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Comparative diversity and composition of cyanobacteria in three predominant soil crusts of the Colorado Plateau

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Abstract

Terminal restriction fragment length polymorphism (TRF or T-RFLP) analysis and 16S rDNA sequence analysis from clone libraries were used to examine cyanobacterial diversity in three types of predominant soil crusts in an arid grassland. Total DNA was extracted from cyanobacteria-, lichen-, or moss-dominated crusts that represent different successional stages in crust development, and which contribute different amounts of carbon and nitrogen into the ecosystem. Cyanobacterial 16S rRNA genes were amplified by PCR using cyanobacteria-specific 16S rDNA primers. Both TRF and clone sequence analyses indicated that the cyanobacterial crust type is dominated by strains of *Microcoleus vaginatus*, but also contains other cyanobacterial genera. In the moss crust, *M. vaginatus*-related sequences were also the most abundant types, together with sequences from moss chloroplasts. In contrast, sequences obtained from the lichen crust were surprisingly diverse, representing numerous genera, but including only two from *M. vaginatus* relatives. By obtaining clone sequence information, we were able to infer the composition of many peaks observed in TRF profiles, and all peaks predicted for clone sequences were observed in TRF analysis. This study provides the first TRF analysis of biological soil crusts and the first DNA-based comparison of cyanobacterial diversity between lichen-, cyano- and moss-dominated crusts. Results indicate that for this phylogenetic group, TRF analysis, in conjunction with limited sequence analysis, can provide accurate information about the composition and relative abundance of cyanobacterial types in soil crust communities. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Approximately 30% of lands globally consist of semiarid and arid landscapes. Biological soil crusts are a predominant feature of many arid landscapes and can almost completely cover the soils between widely spaced vascular plants [1,2]. In some arid landscapes, soil crusts can constitute 70% or more of the living ground cover [3]. Soil crusts play critical ecosystem roles that affect the structure, function and productivity of arid lands, including soil stabilization, water retention, carbon and nitrogen fixation, and soil fertility [4].

On the Colorado Plateau of the western USA, cyanobacteria-, lichen-, or moss-dominated crusts are the common crust types. Their floral and microbial composition is complex and is greatly affected by environmental conditions and length of time an area has been colonized [5,6]. Studies of crust productivity indicate that species composition of crusts affects their morphology and ecological functioning [7–10]. Dark-colored lichen-dominated crusts have higher soil temperatures and lower surface albedo than the lighter-colored cyanobacterial crusts [3]. They have greater surface roughness than cyanobacterial crusts, which reduces wind erosion while increasing water infiltration, soil moisture, and retention of seeds and organic matter [1,7,11]. Lichen crusts are responsible for higher carbon and nitrogen inputs than cyanobacterial crusts, and respond differently to changes in temperature and moisture [12,13]. Moss-dominated crusts have even higher carbon fixation

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than lichen crusts, but very low nitrogen fixation rates (S. Phillips and J. Belnap, unpublished results).

Cyanobacteria are an essential component of cyanobacteria-, lichen-, and moss-dominated crusts of the Colorado Plateau, while green algal components are rare in the alkaline soils of this region [5]. The cyanobacteria contribute to the physical structure of the crust and provide fixed nitrogen and photosynthetically fixed carbon to other crust components and neighboring plants [13,14]. Different species of cyanobacteria have different metabolic capabilities and physiological tolerance of the extreme environmental conditions in arid lands. Thus, knowledge of the diversity and species composition of cyanobacteria in different soil crusts is important in understanding the relationships between the resident cyanobacterial community, soil crust function, and arid land productivity. Information on the cyanobacterial community is also needed for monitoring the impact of soil disturbance and other environmental changes on the stability and functioning of arid land ecosystems.

Microbial diversity in crusts remains poorly understood because of the many types of crusts and the wide variety of environments in which they occur [9]. Cyanobacterial diversity in crusts has been studied (for reviews see [4,15]), however most previous studies have employed laboratory cultivation and/or microscopy to determine species composition. Difficulties in obtaining axenic cultures, morphological identification, taxonomic assignment and cryptic strain variability have all contributed to underestimation and inaccurate determination of species composition [5]. A recent polyphasic study of biological soil crusts by Garcia-Pichel et al. [16] indicated that DNA-based techniques are capable of revealing more extensive species diversity in soil crusts than traditional techniques.

Terminal restriction fragment length polymorphism (TRF or T-RFLP) analysis (reviewed by Marsh [17] and Kitts [18]) is a rapid DNA-based technique for analysis of bacterial communities that permits replicated, field-scale comparisons across variables of space, time, physicochemical gradients, and environmental disturbance. In this method, DNA from microbial communities is first amplified by PCR using fluorescently labeled primers, and the products digested with restriction enzymes to produce terminal fragments characteristic in size of particular sequence types. Laser-based detection of fluorescently labeled fragments on an automated sequencing instrument is then used to provide precise identification of fragment size, quantitative measurement of fragment abundance, and high-throughput capability. TRF analysis has proven useful for comparative analysis of complex microbial communities in a wide variety of environments [17,18]. A shortcoming of the method has been difficulty in assigning species identity to individual peaks in the profiles [19].

