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Spatial and temporal variability of bacterial 16S rDNA-based T-RFLP patterns derived from soil of two Wyoming grassland ecosystems

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Abstract

Spatial and temporal variability of soil bacterial 16S rDNA terminal restriction fragment (TRF) size variation was evaluated in a homogeneous grassland (HG) dominated by the turf-forming grass *Bouteloua gracilis* and in a shrubland (SL) dominated by *Artemisia tridentata* (Wyoming big sagebrush). Temporal variability was also evaluated on the HG site over a growing season. No trends toward dissimilarity were detected with temporal (180 days) or spatial (up to 100 m) distance in the HG system. Terminal-restriction fragment length polymorphism (T-RFLP) profiles of the SL site exhibited pronounced small-scale spatial variability (< 70 cm), although spatial analysis indicated weak spatial autocorrelation to distances greater than 36 cm. While shrub-induced nutrient localization was shown to significantly influence T-RFLP profiles, very little of the variability could be accounted for on the basis of spatial characteristics, suggesting that soil bacterial 16S rDNA composition of this site is predominantly controlled at scales other than those measured. Average dissimilarity values differed greatly between the two sites (0.27 and 0.59 for HG and SL sites, respectively). These results suggest that plant community structure strongly influences bacterial community composition in these semiarid ecosystems, highlighting the importance of considering spatial variability when designing field studies related to bacterial diversity in ecosystems having patchy or heterogeneous plant cover.
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1. Introduction

Although a number of studies have examined spatial and temporal variation of soil properties, processes, and the biomass of microbial communities (e.g. [1–5]), knowledge of how bacteria are distributed in the environment, and therefore what scales are relevant for understanding relationships between bacterial communities and processes, is extremely limited [6,7]. While the combined activities of individual microorganisms mediate critical ecological processes at the microhabitat, field, landscape, regional, and global levels; microbial abundance and diversity is influenced to different degrees by processes operating at a multitude of scales [8]. Determining causes of biological diversity in a given ecosystem or area requires linking the scales of diversity patterns with the scale at which the processes hypothesized to influence biodiversity operate [9,10].

While plant community structure is known to influence microbial community composition, as are soil physical and chemical properties [11,12], these relationships are poorly defined, and knowledge about the scales at which soil microbial interactions and associations become important in any ecosystem is incomplete.

Grasslands of southeastern Wyoming are represented by a number of grass-dominated ecosystems including short- and mixed-grass prairie systems, in addition to shrub-steppe communities characterized by a high diversity of grass, forb and shrub species separated by areas of bare soil. In arid and semiarid ecosystems, shrub cover is known to influence the spatial stratification of soil physical and chemical properties, as well as microbial processes, resulting in nutrient localization that has been termed ‘is-

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lands of fertility' or 'resource islands' [13]. In contrast, homogeneous grass cover would be expected to have a relatively uniform influence on soil properties across a landscape. Plant influences on microbial community composition and distributions may therefore vary greatly over the landscape in this region.

In the present study, temporal variability of bacterial 16S rDNA-based terminal restriction fragment length polymorphism (T-RFLP) profiles derived from soil of an ecosystem having homogeneous grass cover was evaluated to provide insights into seasonal shifts. Spatial analysis of T-RFLP profiles derived from ecosystems having homogeneous and heterogeneous plant cover was also determined to elucidate plant community influences. Knowledge of spatial and temporal bacterial community compositional variability, and its relationship to plant community structure, has important implications for development of sample strategies to investigate a given process, or for determining predominant species compositions, thus providing insights into the number of samples, and the spatial and temporal scale of their collection, required in order to representatively sample an ecosystem.

2. Materials and methods

2.1. Study site and soil collection

Two sites having vegetation cover commonly found in Wyoming short-grass steppe ecosystems were selected to represent areas having homo- and heterogeneous plant cover. Both sites have a semiarid climate and soil moisture availability is low for much of the year. Nearly half the annual precipitation onto these sites occurs in five summer months as sporadic, often intense, thunderstorms, resulting in rapid soil-wetting events.

