayston et al. 2001, 2004), possibly accounting for the higher fungal ribotype number found in unimproved soils in our study.

Although season was much less correlated with changes in fungal community structure than was grassland type, fungal profiles from samples collected in autumn (October) appeared to exhibit some differences from those collected in other seasons. There is evidence that the colder temperatures in autumn can cause a reduction in microbial biomass (Sarathchandra et al. 1988), with a previous study finding that both overall microbial biomass, and fungal biomass, were lowest in grasslands in autumn (Bardgett et al. 1999). These temporal fluctuations may be due to microbial populations responding to seasonal variations in soil temperature, moisture, and substrate availability, as plant productivity and litter deposition change throughout the year (Grayston et al. 2001). Autumn marks the end of the plant growing season, and a resultant decrease in soil concentrations of plant root exudates, which are easily degradable by bacteria. Recalcitrant polymers, which fungi are better positioned to utilize (Newman 1985), would remain in the soil and could be degraded by fungi during the autumn months. Although our samples were taken over one year only, leaving open the possibility of annual variation, comparison of climatic data from this year with averages from the past 30 years indicated that it was representative of typical seasonal variations in this climate (Kennedy et al. 2005b).

In conclusion, our study has shown that although seasonal variation in fungal community structure existed, it did not obscure the measurement of those differences in fungal community structure that were caused by improvement. In conjunction with data regarding bacterial community structure in these grassland sites (Kennedy et al. 2005b), this indicates that the effect of improvement on grassland soil microbial communities is detectable in samples from most seasons. Therefore, even studies relying upon a single time point sampling should be capable of

reaching valid conclusions about how changes in grassland management regime impact upon microbial populations in these ecosystems. On a larger scale, our finding that management regime exerts greater influence than season on soil microbial communities may indicate that agricultural practices have an important role to play in climate change scenarios for temperate regions. It would be of interest to focus future studies on identifying the drivers of shifts in microbial community structure due to improvement, and to elucidate the impact of these shifts on ecosystem function.

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Table 1. Fungal ribotype number, as affected by season and grassland type. Means and standard error of differences (SED) are shown (n=5 for means of grassland type at each season, n=25 for average of grassland types over all seasons). ANOVA p-values shown as *, p <0.05; NS, not significant. U4a, unimproved; MG7b, improved.

	Fungal Ribotype Number (mean ribotypes per replicate ± SED)	
	U4a	MG7b
SEASON		
February	56 ± 13.3	25 ± 8.8
May	24 ± 8.2	10 ± 2.8
July	61 ± 22.4	6 ± 1.4
October	22 ± 6.2	16 ± 4.8
December	14 ± 5.3	15 ± 4.4
Average	35 ± 9.2	14 ± 2.4
p-values		
Season	NS	
Grassland Type	*	
Season*Grassland Type	NS	

Table 2. Abundance rankings and ANOVA results for the top 20 most abundant fungal ARISA (automated ribosomal intergenic spacer analysis) ribotypes, as ranked by average abundance over all samples.

	Abundance			***p*-values**	
FARISA Ribotype (bp)	Abundance Rank	% Abundance	Cumulative % Abundance	Season	Grassland Type
578	1	10.5	10.5	0.461	**<0.001**(MG7b, improved)
579	2	7.2	17.8	0.505	**<0.001**(MG7b, improved)
669	3	3.6	21.4	0.551	**0.01**(U4a, unimproved)
668	4	2.4	23.8	0.452	**0.027**(U4a, unimproved)
664	5	2.4	26.2	0.486	0.228
671	6	2.3	28.5	0.777	0.654
524	7	2.3	30.7	0.324	0.136
577	8	1.9	32.6	0.527	0.191
439	9	1.7	34.3	0.419	0.323
670	10	1.7	36.0	0.879	0.232
665	11	1.7	37.7	0.602	0.134
525	12	1.6	39.3	0.352	0.074
655	13	1.6	40.9	0.379	0.305
1124	14	1.3	42.2	0.419	0.323
760	15	1.2	43.4	0.419	0.323
697	16	0.9	44.3	0.064*	0.153*
412	17	0.9	45.2	0.420	0.326
611	18	0.9	46.1	0.461	0.201
617	19	0.9	46.9	0.442	0.240
685	20	0.9	47.8	0.199	0.077

Bold indicates a significant effect (p <0.05)
Season/grassland type with highest abundance of ribotype is indicated next to significant effect
*Significant interaction with each other

Figure captions

Figure 1. Effect of grassland type and season on fungal community structure, as measured by fungal ARISA (automated ribosomal intergenic spacer analysis) ribotype profiles. Canonical correspondence analysis (CCA) ordination diagram of fungal ARISA data, with grassland types (U4a, unimproved; MG7b, improved) (dashed lines) and seasons (unbroken lines) represented as large arrows and ribotypes represented as black dots. Ribotypes are labeled according to size (base pairs).