In this study we compared the diversity and composition of cyanobacteria assemblages in soil crusts that differ in floral composition, successional stage, and photosynthetic and nitrogen fixing capability [4,12,13]. Combining TRF and clone sequence analyses of cyanobacterial 16S rRNA genes amplified from cyanobacteria-, lichen- and moss-dominated crust DNA, we identified and determined the relative abundance of the predominant cyanobacteria species in each crust type, and correlated the sequence information with the location and relative abundance of peaks in the TRF profiles.

2. Materials and methods

2.1. Crust samples

Crusts were collected on 24 September 1998 at two sites in Canyonlands National Park, UT, USA. Three crust types were collected. Cyanobacterial crust, having the same color as the surrounding sandy soil, was dominated by *Microcoleus vaginatus* and lacked visible lichen structures. Lichen-dominated crust was darker in color due to the presence of darkly pigmented cyanobacterial species and contained visible lichen thalli (predominantly *Collema tenax* [10]). Moss-dominated crust was visibly dominated by mosses (primarily *Tortula* species). All crusts were growing on a red sandstone soil substratum (Typic Torripsamments from the Begay series). Samples were collected by inverting a petri dish over the crust, pushing the dish into the soil, and gently lifting up while supporting the bottom with a spatula. Crust samples were placed on dry ice for transport to the laboratory, and stored at $-70°C$ prior to extraction of DNA.

2.2. DNA extraction

A 0.5-g subsample of each crust type was used in the DNA extraction procedure described by Kuske et al. [20]. The procedure included incubation at 70°C with 1% (w/v) sodium dodecyl sulfate, three cycles of freeze–thaw, bead-mill homogenization, centrifugation, and ethanol precipitation. The DNA was purified of PCR-inhibiting material by CTAB/phenol/chloroform extraction as described by Wilson [21], and precipitated with sodium acetate and ethanol. Pelleted DNA was washed with cold 70% ethanol, and suspended in TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA). The cleaned DNA extracts were quantified on a 1% agarose gel using an uncut lambda DNA mass standard, and were further purified by passage through TE-saturated Sephadex G-200 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) spin columns using a 96 well microtiter method [20]. The purified crust DNA was precipitated with ammonium acetate and ethanol, suspended in 50 μl TE, and quantified on a 1% agarose gel with uncut lambda DNA standards. Extracted samples were stored at $-70°C$.

2.3. PCR amplification of cyanobacterial 16S rDNA from crusts

Cyanobacterial 16S rDNA was amplified from the soil crust DNA using primers CYA359F (*Escherichia coli* nucleotides 359–378) and CYA781R (a mixture of two primers targeting *E. coli* positions 781–805), as described by Nübel et al. [22]. Each 100 µl PCR reaction mixture contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl$_2$, 0.001% (w/v) gelatin (Applied Biosystems, Foster City, CA, USA), 200 µM concentration of each deoxynucleoside triphosphate, 0.01 mg BSA (Boehringer Mannheim, Germany), 50 pmol of each primer, and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR was performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) with the following cycling conditions: 12 min of denaturation at 95°C, followed by 35 cycles of 20 s at 95°C, 1 min at 60°C, 1 min at 72°C, with a final 10 min extension at 72°C. PCR products were gel purified using the QIAquick gel extraction kit (Qiagen, Chatsworth, CA, USA). To avoid quantitative biases in PCR [23], we attempted to maintain reaction conditions below saturating product amounts, and pooled amplicons from 10 separate reactions for clone sequence and TRF analyses.

2.4. Cloning and sequencing cyanobacterial 16S rDNAs

16S rDNA genes amplified from the three crust types were cloned into the pCR4 vector, using the TOPO-TA cloning kit and the manufacturer's protocols (Invitrogen, San Diego, CA, USA). Ribosomal DNA inserts of 34–36 clones from each of the three crust 16S rDNA libraries were sequenced. The clones were grown overnight in LB medium (Sigma, St. Louis, MO, USA) at 37°C, and DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen). Extracted DNA was quantified by UV spectrophotometer. Approximately 500 ng template was used for sequencing reactions performed using BigDye terminator cycle sequencing kit (Applied Biosystems) with sequencing primers T7 and M13R to obtain sequences from both strands of the insert. Sequencing reactions were purified using Centri-Sep spin columns (Princeton Separations, Adelphia, NJ, USA). Sequencing gels were run on an Applied Biosystems model 373 automated sequencer (Applied Biosystems). Consensus sequences were constructed using Auto Assembler software (Applied Biosystems).