The first of these sites, hereafter referred to as the homogeneous grass (HG) site, is located on a gentle slope and dominated ($>90\%$ basal cover) by the turf-forming grass *Bouteloua gracilis* (Blue Grama). Elevation at this site is approximately 2215 m and it annually averages 22.5 cm precipitation. The Laramie Regional Airport meteorological station is located within 300 m of the site, allowing for access to temperature and precipitation data (nndc.noaa.gov). Soils of this site are fine loamy over sandy, Ustic Calciargids of the Stunner series.

In order to determine temporal variability of soil bacterial terminal restriction fragment (TRF) signatures, variance due to spatial influences must be estimated. Therefore, a 1 m^2 sample plot was selected for analysis of small-scale variability, and eight samples initially collected at distances from 5 cm to 1 m. Soil samples were collected to a depth of 7.5 cm using a 2 cm diameter soil core extractor and frozen at -20°C within 30 min of collection.

After analysis of small-scale spatial variability, soil samples for temporal analysis were collected biweekly from this plot throughout the 1999 growing season (March to August). On each sample date four samples were collected from random locations within the plot and pooled prior to analysis. On the June 25 sample date, soil samples for large-scale spatial variability analysis were collected beneath *B. gracilis* turf along a 100 m transect. Starting at the small-scale and temporal analysis sample plot, soil samples were collected from *locations* 0, 10, 25, 50, 75, and 100 m away.

The second site, referred to hereafter as the shrub land (SL) site, is located in the Shirley basin of Wyoming (N,W). Sampling design and a detailed site description are described by Mummey et al. [14]. Briefly, the site is dominated by *Artemisia tridentata* Nutt. ssp. *wyomingensis* (Wyoming big sagebrush) with an understory of small grasses and forbs. In contrast to the HG site, this site averages 6.2 plant species per 0.18 m^2 and exhibits only 28% basal cover. The site has a mean elevation of 2150 m and, on average, receives 28 cm precipitation annually. Soils of the site are classified as fine loamy mixed Ustic Haplargids. Twenty-nine soil samples were collected beneath the pebbly surface layers (5–10 cm depth) from each of two grids centered on individual *A. tridentata* shrubs (Fig. 1B,D). Sample grids were designed to facilitate the use of geostatistical methods by maximizing the number of samples within each potential lag distance (see below).

2.2. DNA extraction and amplification

DNA was extracted from 0.4 g of each soil sample using the UltraClean soil DNA kit (MoBio, Solano Beach, CA, USA) according to the manufacturer's instructions. The concentration of DNA from each sample was estimated using ethidium bromide-stained agarose gels with a puC 18 molecular mass marker (Sigma, St. Louis, MO, USA) as a calibration standard.

Whole community genomic DNA was amplified using flourescently labeled polymerase chain reaction (PCR) primer 27f (5′-Fam (6-carboxyfluorescein)-AGAGTTTGATCCTGGCTCAG) and the unlabeled reverse primer, 927r (5′-CCGTCAATTCCTTTRAGTTT) [15]. The 50 μl reaction mixtures contained 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 2 U *Taq* polymerase (Promega, Madison, WI, USA), and approximately 50 pg template DNA. Thermocycling was carried out on an icycler (Biorad, Benecia, CA, USA) with an initial denaturation step of 94°C for 3 min; 32 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 2 min; cycling was completed by a final extension period of 72°C for 3 min.

2.3. T-RFLP analysis

PCR products were purified using the Ultraclean PCR cleanup kit (MoBio) and approximately 50 ng digested in

