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Bacterial Community Composition in Brazilian Anthrosols and Adjacent Soils Characterized Using Culturing and Molecular Identification

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Abstract Microbial community composition was examined in two soil types, Anthrosols and adjacent soils, sampled from three locations in the Brazilian Amazon. The Anthrosols, also known as Amazonian dark earths, are highly fertile soils that are a legacy of pre-Columbian settlement. Both Anthrosols and adjacent soils are derived from the same parent material and subject to the same environmental conditions, including rainfall and temperature; however, the Anthrosols contain high levels of charcoal-like black carbon from which they derive their dark color. The Anthrosols typically have higher cation exchange capacity, higher pH, and higher phosphorus and calcium contents. We used culture media prepared from soil extracts to isolate bacteria unique to the two soil types and then sequenced their 16S rRNA genes to determine their phylogenetic placement. Higher numbers of culturable bacteria, by over two orders of magnitude at the deepest sampling depths, were counted in the Anthrosols. Sequences of bacteria isolated on soil extract media yielded five possible new bacterial families. Also, a higher number of families in the bacteria were represented by isolates from the deeper soil depths in the Anthrosols. Higher bacterial populations and a greater diversity of isolates were found in all of the Anthrosols, to a depth of up to 1 m, compared to adjacent soils located within 50–500 m of their associated Anthrosols. Compared to standard culture media, soil extract media revealed diverse soil microbial populations adapted to the unique biochemistry and physiological ecology of these Anthrosols.

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Introduction

Amazonian dark earths (ADE) contain organic amendments, such as charcoal, that were incorporated into them in pre-Columbian times. This has resulted in soils prized for their sustained fertility. The existence of these Anthrosols in close proximity to adjacent, nutrient-poor soils formed from the same parent materials provided an opportunity to study variables, other than their inherent mineralogy, that influence their bacterial diversity. Cloning and sequencing have revealed distinctly different bacterial communities in the Anthrosols compared to adjacent soils [22], but the factors controlling these differences and the unique roles that microbial communities may have in ADE are not well-understood [41].

The high levels of black carbon (BC) present in ADE have existed for several hundred to thousands of years since

their incorporation, in spite of intense weathering and conditions highly favorable for microbial decomposition [20]. Soil temperature, moisture, texture, carbon (C) content, nutrient availability, and pH, along with land use history, plant species, and seasonality are among the factors that have been shown to affect soil microbial community composition [6, 7, 17, 49]. ADE have higher concentrations of key nutrients, such as phosphorus (P), calcium (Ca), and soil organic C, but lower respiration rates (carbon dioxide evolution) compared to adjacent soils (Liang, Ph.D. thesis). Understanding the interaction of diverse microbial populations with the many dynamic biogeochemical factors in soil remains a great challenge [18, 21, 45, 49] and is central to modeling rates of nutrient turnover in soil ecosystems [2]. A key question for nutrient cycling in ADE is whether the pool of BC itself is cycling at a greatly reduced rate or if the presence of BC has altered nutrient and C cycling throughout the soil ecosystem. Furthermore, while soil horizon depth dictates the volume in which key microbial processes predominate [3], especially in tropical soils, the increased fertility at depth in the ADE may greatly expand the extent of microbial activity relevant to modeling nutrient dynamics.

To compare soil microbial communities in Anthrosols and adjacent soils, we used the differences in soil chemical composition as the selective agent to isolate bacteria. Bacterial culturing techniques can be tedious and reveal only a small proportion of extant organisms in soil, but they are an excellent method for examining the physiological and metabolic diversity of bacteria potentially active in soil ecosystems [23]. Combined with powerful molecular techniques, culturing methods work synergistically to capture and identify a distinct proportion of the microbial diversity in soil and more completely describe its community composition [24, 45, 48].

In this study, we examined bacterial population size and composition in paired tropical soil types: the Anthrosols created during pre-Columbian settlement and adjacent "unmodified" soils from three locations in the Brazilian Amazon. Both soil types at each location were derived from the same parent material and were subject to the same climate, but differed by the presence/absence of BC and the associated high P and Ca contents in the Anthrosols. We cultured and isolated bacteria on a minimal medium and on soil extract media prepared from each soil sample and used colony polymerase chain reaction (PCR) [34] to amplify 16S rRNA genes from each isolate [42]. Using restriction fragment length polymorphisms (RFLP) and direct sequencing of 16S rRNA, we identified and compared the dominant, culturable bacteria residing in each soil type at each site at multiple soil depths. We hypothesized that the Anthrosols would contain higher numbers of culturable bacteria in multiple soil horizons and a greater diversity of bacteria and that the culturable bacterial communities would be more similar among the Anthrosols than between the Anthrosols and the adjacent soils sampled from the same site. In comparison with adjacent soils, a better understanding of the size and diversity of the bacterial population in ADE should inform future inquiry into the physiological ecology of BC-rich soils.

Materials and Methods

Site Descriptions and Soil Sampling

Soils were collected from three sites within the Amazon Basin each located within 50 km of the city of Manaus, Brazil (3°08′ S, 59°52′ W, 4–50 m above sea level). The natural vegetation is tropical lowland rain forest with a mean annual rainfall from 2,000 to 2,400 mm [37]. The sampled soils were Anthrosols [29] and their adjacent, naturally occurring, nutrient-poor soils, which are derived from tertiary sediments, primarily laterite. Three sites were sampled in this study: Hatahara (Hat), Lago Grande (LG), and Açutuba (Acu). Horizon classification in the Anthrosols was determined by factors such as density of pottery sherds, indicative of human settlement. Sampled Anthrosols and adjacent soils were 50–500 m apart at each site and were formed from the same parent material. Anthrosols were sampled from existing archeological excavations by taking multiple auger cores within each soil horizon and compositing the cores. The unexcavated adjacent soils at each site were sampled by use of a soil auger and separated by soil horizons. Multiple cores from each horizon were mixed together. Approximately 1.0 kg total sample weight was taken from each horizon. Samples were stored in sealed plastic bags at room temperature for 1 week and then at 4°C for 12 weeks prior to culturing. Soil characteristics of paired A horizons at each location are presented in Table 1.