2.5. Phylogenetic analysis

Sequences were analyzed for the presence of chimeric artifacts using the Check Chimera program of the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/html/analyses.html [24]). Sequences were initially analyzed for phylogenetic affiliation using the BLAST 2.0 program (http://www.ncbi.nlm.nih.gov/blast/ [25]). Reference sequences were obtained from GenBank, and preliminary alignment was performed using the web-based Sequence Aligner program of the RDP [24]. Final alignment of sequences was accomplished manually with the GDE multiple sequence editor [24], using conserved primary sequence regions and secondary structure folding as guidelines. Sequence regions which could not be aligned with confidence were excluded from analyses.

Initially, a phylogenetic tree of nearly full-length (> 1000 nt) reference sequences was constructed and optimized. Previous analyses have determined that the relatively rapid rate of sequence change found in cyanobacterial rDNA sequences can lead to artifacts in phylogenetic analysis [26,27], so correction for site-to-site rate heterogeneity was employed. An initial maximum likelihood tree was constructed using fastDNAml (version 1.1, distributed by RDP [28]) with empirical base frequencies, and a transition–transversion ratio ($T = 1.4$), optimized by comparing likelihoods under a T of 1.0–2.0. The tree of highest likelihood was found by repeated (100×) tree building using random sequence input orders, with the best tree optimized by global rearrangement of branches. With the program DNArates [28], this tree was used to estimate the evolutionary rate of change for each nucleotide position in the sequence alignment, partitioned into 35 rate categories. The resultant dataset, including the rate categories, was used in another round of tree building (100×) with random sequence addition order, and the best tree optimized by global branch swapping. The transition–transversion ratio was reoptimized using this tree ($T = 1.7$), and another 100 trees constructed, with the best tree optimized. Insertion of partial sequences (< 1000 bp), including all of those determined from rDNA clones in this study, into the optimized tree of longer sequences was performed 100 times using the 'restart' script of fastDNAml, with random addition of taxa, without rearrangement of branches in the starting tree.

2.6. TRF analysis

Cyanobacterial 16S rDNA for TRF analysis was amplified by PCR using CYA359F primer that was fluorescently labeled with FAM (6′-carboxyfluorescein, Life Technologies, Carlsbad, CA, USA) and CYA781R [22]. PCR conditions were as described above. Approximately 200 pg of crust DNA template per reaction was used. *Synechococcus* sp. (ATCC 27194) DNA was used at 2 ng per reaction as a positive control. PCR for each crust DNA sample was performed in triplicate using three independent PCR reactions and combined prior to purification of the PCR product.

The pooled PCR reactions for each sample were run on a 2% agarose gel, and DNA was visualized by UV illumination following staining with ethidium bromide. The correct fragment sizes, approximately 420–436 bp, were excised from the gel and purified using a QIAquick gel

extraction kit (Qiagen). Samples were quantified by analysis of the purified products on a 2% agarose gel with an uncut lambda DNA mass standard. DNA quantities were calculated by Image Gauge v3.3 software (Fujifilm, USA). Approximately 70 ng of purified amplicon DNA was digested overnight at 37°C with 20 U *Msp*I plus 10 U *Rsa*I (New England Biolabs, Beverly, MA, USA) in 20 μl reactions. Following restriction digestion, the DNA was cleaned using Centri-Sep spin columns (Princeton Separations). One μl of the digest mixture was dried by incubation at 94°C for 2 min. Samples were then suspended in 2 μl of loading buffer containing 0.25 μl of Genescan 500 TAMRA size standards (Applied Biosystems), 1.4 μl of deionized formamide, 0.35 μl of a 3% (w/v) blue dextran solution, and 25 mM EDTA, denatured for 2 min at 94°C and immediately placed on ice. Fragments were separated by electrophoresis in denaturing 4% polyacrylamide Long Ranger gels (FMC BioProducts, Rockland, ME, USA) on an ABI 377 DNA sequencer (Applied Biosystems). TRF sizes between 35 and 500 bp, with peak heights > 30 fluorescence units were analyzed using Genescan analytical software v3.3 (Applied Biosystems).

A preliminary TRF gel was used to standardize the DNA quantity loaded on TRF gels between the different environmental samples. The sum of all profile peak heights ≥25 fluorescence units in each sample was calculated as an indication of the total DNA quantity represented by each TRF profile. DNA quantity was standardized by calculating the amount of sample volume that would give a sum-of-peak-heights value between 8000 and 10 000 fluorescence units for each sample [19]. Three replicate TRF profiles were run from the digested DNA by loading three aliquots of standardized digested DNA (0.5–4 μl) on each of three separate polyacrylamide gels. One sample of undigested (uncut) cyanobacterial 16S rDNA amplicon was run on a TRF gel for each crust sample.

