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Comparison of Crenarchaeal Consortia Inhabiting the Rhizosphere of Diverse Terrestrial Plants with Those in Bulk Soil in Native Environments

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To explore whether the crenarchaeal consortium found in the rhizosphere is distinct from the assemblage of crenarchaeotes inhabiting bulk soil, PCR–single-stranded-conformation polymorphism (PCR-SSCP) profiles were generated for 76 plant samples collected from native environments. Divergent terrestrial plant groups including bryophytes (mosses), lycopods (club mosses), pteridophytes (ferns), gymnosperms (conifers), and angiosperms (seed plants) were collected for this study. Statistical analysis revealed significant differences between rhizosphere and bulk soil PCR-SSCP profiles (Hotelling paired T^2 test, $P < 0.0001$), suggesting that a distinct crenarchaeal consortium is associated with plants. In general, phylotype richness increased in the rhizosphere compared to the corresponding bulk soil, although the range of this increase was variable. Examples of a major change in rhizosphere (versus bulk soil) PCR-SSCP profiles were detected for all plant groups, suggesting that crenarchaeotes form associations with phylogenetically diverse plants in native environments. In addition, examples of minor to no detectable difference were found for all terrestrial plant groups, suggesting that crenarchaeal associations with plants are mediated by environmental conditions.

The rhizosphere effect is described as a characteristic increase in abundance and a change in the distribution of the microorganisms associated with terrestrial plant roots in comparison with the surrounding bulk soil (6). Exploring this dynamic soil habitat by culture-independent techniques has revealed that the majority of microorganisms inhabiting terrestrial roots have not been cultured (5, 8). Recent studies suggest that members of the archaeal division *Crenarchaeota*, including clades C1a, C1b, and C1c (3, 4), colonize the rhizosphere. Previous work by our laboratory has shown that crenarchaeotes colonize tomato roots in growth chamber experiments and at a Wisconsin field site (9); phylogenetically stained cells were visualized on the rhizoplane by fluorescent in situ hybridization of crenarchaeal probes, followed by epifluorescence microscopy. The resulting cell counts revealed that crenarchaeotes are present throughout the tomato root system, with higher numbers (up to 10-fold) on senescent roots. The authors also produced washed tomato root 16S rRNA gene (rDNA) clone libraries from which only C1b sequences were recovered. In a second Wisconsin study, Chelius et al. recovered C1a sequences associated with washed roots of field-grown maize (2). These rDNA sequences grouped closely with crenarchaeal sequences recovered from marine environments. A third study found C1c sequences in microcosms containing mycorrhizal pine seedlings planted in Finnish pine forest humus (1); comparison of their clone libraries by amplified rDNA restriction analysis (ARDRA) led these authors to suggest that crenarchaeal diversity in the mycorrhizosphere is greater than that in bulk forest humus. In the mycorrhizosphere, they found five ARDRA patterns from 173 clones screened versus two ARDRA patterns in bulk forest humus from 84 clones screened. These studies suggest that crenarchaeotes associate with a variety of terrestrial plant root systems.

In this study, we sampled a broad range of phylogenetically diverse terrestrial plants growing in native environments, including representatives of the mosses, club mosses, ferns, conifers, and seed plants (including both monocotyledonous and dicotyledonous plants). To determine if the rhizosphere crenarchaeal C1b consortium associated with these plants is significantly different from that of the corresponding bulk soil samples, we generated PCR–single-stranded-conformation polymorphism (PCR-SSCP) profiles for each sample. For statistical testing, these data were converted into numerical matrices containing two components of diversity, richness (matrix columns represented unique phylotypes) and evenness (matrix rows contained relative abundance of each phylotype).

Terrestrial plant roots were collected from 12 sampling locations in Wisconsin (Table 1) chosen at locations harboring divergent flora. Hancock samples were collected from the University of Wisconsin Hancock Agricultural Research Station in mixed pine and oak stands surrounding agricultural fields. Stone's Pocket Road is located in the Baraboo Hills near the town of Baraboo, Wis. Simpson Road is located near Mirror Lake, Wis. Both of these locations are forested sites containing predominately oak with interspersed pine trees. Picnic Point samples were collected from a forested site located in the University of Wisconsin Campus Natural Areas. These sampling plots were located near a predominately maple wooded area with interspersed turf fields. The soil classification at each sampling plot is listed in Table 1. The soils at these locations had a low clay content and provided an easy medium from which to remove roots without copious amounts of adhering soil.