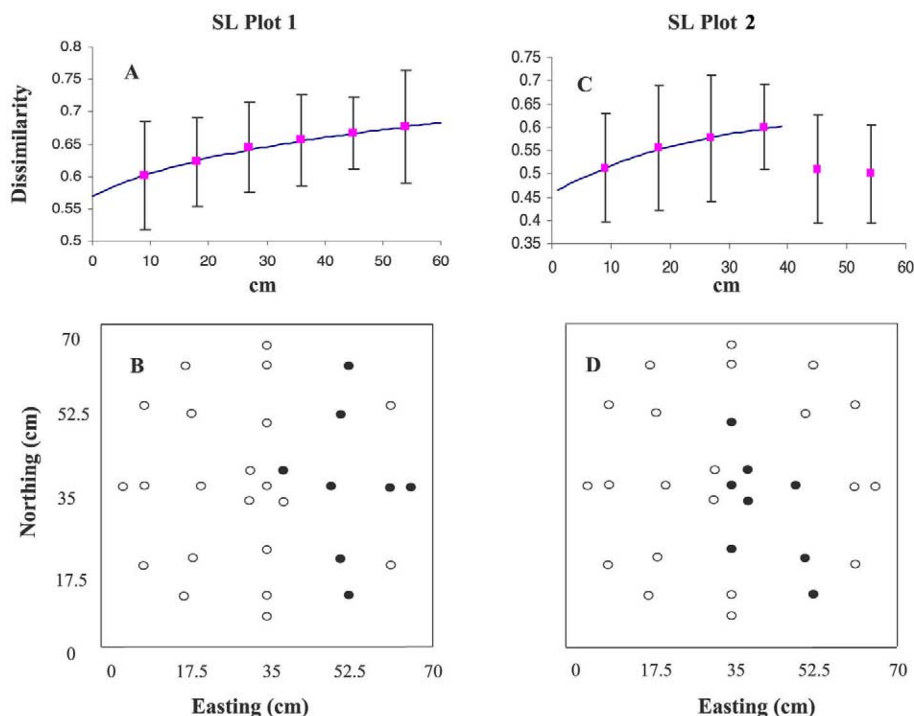

Fig. 1. Plots of relative dissimilarity with spatial distance and sample grid patterns for plots 1 (A and B, respectively) and 2 (C and D, respectively). Data points on dissimilarity graphs represent the average of data grouped within 9 cm lags and continuous lines represent modeled estimates. Error bars represent standard deviation. Sample grids were centered on individual *A. tridentata* shrubs. Filled circles indicate 'resource island' sample points (> 75% quartile OM and 50% NH_4^+). Open circles indicate non-resource island sample points.

20 µl reaction volumes with 6 U *Hha*I (New England Biolabs, Beverly, MA, USA) for 2 h in the manufacturer's recommended reaction buffer. Digests were then purified by passage through gel filtration cartridges (Edge Biosystems, Gaithersburg, MD, USA) and subsequently lyophol-ized. Three replicates from each sample were resuspended in 2 µl deionized formamide, 0.5 µl loading buffer, and 0.3 µl Genescan 500 ROX (Applied Biosystems Inc., Fremont, CA, USA) size standards, denatured at 96°C for 2 min, then placed on ice until analysis. TRF size composition of each sample replicate was analyzed by electrophoresis on denaturing 5% polyacrylamide gels (6 M urea) using a ABI 377 DNA sequencer. TRF lengths of between 50 and 500 bp were determined using Genescan analytical software (ABI). To quantify electropherogram output, a baseline threshold value of 50 fluorescence units, Local Southern size matching, and heavy smoothing were used [16].

Replicate profiles were aligned and standardized to eliminate spurious peaks using methods similar to those of Dunbar et al. [17]. Briefly, relative fluorescence of replicate profiles was standardized to the smallest quantity by proportionally reducing each peak area in larger profiles. After proportional reduction of larger profiles, peaks having fluorescence values less than the threshold value were eliminated. Peaks not appearing in all replicate profiles were also eliminated.

Comparison of standardized replicate T-RFLP profiles from different samples also requires standardization of relative fluorescence between samples [17]. Therefore, samples exhibiting greater total relative fluorescence were proportionally reduced as above and peaks having fluorescence values less than the threshold value eliminated.

2.4. Data analysis

Relative similarities between T-RFLP profiles of sample pairs within each site were calculated using Jaccard's coefficient:

$$\text{sim} = A/(A+B+C)$$

where A = the number of TRF sizes present in both samples, B = the number of TRF sizes found in sample 1 but not sample 2, and C = the number of TRF sizes present in sample 2 but not sample 1. Similarity values calculated in this way were converted to relative dissimilarity values (dissimilarity = 1−similarity) prior to analyses below.

For the HG site, variation in community profiles with spatial or temporal distance was determined by plotting dissimilarity values for each sample pair against their spatial or temporal separation distance.