2.7. Prediction of clone TRF sizes

The 16S rDNA clone library sequences produced from the crust cyanobacterial amplicons were used to predict terminal restriction sizes using PatScan software (Scan for Matches, R. Overbeek, Argonne National Laboratory, Argonne, IL, USA). Clone library consensus sequences were aligned in the forward primer orientation. TRF sizes were predicted individually for the enzymes *Msp*I and *Rsa*I, then combined to predict TRF products of a double digest with the two enzymes.

3. Results and discussion

3.1. Phylogenetic analysis of rDNA clone sequences

Initial BLAST analysis of 34–36 clone sequences from each crust sample indicated that all rDNA clones obtained

were of cyanobacterial or moss chloroplast origin (data not shown). The phylogenetic tree of the highest likelihood from our analysis of soil crust clone and database sequences is shown in Fig. 1. A wide variety of cyanobacterial and chloroplast phylotypes were obtained in the libraries. Sequences that were clearly related to those of cultivated species clustered with the *Oscillatoria, Phormidium, Nostoc, Pseudanabaena* and plastid sequence groups, defined by Turner et al. [27]. However, many of the crust clone sequences were only distantly related to any cultivated species, and may represent new genera or groups within the Cyanobacteria division.
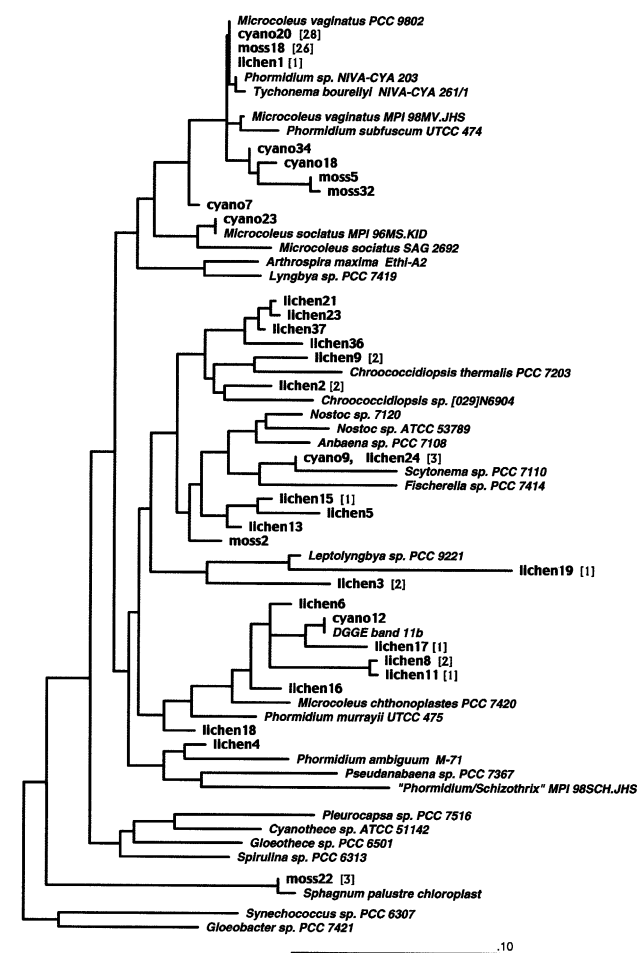

Fig. 1. Phylogenetic tree of soil crust 16S rDNA sequences and reference sequences, inferred by maximum likelihood analysis. Cyano#: clones obtained from cyanobacterial soil crust sample; lichen#: clones from lichen crust; moss#: clones from moss crust sample. Numbers in brackets indicate additional clones obtained from a sample that have sequences identical with the clone listed. Database sequences were most closely related to soil crust clone sequences, together with sequences representative of major cyanobacterial phylogenetic groups, are included for reference. Scale bar represents number of nucleotide substitutions per sequence position. Sequences obtained in this study have been deposited in GenBank under accession numbers AF428473–AF428578.