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TABLE 1. Rhizosphere sampling plots

Sampling plot[a]	Location	Soil series (classification)	Plant groups sampled[c]	No. of samples[b]	UTM[d] (zone 16) coordinates	Collection date
A	Hancock	Plainfield sand (Typic Udipsamment)	2b, 3d, 1g	6	4,888,042.3 296,967.1	10/11/01
B	Stone's Pocket Rd.	Baraboo silt loam (Typic Hapludalf)	1p, 1d, 1m	3	4,807,494.2 273,380.4	10/19/01
C	Simpson Rd.	Marshan loam (Typic Haplaquoll)	1b, 1l, 1p, 1g, 1d, 1m	6	4,829,635.3 265,720.1	10/19/01
D	Hancock	Plainfield sand (Typic Udipsamment)	2g, 2d, 2m	6	4,888,134.2 296,995.3	11/15/01
E	Hancock	Plainfield sand (Typic Udipsamment)	2b, 2g, 2d, 2m	8	4,888,174.1 296,990.6	11/16/01
F	Hancock	Plainfield sand (Typic Udipsamment)	1b, 2g, 2d, 2m	7	4,888,167.1 297,023.5	11/16/01
G	Picnic Point	Kidder silt loam (Typic Hapludalf)	4d, 4m	8	4,773,268.2 302,360.0	11/16/01
H	Picnic Point	St. Charles silt loam (Typic Hapludalf)	2b, 3d	5	4,773,223.5 302,369.4	11/20/01
I	Stone's Pocket Rd.	Baraboo silt loam (Typic Hapludalf)	1b, 1l, 1p, 1g, 1d, 1m	6	4,807,494.3 273,378.4	11/21/01
J	Stone's Pocket Rd.	Baraboo silt loam (Typic Hapludalf)	1b, 2l, 1p, 1g, 1d, 1m	7	4,807,573.2 273,414.0	11/21/01
K	Simpson Rd.	Boone sands (Typic Quartzipsamment)	2l, 1p, 1g, 1d, 1m	6	4,830,035.3 265,767.1	11/26/01
L	Stone's Pocket Rd.	Baraboo stoney silt loam (Typic Hapludalf)	2b, 1l, 2p, 1g, 1d, 1m	8	4,807,479.2 273,395.4	11/28/01

[a] Nonoverlapping sampling plots were no larger than 50 m^2.
[b] Number of paired samples (rhizosphere and corresponding bulk soil). Total, 76.
[c] b, bryophyte (moss); l, lycopod (club moss); p, pteridophyte (fern); g, gymnosperm (conifer); d, dicotyledonous (dicot); m, monocotyledonous (monocot).
[d] UTM, Universal Transverse Mercator.

To minimize variability introduced by the spatial heterogeneity of crenarchaeal assemblages in soil (10), we collected paired rhizosphere and bulk soil samples. For each root system collected, a corresponding 2-g (fresh weight) bulk soil sample was collected from the center of the mixed soil disturbed by collection of the root specimen. This sampling strategy allowed the comparison of a PCR-SSCP rhizosphere profile to the profile of the soil immediately surrounding that root system. Digital photo vouchers were taken for each plant, followed by collection of the root system by loosening the surrounding soil with a shovel. Root material not attached to the collected plant stem was discarded. The root system was shaken to remove loosely adhering soil and placed into a plastic storage bag. Plants were identified to the genus level on the basis of vegetative morphology. Root samples included species within the following genera: *Lycopodium*, *Dryopteris*, *Polystichum*, *Pinus*, *Acer*, *Viola*, *Taraxacum*, *Ribes*, *Leonurus*, *Geum*, *Pyrola*, *Carex*, and *Poa*. The paired rhizosphere and bulk soil samples were frozen immediately in liquid nitrogen and stored at −80°C in the laboratory until processing.