For the SL site, spatial continuity of bacterial T-RFLP profiles were analyzed using geostatistical methods. The principal of spatial continuity is based on the premise that pairs of random variables separated by short distances are more similar than other pairs of the same variables spaced further apart. If spatial continuity is present for

continuous variables having known locations, it can be modeled by the non-ergodic autocorrelation function [18]. However, because multivariate, non-parametric T-RFLP profile data was used in our analyses, lag correlation coefficients could not be calculated for the traditional autocorrelation function; instead, values for relative dissimilarity between each sample pair were used. Relative dissimilarity values, when plotted with distance, analogously take the form of the traditional correlogram [19,20].

To determine spatial continuity, dissimilarity values were segregated into groups based on spatial separation (lags), averaged within each lag, and fitted to continuous models [18] by least-squares approximation. Exponential models fit the data of both SL plots:

$$y = C_0 + C_1(1-\exp(-3h/a))$$

where y is the dissimilarity term, C_0 (nugget variance) is the dissimilarity at lag 0 and represents the variation that cannot be resolved due to errors of measurement or spatial variation at scales smaller than measured, C_1 is a structural component, h is the spatial separation distance, and a is the distance beyond which variance is accounted for by spatial separation. C_0, C_1, and a were estimated from the model. The sill (C) is the y value at which the plot levels off and is calculated as: $C = C_0 + C_1$. Because exponential models reach the maximum asymptotically, the range was defined as the distance where y equals 95% of the sill.

The proportion of the total model variance ($C_1 + C_0$) represented by structural variance can be estimated using the equation $C/(C+C_0)$, thus providing a measure of spatial dependence. Values approaching 1.0 indicate a spatially structured system, while values approaching 0.0 indicate either poor spatial structure or that spatial structure occurs at scales different from those measured [5,19].

Due to suspected spatial dependence amongst sample pairs, which would violate the assumption of data independence necessary for analysis of variance (ANOVA) [1], non-parametric statistical analysis was used to detect differences between sample groups of each site. Dissimilarity between all HG site sample pairs were ranked and separated into temporal, small- and large-spatial analysis groups. Dissimilarities of SL site sample pairs were ranked and separated into three groups of sample pairs having separation distances < 30 cm; pairs outside resource islands ($< 75\%$ organic matter content and $<$ average NH_4^+ contents), pairs inside resource islands ($> 75\%$ organic matter content and $>$ average NH_4^+ contents), and pairs having a inner- and outer-resource island member. These data were analyzed by one-way ANOVA (Bonferroni adjustment).

Table 1
Summary of statistics for all sample sites

Site and analysis	n samples	n sample pairs	Mean dissimilarity[a]	S.D.
Homogeneous plant cover				
Small-scale spatial	8	29	0.26	0.08
Large-scale spatial	12	66	0.27	0.09
Temporal	12	66	0.29	0.09
Heterogeneous plant cover				
Plot 3 spatial	29	406	0.63	0.08
Plot 4 spatial	29	406	0.55	0.12

[a]Mean similarity is based on Jaccard coefficients.

3. Results

Overall, dissimilarity values for T-RFLP profiles of temporal and small- and large-scale spatial sample pairs of the HG site averaged 0.27 (Table 1). No trends towards dissimilarity were found with increased temporal or spatial distance, nor were significant differences found between small- and large-scale spatial or temporal analysis sample groups (data not shown).

In contrast to the HG site, dissimilarity values averaged 0.59 amongst all samples collected at the SL site. Plots of dissimilarity with distance are presented in Fig. 1A,C for SL plots 1 and 2, respectively. Exponential model fits were significant for both plots ($P < 0.001$) and spatial continuity was found to a distances of at least 36 cm. However, calculated spatial dependence was low for both plots (0.16 and 0.25 for plots 1 and 2, respectively), and very little of the overall variability in dissimilarity scores was accounted for by the models ($R^2 = 0.07$ and 0.05 for plots 1 and 2, respectively). Sample pairs within both SL plots having resource island and non-resource island members were found to be significantly less similar ($P < 0.05$) than sample pairs consisting of only resource island or non-resource island sample pairs (Fig. 2).

Amongst HG samples, TRF numbers ranged from 20 to 32 and averaged 26 per sample, while SL TRF numbers ranged from 21 to 44 and averaged 33.