3.1.1. Cyanobacterial crust clone sequences

The clone library generated from the cyanobacterial crust sample comprised seven different sequence types. The library was heavily dominated (29 of 35 clones) by virtually identical rDNA sequences having >99.2% sequence identity to that of *M. vaginatus* PCC 9802 (data not shown), an isolate from nearby Arches National Park, UT, USA [16]. This result was expected, as previous studies identified *M. vaginatus* Gomont as the predominant colonizer of cyanobacterial soil crusts in the Colorado Plateau region, often comprising up to 95% of the biomass of crusted soils [29]. A recent study of cyanobacterial crusts from this region using microscopy, denaturing gradient gel electrophoresis (DGGE) and sequencing analyses also identified this species as being dominant in different soil types [16]. Phylogenetic tree inference (Fig. 1) identified three other sequences that were closely related to, but not identical with, that of *M. vaginatus*, ranging from 97.2 to 98.2% in sequence identity. This result has been seen commonly in rDNA sequence-based analysis of many microbial communities [30–32], and may indicate the presence of a group of closely related strains or species within the community, or variability among rRNA genes within a species [33]. The three remaining clone sequences were also most closely related to those of cyanobacterial species previously identified in soil crusts. The sequence of clone cyano23 was identical with that of *Microcoleus sociatus* strain MPI 96MS.KID isolated from a desert soil crust in Israel [16], while that of cyano12 matched the sequence obtained from DGGE analysis of a sandy soil crust from Arches National Park (DGGE band 11b [16]). The remaining clone sequence, cyano9, was virtually identical with that obtained from four clones of the lichen crust sample (e.g. lichen24 in Fig. 1), and was most closely related to the sequences of *Nostoc* spp. ATCC 53789 and PCC 7120.

3.1.2. Lichen crust clone sequences

In contrast to the cyanobacterial soil crust clone library, a very heterogeneous group of rDNA sequences was obtained from the lichen crust clone library. Twenty distinct phylotypes were identified among the 35 sequences analyzed. This distribution suggests that many unsampled phylotypes may be present in the lichen crust, and would be revealed by analysis of additional clones. Although it is possible that some of the observed sequence diversity arises from multiple different rDNA operons within a single species [33], most lichen-derived sequences differ from each other by >5%. Thus, variability between operons likely accounts for only a portion of the diversity seen in the lichen sample.

The sequences of only two clones matched that of *M. vaginatus*. These were identical with most of those obtained from the cyanobacterial crust sample. The remaining clone sequences obtained from the lichen crust were phylogenetically very diverse, spanning numerous genera and groups of cyanobacteria (Fig. 1). Ten sequences clustered most closely with those of two *Chroococcidiopsis* spp. (PCC 6904 and 7203), having 91.8–98.6% sequence identity with the cultured species. Another group of 10 clone sequences clustered with sequences of the *Oscillatoria* group [27], and these clone sequences were most closely related to that of *Microcoleus chthonoplastes* PCC 7420. Several sequences in this group were also recovered from the DGGE analysis of Utah soil crusts (Cluster B [16]). The clones in the next largest group (eight clones) were somewhat related to members of the *Nostoc* group [27], including species of *Nostoc* and *Scytonema* commonly found in these crusts by culture-based and microscopy analyses [4]. These may derive from heterocystous species that are capable of nitrogen fixation and are thus important in crust function and productivity. The remaining clone sequences were distantly related to *Leptolyngbya* sp. PCC 9221 or *Phormidium ambiguum*.

Many different cyanobacterial species have been recorded in previous studies of lichen crusts of this region [6,7,34]. However, with the exception of those matching the sequence of *M. vaginatus*, none of the rDNA clone sequences we obtained from the lichen crust were identical with that of any cultured cyanobacterial species available in the database. Cultivation techniques generally fail to recover the majority of microbial species present in environmental samples, presumably due to inadequate culture conditions and/or obligate symbiotic interactions between species. In addition, sequence data have still not been obtained from many cultured cyanobacterial isolates. Finally, analyses of rDNA sequences from cyanobacteria have shown that many previously described genera are not phylogenetically coherent, and are in need of taxonomic revision [26,27], thus we cannot assign our environmental sequences to established genera with confidence. Further sequencing of rDNA genes from soil crust cyanobacterial isolates will be necessary to resolve this issue, and to indicate whether studies of available, cultured species may be useful in exploring the functional diversity of cyanobacteria present in these crusts.

The composition of cyanobacterial phylotypes from the lichen crust was unexpectedly diverse. Lichen soil crusts establish on soils that have been previously stabilized by *M. vaginatus*-dominated cyanobacterial crusts [10,35], and represent a later successional stage in crust development. Our data suggest that this process involves displacement of *M. vaginatus* by diverse other cyanobacterial species. Several possible explanations exist for the presence of so many photosynthetic taxa in a single ecological niche. It is thought that species are brought to crusts by wind dispersal, and colonize available space in the existing crust. Lichen-containing crusts require at least several decades to develop [36], allowing time for multiple cyanobacterial species to arrive and establish. Once established, low moisture and nutrient levels greatly restrict the growth rate of

Table 1
Comparative analysis of cyanobacterial 16S rRNA genes from three types of soil crust using TRF and clone library sequence approaches