The distribution of plant groups at each sampling plot is described in Table 1. Woody perennials were collected as seedlings, and herbaceous plants were collected over a range of developmental stages. Rhizomes and corms were excluded from the analysis. Mosses, representing the most primitive terrestrial plants sampled, were included in this study even though they do not produce a typical root system. The moss rhizoid does not play a primary role in water and mineral absorption, but it does anchor plantlets to a substrate and is capable of adsorption. Moss associations with microorganisms have been documented (7), suggesting that even this primitive plant is capable of interactions with microorganisms. Prior to DNA extraction, only the moss samples were rinsed with sterile, MilliQ-purified water to further remove adhering soil. In this report, we use the term moss rhizosphere to indicate samples of the entire moss plantlet, including both rhizoid and leafy gametophyte portions. For the other rhizosphere samples, only below-ground root segments, cut into <1-cm fragments, were used for DNA extraction. This included possible endophytes, rhizoplane microorganisms, and any organisms within soil particles remaining attached to the root after specimen collection.

Approximately 0.1 g of each root or bulk soil sample was used for DNA extraction. Nucleic acids were extracted and purified as described previously (10). Briefly, root or soil samples were mixed with 1 ml of TEND (50 mM Tris, 50 mM EDTA, 100 mM NaCl, 1× Denhardt's reagent) and zirconium-silica homogenization beads. Samples were sonicated for 1 min in a bath sonicator and then processed in a Bio 101 Fast Prep bead beater for 30 s at 5.5 m/s. DNA was purified by silica binding, followed by Sepharose CL-2B spin column chromatography (two rounds of purification were performed in succession). PCR-SSCP was conducted as described previously (10). Crenarchaeote-biased primers 133FN6F and 248R5P were used for PCR-SSCP to produce a profile of the most abundant phylotypes present in rhizosphere and bulk soil samples. Every extracted DNA sample (152 samples) yielded amplified PCR products of the expected size detected by agarose electrophoresis.

Each PCR-SSCP electropherogram is a representation of the crenarchaeal relative diversity present in a sample in terms of richness (number of unique PCR-SSCP peaks) and evenness (relative peak area within an electropherogram). A numerical matrix was generated with these data for the rhizosphere (rmatrix) and bulk soil (smatrix) samples. The matrix columns represented each unique phylotype found in both rhizosphere and bulk soil electropherograms. The rows represented each extracted DNA sample (a total of 76 rmatrix and smatrix samples). Values within a matrix corresponded to the relative abundance of each phylotype within a sample. To quantify the difference between bulk soil and rhizosphere relative diversity, the rmatrix was subtracted from the smatrix. This produced a single multivariate data set (dmatrix) containing the calculated difference between paired samples. That is, the dmatrix contained the bulk soil relative abundance values minus the rhizosphere relative abundance values for every phylotype. A calculated difference of zero represents identical paired samples; the rhizosphere electropherogram is identical to the bulk soil electropherogram. Quantification of the PCR-SSCP pro-