4. Discussion

Most temperate soils generally exhibit marked seasonal fluctuations in microbial biomass and activity [21]. Although soil nutrient concentrations and other characteristics that would be expected to influence bacterial community composition were not analyzed for the HG site, a number of studies have documented seasonal changes in soil properties under *B. gracilis*. For example, Dormaar et al. [22,23] found organic matter characteristics, root biomass, C, N, methoxyl groups, soluble carbohydrate, and lignin contents to fluctuate significantly from season to season in soil of a site dominated by *B. gracilis*, and Epstein et al. [24] found net nitrogen mineralization rates to vary significantly during three monthly time periods (May,

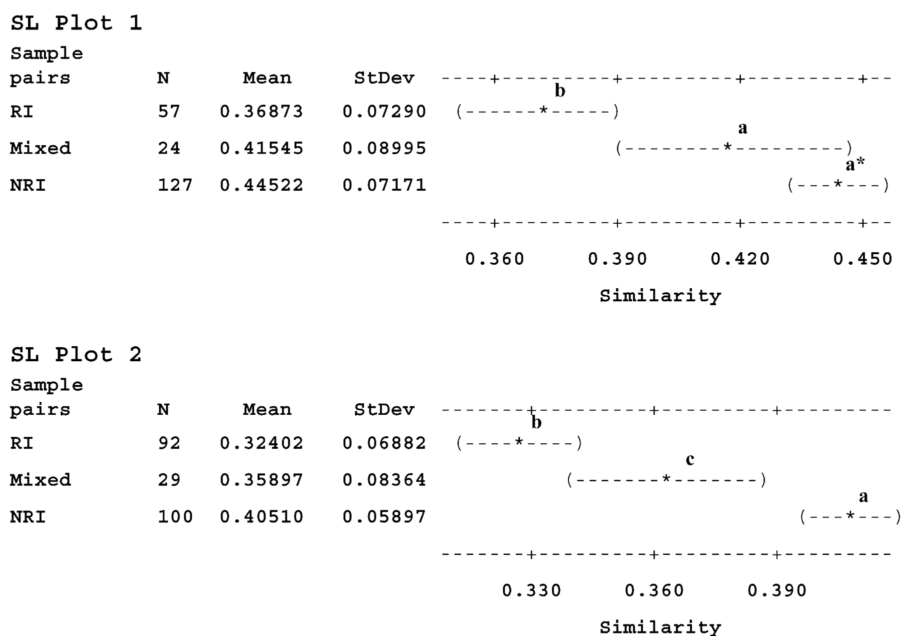

Fig. 2. Analysis of differences between groups consisting of sample pairs separated by less than 30 cm from resource islands (> 75% quartile OM and 50% NH_4^+), non-resource island (< 75% quartile OM and 50% NH_4^+) and sample pairs having a resource island and non-resource island member. *Different letters signify significant differences at the 0.05 level of probability.

July, and September) in a *B. gracilis*-dominated Colorado ecosystem.

Despite marked changes in temperature (−22 to 33°C), precipitation, and likely nutrient availability over the growing season, our results indicate no trends toward dissimilarity in T-RFLP profiles with temporal distance. Rapid soil drying and rewetting, such as is the norm for the HG site, represents a physiological stress that likely exerts intense selective pressures on surface soil microbial communities. A recent study [25] suggests that soils frequently exposed to wet–dry cycles select for bacterial communities which exhibit high stability under conditions of rapid alteration of moisture availability. Our results suggest high bacterial 16S rDNA compositional stability even 3 days after a 2.6 cm precipitation event unto desiccated soil. Measurement at greater temporal scales than measured here would therefore be required to detect substantial alteration in T-RFLP signatures of this ecosystem.