Crust type	TRF fragment size (bp)[a]	Predicted fragment size (bp)[b]	Mean TRF peak height[d]	TRF peak percent of total profile	Percent of predicted clone TRFs	Representative clones	Phylogenetic affiliation[e]
Cyano	103	104	120 (16)	3.9	5.7	cyano 9, 18	multiple genera
	105	106	320 (35)	10.3	8.6	cyano 12, 23, 34	multiple genera
	116	117	2385 (293)	76.7	85.7	cyano 20	*M. vaginatus*
	173	n.f.[c]	53 (9)	1.7	n.f.	n.f.	n.f.
	175	n.f.	48 (2)	1.5	n.f.	n.f.	n.f.
	186	n.f.	184 (30)	5.9	n.f.	n.f.	n.f.
Lichen	103	104	421 (47)	14	19.4	lichen 4, 18, 24	*Nostoc, Phormidium*
	105	106/107	1261 (134)	42	66.7	lichen 2, 6, 8, 15	multiple genera
	116	117	140 (12)	4.7	5.6	lichen 1	*M. vaginatus*
	173	173	481 (45)	16	8.3	lichen 3	*Leptolyngbya*
	175	n.f.	416 (45)	13.9	n.f.	n.f.	n.f.
	186	n.f.	59 (10)	2	n.f.	n.f.	n.f.
	423	uncut	44 (16)	1.5	n.f.	n.f.	n.f.
	424	uncut	88 (1)	2.9	n.f.	n.f.	n.f.
	425	uncut	91 (6)	3	n.f.	n.f.	n.f.
Moss	105	107	198 (5)	8.7	2.9	moss 2	*Nostoc*
	116	117	1584 (67)	69.8	79.4	moss 18	*M. vaginatus*
	173	n.f.	100 (10)	4.4	n.f.	n.f.	n.f.
	175	175/176	61 (2)	2.7	17.6	moss 5, 22, 32	chloroplast, *M. vaginatus*
	186	n.f.	239 (18)	10.5	n.f.	n.f.	n.f.
	243	n.f.	50 (8)	2.2	n.f.	n.f.	n.f.
	436	uncut	38 (5)	1.7	n.f.	n.f.	n.f.

[a] 16S rDNA fragment sizes as measured by TRF analysis.
[b] Predicted TRF size for clones in soil crust 16S rDNA libraries.
[c] Fragment or clone not found among the 34–36 clones that were sequenced.
[d] Height of TRF peaks calculated as the average of three subsamples run on three electrophoresis gels. The standard deviation for each mean peak height is given in parentheses following the mean.
[e] The closest reference sequence from Fig. 1.

organisms, perhaps leading to physical isolation of species and little or no direct competition for resources, allowing coexistence of diverse taxa. In addition, it has been shown in cyanobacterial crusts that species (and even strains within species) of cyanobacteria adapt to different light intensities and wavelength optima, and have different UV tolerance [37,38]. As a result, cyanobacterial communities in crusts are layered, with more light- or UV-tolerant species on the surface, while less-tolerant species occur below. Other essential environmental parameters, such as moisture availability, oxygen tension and nitrogen availability, also vary through the crust [10], and may contribute to microscale differences in species distribution and/or colonization.

The added dimension of symbiosis as lichen photobionts also provides another source of potential species diversity in the cyanobacteria. The lichen crust analyzed in this study contained visible lichen structures of *Collema tenax*, which is common in this region [14]. This lichen has *Nostoc* spp. as its photobiont [14], and it is possible that the four clones with identical sequences (represented by lichen24, Fig. 1) that were most closely related to that of *Nostoc* spp. (ATCC 53789) derived from the lichen symbiont.

3.1.3. Moss crust clone sequences

The moss crust clone library contained five phylotypes. In a pattern similar to the cyanobacterial crust library, most of the moss soil crust clone sequences were closely related to *M. vaginatus* (27 of 34 clones), while two additional sequences were more distantly related members of this group (Fig. 1). The next most abundant sequence type (four clones) was 98.8% identical with that of the chloroplast of the moss *Sphagnum palustre*. It is likely that these sequences derived from the rDNAs of the chloroplasts of *Tortula*, *Bryum*, *Pterygoneurum*, and/or *Ceratodon* species that dominate moss crusts in this region. Only one additional sequence type was recovered (clone moss2), somewhat related to several sequences obtained from the lichen crust library (94.0–97.9% sequence identity), and more distantly related to cultivated *Nostoc* spp. (93.2–96.3% sequence identity).