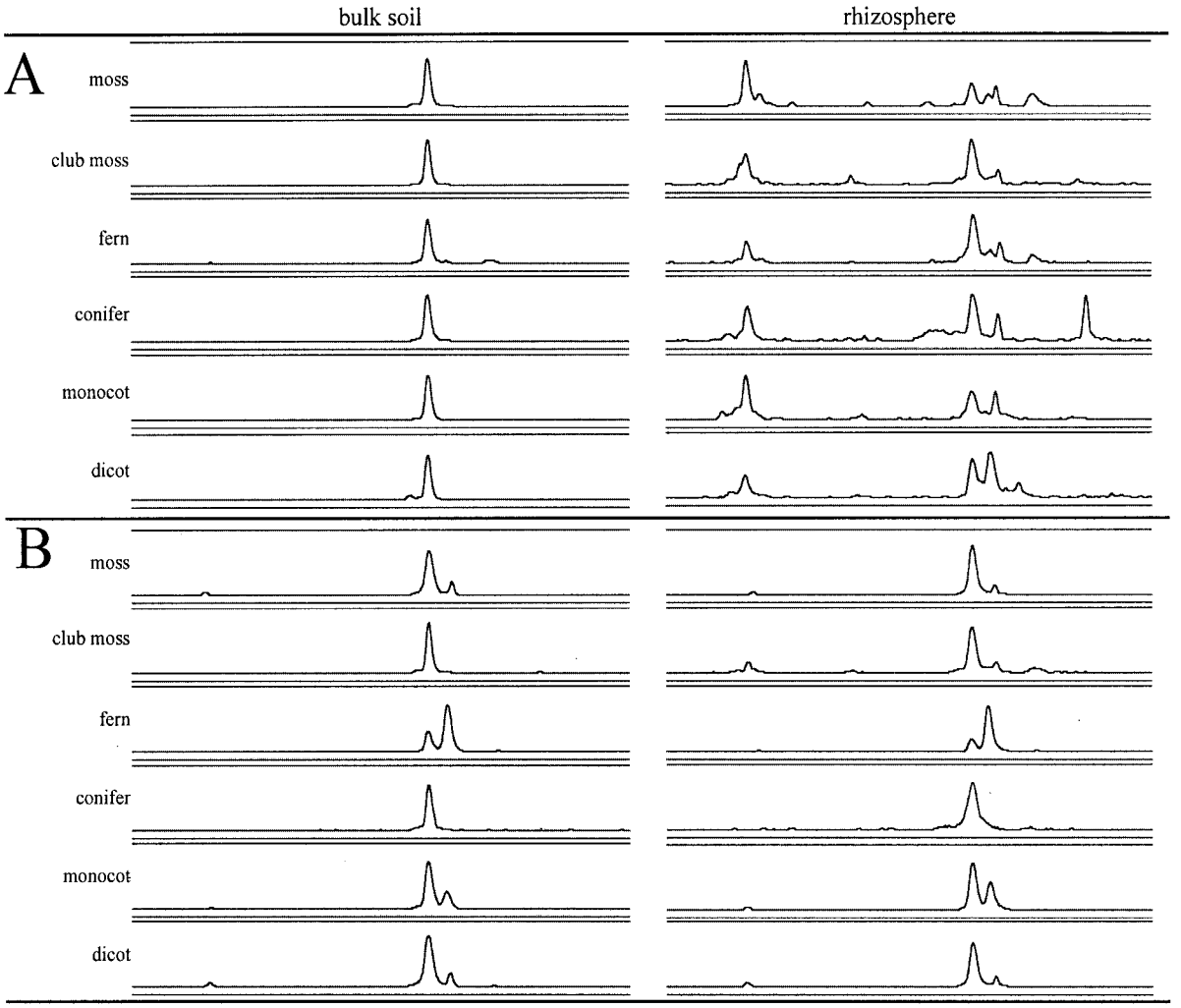

FIG. 1. PCR-SSCP electropherograms representing the range of bulk soil to rhizosphere comparisons found in this study. Paired samples exhibiting major differences in relative diversity were found for all of the terrestrial plant groups sampled (A), and paired samples exhibiting very similar PCR-SSCP profiles were also found for all of the plant groups sampled (B). The *x* and *y* axes of each electropherogram represent relative migration distance and relative fluorescence intensity, respectively.

files in this manner produced a multivariate data set suitable for statistical testing.

A Hotelling paired T^2 test, conducted in R (version 1.6; The R Development Core Team [http://cran.r-project.org/]), provided evidence for a significant difference between rhizosphere and bulk soil samples (H_o: dmatrix = 0, $P < 0.0001$). The nature of this difference was examined by comparing the range of PCR-SSCP profiles for each plant group (Fig. 1). It was found that all plant groups produced examples of a major difference in rhizosphere relative diversity compared to the associated bulk soil. Moss samples (no true roots) exhibited differences of the same order of magnitude as the other plant groups, suggesting that moss associations with crenarchaeotes are independent of root exudates. The range of profiles also included examples of little to no detectable difference for all plant groups. The lycopod samples always produced at least a small difference between root-associated relative diversity and bulk soil relative diversity, although this may be a result of the low sample number. Only six lycopod rhizosphere samples were examined, which may not encompass the full range of associations present in natural environments. In general, there was no trend suggesting that crenarchaeal relative diversity is specific to plant phylogenetic lineage.