The high stability and similarity exhibited amongst T-RFLP profiles suggests that the dominant groups detected may consist of oligotrophic bacteria which would be expected to respond very slowly to changes in the environment [26], or organisms in a state of very low activity, possibly sequestered in the soil matrix away from the relatively nutrient-rich rhizosphere environment. Although estimation of energy inputs that drive gross microbial turnover rates were not determined, literature reports estimating soil microbial turnover rates for different ecosystems range from 0.07 to 0.32 per year [26]. Estimates for semiarid grassland soils are typically on the low end of this range, suggesting energy inputs into the study system are insufficient to allow the bacterial population as a whole to divide more than a few times a year [26,27]. Due to the heterogeneous nature of soil bacterial populations and their microhabitats, however, different segments of the bacterial population would be expected to exhibit pronounced differences in the ability to access, and rapidly use, nutrients. Therefore different segments of the population would be expected to turnover at rates quite different than energy inputs would dictate for the whole population. Although T-RFLP profiles were highly similar across the growing season, changes in the relative abundance of 16S rDNA for different segments of the population likely occurred that were not detected due to the resolution of the methods used. Our analyses considered only TRF presence or absence and in order for a given TRF size to be detected by our methods it must be represented by approximately 0.5% and 1% of the sequences within a sample that have homology to primer sequences (data not shown). Additionally, our use of T-RFLP analysis is based upon a single restriction enzyme. Because different bacterial strains can have the same TRF size after digestion with a given restriction enzyme [17], these analyses likely underestimate community compositional changes.

Although temporal analysis of predominant bacterial community compositions based on 16S rDNA data from similar ecosystems is not available, the results of this analysis are not without precedent. Krave et al. [28] used DGGE (denaturing gradient gel electrophoresis) of 16S rDNA PCR products to determine bacterial community temporal variability in litter, fragmented litter, and mineral soil fractions of a forest soil in Java. Despite significant

seasonal effects on soil moisture, pH, and nutrient contents, no apparent differences in bacterial community structure were found over time in either fragmented litter or mineral soil fractions. Other researchers have used culture-independent techniques to analyze temporal variability of soil and rhizosphere communities and found little difference in bacterial 16S rDNA signatures with time [29,30].

The extent to which DNA from non-living material contributes to these findings is not known, but could be significant. Extracellular DNA can remain absorbed to soil particles for long periods [31,32] and may be coextracted with nucleic acids from cells lysed during extraction, potentially obscuring changes in bulk soil bacterial community composition. Detection of salient changes in bacterial community composition may require analysis of soil subfractions. Since 16S rDNA of bulk soils may be largely dominated by a relatively few species, abundance changes in many important bacterial groups may require group-specific analyses.

Spatial analysis of samples collected at distances up to 100 m yielded approximately the same range of similarity values as small-scale spatial and temporal analyses (Table 1), suggesting relatively similar and uniform controls on bacterial communities across this site. *B. gracilis* is known to develop a high density of laterally spreading, shallow roots that allow the plants to benefit from light precipitation events [33], potentially resulting in similar rhizosphere influences at the scales measured.

Felske and Akkermans [34] also found low bacterial 16S rDNA signature spatial variability in soil under homogeneous grass cover. Using TGGE (temperature gradient gel electrophoresis), these authors found very similar banding patterns between samples collected at spatial scales of up to several hundred meters. Similar to our results, these authors concluded that analysis of a single gram of soil was sufficient to represent the predominant bacterial 16S rDNA composition across their study plots.

In contrast to the highly similar bacterial community structures of the HG site, T-RFLP profiles of the SL site varied greatly between samples. Despite high dissimilarity values for the SL site, spatial autocorrelation was found to distances of 36 cm or more (Fig. 1A,C). However, very little of the variance could be explained by spatial characteristics on the spatial scales sampled. Estimation of spatial dependence (the proportion of model variance that could be explained by spatial separation) for plots 1 and 2 yielded values of only 0.16 and 0.25, respectively, suggesting that the predominant influences on bacterial biodiversity operate at scales other than those measured. The high nugget variance of both plots suggests that these scales are predominantly smaller than those measured.

In a previous study [14] we found distinct spatial stratification of soil organic matter and NH_4^+ in relation to shrub locations on both SL plots. Woody plants typically have pronounced effects on spatial patterns of organic matter and nutrients in arid and semiarid ecosystems, due to localization of phytomass, and alteration of decomposition rates and hydrology (e.g. [13]). Shrub-induced changes in soil properties are known to strongly influence microbially mediated processes such as nutrient cycles and N_2O evolution [2,3]. Our results indicate that shrub-induced changes in soil properties also measurably influence bacterial community composition (Fig. 2).