Given suitable site conditions, moss crusts are of a later successional stage. They develop in areas where the soil has been previously stabilized by *M. vaginatus* colonization [10,35]. They typically occupy different microclimates than the other two crust types, preferring tree/shrub-shaded or north-sloping environments to full-sun conditions. In contrast to the lichen crust, our clone sequence
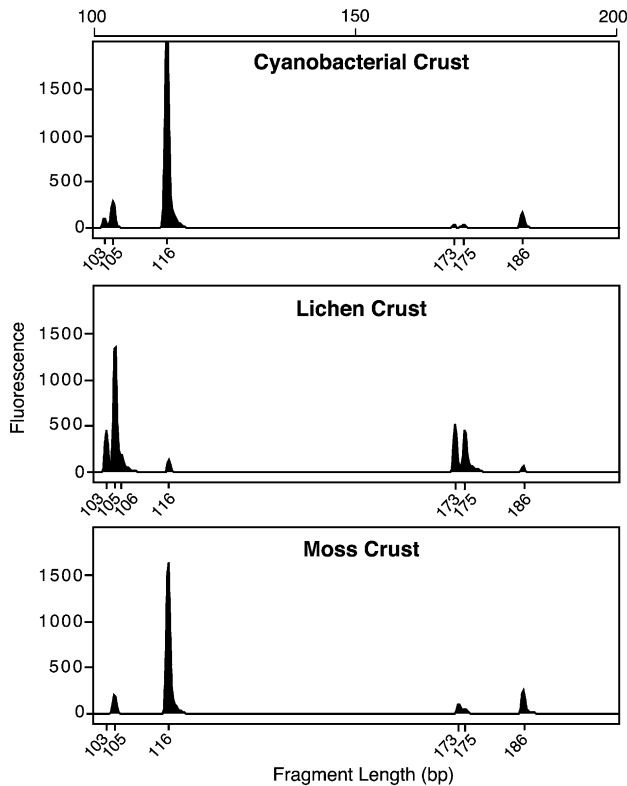
Fig. 2. TRF profiles of cyanobacterial, lichen and moss soil crust cyanobacterial rDNAs. Fragments in the size range 100–200 bp are shown. For each profile, the *x* axis displays the size of the TRFs (in bp), while the *y* axis displays fragment abundance, as measured by fluorescence intensity. The median profile of three replicate profiles for each crust type is illustrated. The lichen crust profile contains an additional small peak at 106 bp that was only observed in this replicate and is not shown in Table 1.

and TRF data (below) indicate that cyanobacterial diversity in moss crusts is low. This may be due to spatial exclusion of cyanobacteria by mosses, whose growth form is very dense and raised above the soil surface. This hypothesis is supported by microscopy studies which found very few cyanobacterial species in moss crusts of the Colorado Plateau region (J. Belnap, unpublished data).

3.2. TRF and PatScan analysis

3.2.1. PCR and analysis of uncut rDNA amplicons

TRF analysis has the potential of providing information about community diversity and relative abundance of different groups, by comparison of the number of different TRFs in a profile and their peak heights. The use of automated sequencing equipment for analysis of TRFs allows both high-precision and high-throughput analysis of samples, making the technique attractive for field-scale comparative analyses.

Undigested rDNA amplicons from each crust sample were analyzed to verify actual PCR amplicon size (data not shown). For each of the three crust types, the cyano-

bacterial 16S rDNA primers produced two DNA fragments, 424 and 436 bp in length. The relative proportion of the fragments differed among the crust types. The TRF profiles of moss and cyanobacterial crusts were very similar in peak distribution, with a less-abundant fragment at 424–425 bp and a more-abundant fragment at 436 bp. In contrast, the 424–425-bp fragment was the most abundant in the lichen crust TRF profile and the 436-bp fragment was less abundant. The relative abundance of undigested cyanobacterial rDNA amplicons correlated well with the frequencies of sequence types observed in the clone libraries. As noted above, the cyanobacterial and moss crust libraries were dominated by clones having insert sequences nearly identical with that of *M. vaginatus*. These rDNAs contain an 11–12-bp insert in their sequences, relative to the sequences of other cyanobacteria, between *E. coli* positions 463 and 468 ([16,39], and this analysis). This insert appears to be diagnostic for the *M. vaginatus* group, as even very closely related sequences (i.e. cyano34) lack this sequence feature (data not shown). Thus, the presence of a larger peak at 436 bp in the cyanobacterial and moss crust samples is most readily explained by the predominance of *M. vaginatus* rDNA sequences in these samples, in contrast to the lichen crust sample, which yielded amplicons mostly of 424–425 bp size.

3.2.2. TRF and clone restriction fragment analysis

*Msp*I/*Rsa*I-digested amplicons of cyanobacterial, lichen and moss crust rDNAs were run in triplicate. The majority of peaks were present and identical in the three replicates of each crust type. A few small peaks (33–191 absorbance units) were present in only one of the three replicates for each crust type (one, one and four spurious peaks in the cyanobacterial, moss and lichen TRF profiles, respectively). Peak heights and peak areas were averaged across the three replicates, and provided similar measures of relative abundance (data not shown). The percent of the total profile calculated from individual peak height or peak area data was within 0.5%, 3.6% and 2.2% for cyanobacterial, lichen and moss crusts, respectively, and the largest deviations were between measurements of the largest peak in each profile. Peak height means and standard deviations for cyanobacteria, lichen and moss crust TRF profiles are presented in Table 1. In the lichen and moss crust profiles, small peaks representing full-length amplicons (423–425 and 436 bp, respectively) were observed (Table 1). Their presence indicates either sequences that lacked the enzyme recognition sites or an incomplete digestion reaction. A few cyanobacterial 16S rDNA sequences in the database do not have restriction sites for either of the enzymes used in this study. No full-length amplicons were detected in the digested cyanobacterial TRF crust profiles.