The crenarchaeal phylotypes detected in this study were not obligate rhizosphere colonizers. They could be found as dominant members of either soil or rhizosphere samples (data not shown). Even though a specific plant-phylotype interaction was not detected, the plant samples exhibiting a distinct crenarchaeal consortium were typified by an increase in richness beyond the number of phylotypes detected in the corresponding bulk soil sample. The increased richness associated with plants may indicate a greater number of plant-associated microniches available for colonization by different phylotypes. That is, the rhizosphere may be capable of supporting a greater number of diverse phylotypes in close proximity that cannot coexist within the same space in bulk soil. This observation is

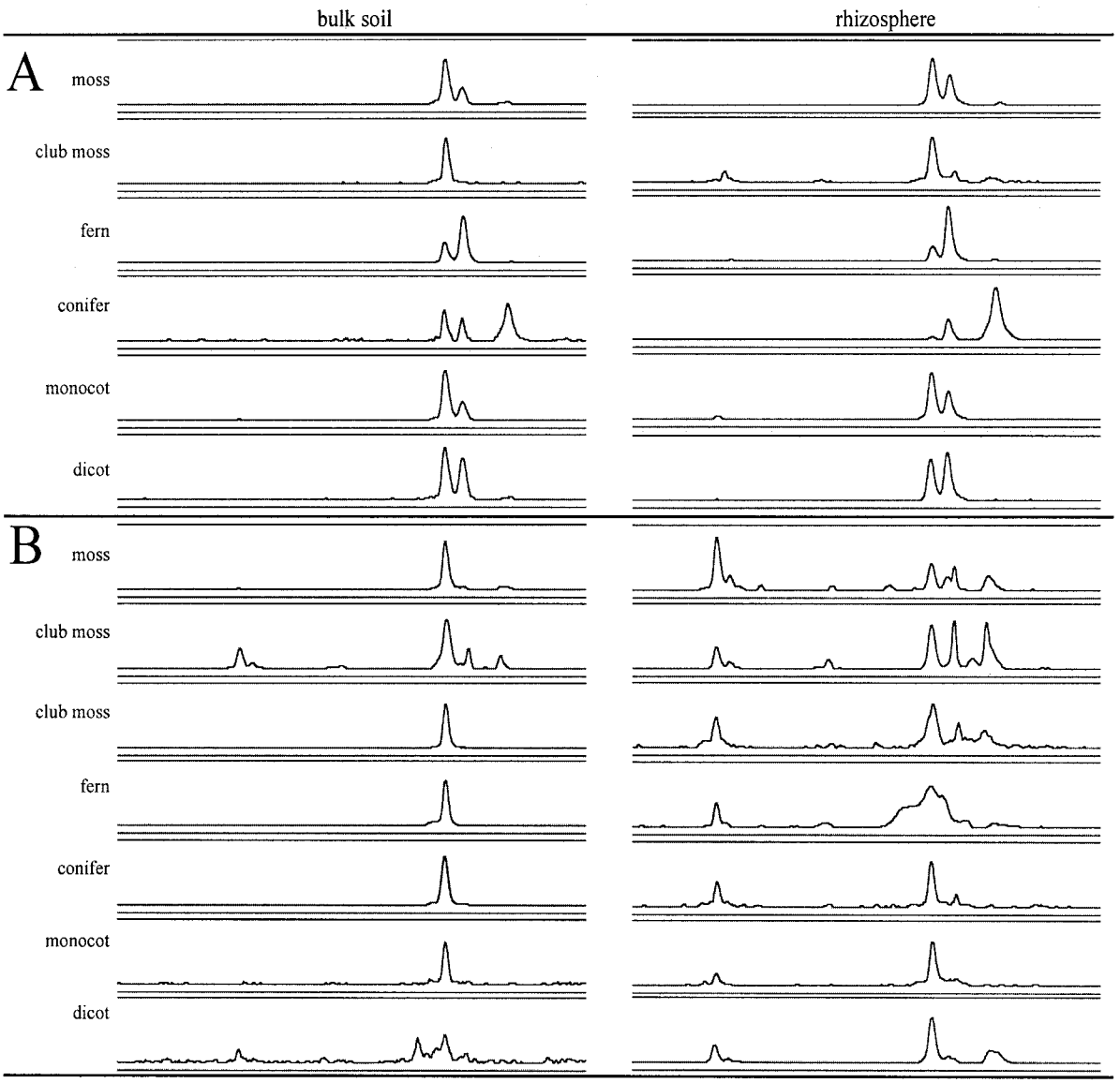

FIG. 2. PCR-SSCP electropherograms of bulk soil and rhizosphere samples collected at two sampling plots located by Stone's Pocket Road. Six paired samples were collected from sampling plot I (A), and seven paired samples were collected from sampling plot J (B). Plot I samples produced minor differences between the rhizosphere and the corresponding bulk soil. In contrast, plot J samples exhibited major differences. The *x* and *y* axes of each electropherogram represent relative migration distance and relative fluorescence intensity, respectively.

in agreement with the Bomberg et al. mycorrhizosphere results discussed previously.