Plotting bacterial dissimilarity values with distance also suggested shrub-induced bacterial 16S rDNA compositional changes. T-RFLP profiles of plot 1 exhibited increased dissimilarity to distances nearly spanning the plot area, while samples deemed to be ‘resource island’ associated were concentrated on the eastern side of the plot (Fig. 1A). Plot 2 dissimilarity values increased to only about 36 cm and decreased at greater distances. ‘Resource island’ samples were located in more central locations in this plot and sample pairs having distances greater than the maximum at which dissimilarity increased were

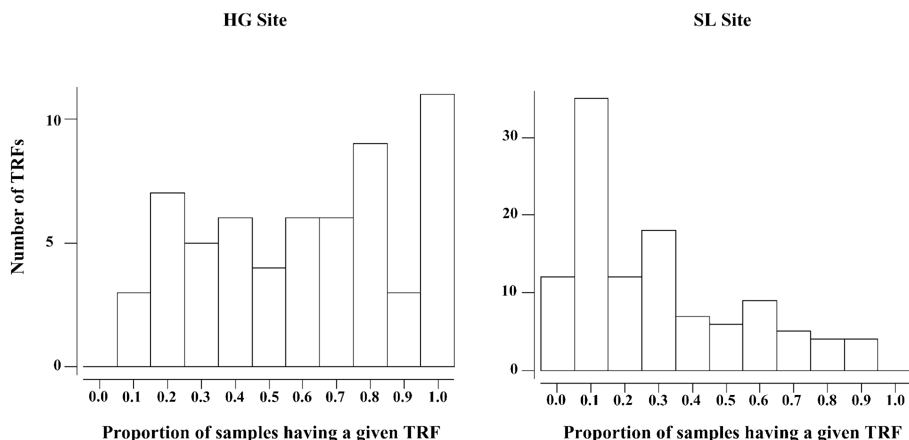

Fig. 3. Graphical presentation of the proportion of samples in HG and SL sites having a given TRF size. The number of samples within which each fragment size was found was determined and is represented on the x-axis as the proportion of the total number of samples collected at each site.

found to be predominantly non-resource island samples (Fig. 1C). Therefore, in contrast to plot 1, predominantly non-resource island samples were compared at greater lags in plot 2, resulting in relatively low dissimilarity values at the two greatest distance lags.

Distributions of plants other than shrubs may have also influenced the high variability of T-RFLP profiles derived from the SL plots. Differences in rooting patterns of the different plants found on the SL site, as well as differences in exudate and litter quality and quantity, would be expected to influence the composition of microbial populations through alteration of soil chemical properties, as well as soil structural characteristics [35,36,37]. Areas adjoining bare soil and plant cover would therefore be expected to represent areas of niche character gradation. While the scales sampled allowed for elucidation of a shrub cover influence on bacterial T-RFLP profiles, the influence of individual grass and forb species on bacterial community patterns will require measurement at smaller scales relevant to individual plants (i.e. canopy cover or rhizosphere) or microorganisms. Analysis of physical, chemical and biological properties at the microsite level may be required to elucidate controls on bacterial community composition, potentially providing the means to scale up to the level of the bulk soil sample, plot and/or ecosystem.

The results of this study suggest that plant community structure strongly influences bacterial T-RFLP variability in these semiarid ecosystems. To graphically represent differences in within-site variability between HG and SL sites, the proportion of samples in each site sharing a given TRF size was plotted (Fig. 3). These plots indicate that a given HG TRF size is most likely to be found in nearly all HG samples, while a given SL TRF size is most likely to be found in only about 10% of SL samples, indicating that a much more extensive sampling effort would be required to characterize the predominant bacterial community of the SL site.

Although the methods used in these analyses were highly reproducible (data not shown), potential bias inherent in nucleic acid extraction and PCR amplification should be noted [38–40]. Differences in soil characteristics may have influenced our results, complicating direct comparisons between sites. In addition, differences in sample collection strategies may complicate direct comparisons between ecosystems. Due to differences in surface characteristics, soils were collected at the 5–10 cm depth on the SL site, while samples were collected to the 7.5 cm depth on the HG site and changes in bacterial community composition with depth are unknown for either system.

This study represents one of the first attempts to characterize bacterial 16S rDNA spatial distributions in an ecosystem having heterogeneous plant cover using molecular methods. Analyses such as this could form the basis for determining influences of disturbance, including climate change, on ecosystem structure and function.

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