In an effort to infer taxonomic identity of the species represented by TRF peaks, fragment sizes were predicted for sequences obtained in our clone libraries. An excellent correlation between the fragments predicted from clone

library sequences and the fragment sizes observed in TRF profiles was observed (columns 2 and 3 in Table 1). All fragments predicted for the clone sequences are evident in the TRF analyses. Precision of TRF fragment sizing is generally within 3 bp of actual fragment length [18,40,41] and all TRF fragments from the crust profiles are either exact matches or are within 1–2 bp of the predicted fragment size. The relative abundance of individual TRFs as measured by peak height percent of total fluorescence was positively correlated with the frequency in our clone libraries (columns 5 and 6 in Table 1), up to the limit of resolution of our clone libraries of 34–36 members (frequency of 2.8–2.9%).

Of the three replicate profiles for each crust type, the profile with median peak heights for most/all of the peaks was designated the median profile. This profile is illustrated in Fig. 2 for each of the three crust types. The TRF profiles of the cyanobacterial and moss crusts were very similar (Fig. 2). For both profiles, an abundant fragment was found at 116 bp that comprised 69.8–76.7% of the total peak height fluorescence for the profiles (Table 1). This peak correlates well with the size and frequency predicted for the *M. vaginatus* phylotype, the sequence that dominated clone libraries from both samples. In the cyanobacterial crust profile, additional small peaks were detected at 103 and 105 bp, and these may correspond to clones related to *M. vaginatus*, *M. sociatus*, and DGGE band 11b (Fig. 1). The moss crust profile lacks the peak at 103 bp, but contains the 105-bp peak. In the moss rDNA clone library, one sequence was obtained that is predicted to give this size fragment, and is distantly related to *Nostoc* species sequences (moss2; Fig. 1). However, assigning identity to small peaks corresponding to low-frequency library clones should be regarded with caution.

As might be predicted from the clone library results, the TRF profile for the lichen crust sample is quite different from the profiles of cyanobacterial and moss crusts (Fig. 2). The 116-bp fragment peak is small, comprising only 4.7% of the total peak height fluorescence for the profile. Since only two *M. vaginatus*-related clones were obtained in the lichen crust library, this result can reasonably be explained by the relatively low frequency of rDNAs from this species in the lichen crust sample. In contrast, the dominant peak of this profile was at 105 bp, and comprises 42% of the total peak height percent of total fluorescence for the profile. Analysis of sequences obtained in the clone library suggest that this peak derives from rDNAs of multiple genera of cyanobacteria, including sequences most closely related to those of *Chroococcidiopsis, Nostoc, M. chthonoplastes* and *Leptolyngbya* species. Another distinct peak is present at 103 bp, comprising 14.3% of the amplified DNA, and may correspond to clones related to *Phormidium* and *Nostoc* species.

All three profiles contain small peaks of sizes 173, 175 and 186 bp. Clones were obtained at low frequency in the libraries whose sequences, in some cases, were predicted to produce peaks of these sizes. The 173-bp peak in the lichen TRF profile may be a *Leptolyngbya* species, and the 175-bp peak in the moss profile may derive from moss chloroplast rDNAs. However, in other cases, no clones were obtained that were predicted to yield fragments of these sizes in their respective libraries, and the source of these peaks is unknown.

Our analysis shows that the phylogenetic specificity of cyanobacterial TRF peaks varies. Although more readily applicable to large-scale comparative studies, TRF analysis is inherently lower in resolution than is sequencing analysis, and therefore underestimates species diversity. Our study demonstrates that the extent to which such diversity is underestimated varies with community composition, but can be usefully examined with limited parallel sequencing analysis. Despite the relatively small size of the clone libraries analyzed (approx. 35 clones per sample), excellent correlation was obtained between predicted and observed TRF profiles, indicating that even small sequencing efforts can be very helpful in interpretation of TRF peaks. This comparative analysis of cyanobacteria distribution in three arid soil crust types has yielded important baseline information about species diversity and composition, and has provided insight on taxonomic interpretation of TRF profiles for the cyanobacteria. This information will allow us to go forward with comparative, landscape-scale studies of the effects of soil disturbance and climate change on soil crust microbial communities and crust productivity on the Colorado Plateau.

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