A permutation test based on the total squared distance from group means provided strong evidence that the differences between the rhizosphere and bulk soil are more similar within sampling plots than between sampling plots ($P < 0.001$, 1,000× resampling, conducted in R). This suggests that environmental conditions specific to each sampling plot influence how the rhizosphere consortium differs from that of the bulk soil. The factors responsible for differentiating sampling plots were not apparent. This is exemplified by comparing electropherograms from plots I and J (Fig. 2). These two plots were in close spatial proximity. Both were located at the Stone's Pocket Road sampling location, and both contain the same soil classification (Table 1). The PCR-SSCP profiles generated from plot I samples exhibited minor differences between the rhizosphere and bulk soil (dmatrix variables close to zero). In contrast, the paired profiles generated from plot J samples exhibited greater differences (dmatrix variables farther from zero).

To quantify how the rhizosphere crenarchaeal relative diversity differs across sampling plots, principal-component analysis (PCA) was used to transform the dmatrix data into a reduced set of variables. PCA was conducted with the SAS procedure PRINCOMP (release 8.2; SAS Institute, Cary, N.C.). Ordination of principal component one (prin1) and prin2 provided a visual description of the dmatrix values at each sampling plot (Fig. 3). If the sampling plot had no effect on rhizosphere crenarchaeal relative diversity, the sampling

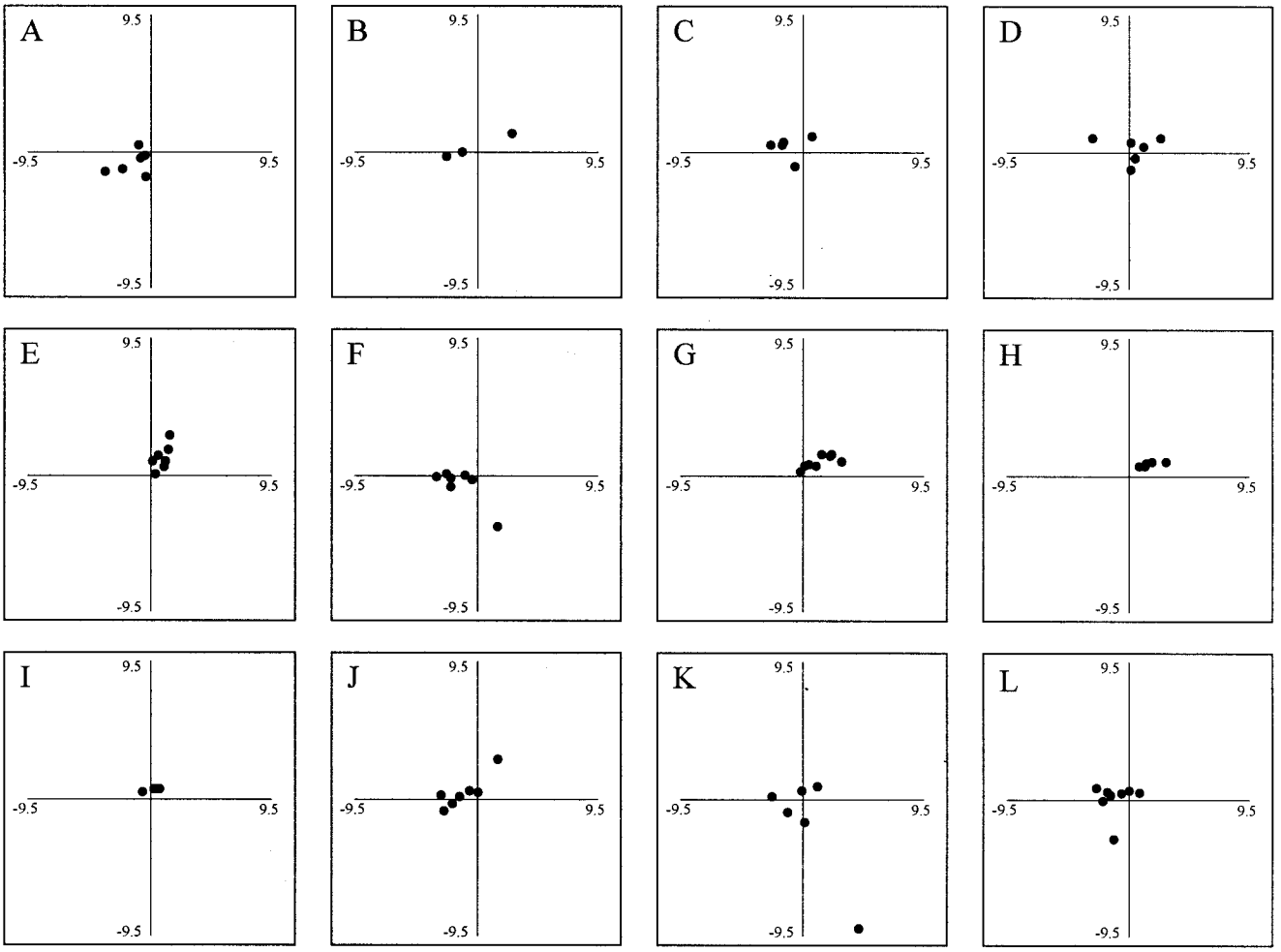

FIG. 3. PCA ordination of the difference between rhizosphere and bulk soil PCR-SSCP profiles (dmatrix) by sampling plot. Plot designations A to L are defined in Table 1. prin1 (17% variance explained) is plotted along the *x* axis, and prin2 (13% variance explained) is plotted along the *y* axis. The origin represents identical paired PCR-SSCP rhizosphere and bulk soil profiles (dmatrix = 0). Each data point represents a single paired rhizosphere and bulk soil sample ($n = 76$ across all plots).

plot PCA graphs would be indistinguishable. This was not the case, as the graphs revealed examples of plot-specific clustering. For instance, plot A samples form a distinct cluster different from E, G, and H. Also, samples collected at plots E, G, H, and I formed tighter clusters than did those from the other plots, indicating less variability among samples at these plots. In general, the clustering patterns were not correlated with spatial distance, sample collection time, or soil type. Both permutation testing and PCA suggest that external environmental factors influence crenarchaeal relative diversity in the rhizosphere, but the identity of these factors is not apparent.

In summary, we have found significant evidence that crenarchaeal rhizosphere PCR-SSCP profiles are distinct from corresponding bulk soil PCR-SSCP profiles, suggesting that crenarchaeal relative diversity in the rhizosphere is distinct from that in the surrounding bulk soil. This difference was typically manifested as an increase in rhizosphere richness compared to bulk soil richness and appeared to be plant lineage independent. Also, as-yet-uncharacterized sampling plot effects were found to significantly influence how the rhizosphere differed from bulk soil. Crenarchaeal associations with plants in native environments are not only the result of interactions between plant and microbe but are mediated by environmental conditions.

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