Media Preparation and Culturing

Soil extracts were prepared from each soil type, depth, and site used in the culturing experiments. These extracts were used to prepare agar-based media according to Burlage et al. [8]. To one part of each soil sample, two parts of double-distilled water were added (*w*/*v*) in milk dilution bottles. Soil slurries were sterilized for 1 h and then allowed to settle and cool to room temperature. Once cooled, the liquid soil extracts were poured off and frozen at −20°C. The soil extracts were then thawed and vacuum-filtered through a 25-μm Whatman® no. 41 filter paper. For each liter of soil extract agar (SEA), 50 mL of soil extract were added to 950 mL distilled water. Bacto Agar (1.8%, BD Difco™, Franklin Lakes, NJ, USA) was added and the medium was sterilized at 121°C for 20 min. Liquid soil extract media were prepared in the same manner and used to analyze

Table 1 Chemical and physical characteristics of paired A horizons for Anthrosols and adjacent soils

Site	Type	Land feature	Age (years)	pH (1:2.5 H_2O)	Sand (%)	Silt (%)	Clay (%)	Organic C (mg g^{-1})	N[a] (mg g^{-1})	P[a] (mg g^{-1})	Ca[a] (mg g^{-1})	C/N	CEC ($mmol_{Charge}$ kg^{-1})	Graphitic BC[b] (%)
Hat	Anth	Under pasture dense pot shards	600–1,000	6.4	51.3	21.7	27.0	22.0	1.0	9.1	17.6	23	211.3	5.82
	Adj	Under early secondary forest, deep orange		4.6	60.4	3.8	35.9	21.8	1.6	0.3	0.1	14	88.4	2.87
LG	Anth	Under secondary forest few pot shards	900–1,100	5.9	47.9	29.6	22.6	31.5	1.8	5.0	6.4	18	222.4	2.32
	Adj	Under secondary forest few concretions		4.2	69.4	3.9	26.7	17.5	1.3	0.3	0.1	14	59.2	1.88
Acu	Anth	Near ag. field dark w/dense pot shards	2,000–2,300	5.6	81.9	7.7	10.4	15.7	1.0	0.8	0.3	16	56.3	5.71
	Adj	Under secondary forest yellow latosol-like		4.7	87.9	3.6	8.5	15.4	0.8	0.2	0.1	20	52.0	2.41

Abbreviated from Liang et al. [26]

[a] Total N, P, and Ca

[b] Percent graphitic BC out of total organic C, determined by thermal oxidation

ammonium N (NH_4^+–N) and nitrate (NO_3^-–N) contents on a continuous flow autoanalyzer (SEAL Analytical, Mequon, WI, USA). Elemental compositions of the soil extract media were determined by use of inductively coupled plasma spectroscopy (ICP, Spectro Ciros CCD, Kleve, Germany) at the Cornell Nutrient Analysis Laboratory in Ithaca, NY, USA. The pH of the extracts was measured for all soil samples at the diluted concentrations used for culturing. Solid R2A was prepared using BD Difco™ (Franklin Lakes, NJ, USA) dehydrated R2A medium (product no. 218261). A modified liquid medium (R2L) was prepared according to Fries et al. [19]. Sterile, molten R2A and SEA were poured into gridded, 150-mm diameter Petri plates. Aliquots of 10 mL of R2L and SEL media (five replicates for each of five dilutions per sample) were added to 50 mL screw-top tubes, capped, and autoclaved for 15 min at 121°C. Soil inocula for culturing were prepared using 10 g soil, extracted for 10 min at room temperature with gentle agitation in 90 mL sterile 0.1 M sodium phosphate buffer, pH 6.8. Five, tenfold, serial dilutions from each soil sampled were prepared. Aliquots from each dilution were used to inoculate: (1) Petri plates containing SEA, (2) Petri plates containing R2A, (3) five replicate tubes of R2L, and (4) five replicate tubes of SEL for most probable number (MPN) estimations. Inoculated liquid and solid media were incubated in the dark at 30°C. For liquid cultures, growth (turbidity) was recorded biweekly for 120 days, although no additional positive tubes were observed after 42 days. MPN estimates were made using the MPNES software [46]. Colonies formed on R2A and SEA were counted daily over the first week and biweekly thereafter, with any new colonies denoted by color-coding on the plate. During 4.5 months of incubation (approximately 135 days), approximately 20 colonies arising on SEA and R2A were selected from plates at multiple dilutions for each sample (generally three plates out of five dilutions contained colonies at a spacing suitable for single-colony isolation). Colony selection was based on morphological differences and the week in which the colony first appeared after plating, with preference given to slower-forming colonies. Selected colonies were subcultured on the same medium on which they were initially cultured and on the alternate medium (i.e., colonies arising on SEA were tested for growth on R2A and those arising on R2A were tested for the ability to grow on SEA for each soil extract) and growth noted after incubating for up to 120 days at 30°C in the dark.

PCR-Restriction Fragment Length Polymorphism Analysis

Cells from purified colony isolates (verified by microscopy) were used directly for DNA amplification by PCR using the forward primer 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 1492r (5′-GGT TAC CTT GTT

ACG ACT T-3′) (Integrated DNA Technologies, Coralville, IA, USA), which target the bacterial 16S rRNA genes [28]. Isolate DNA was amplified using a PTC 100 thermal cycler (MJ Research, Waltham, MA, USA) and the following program: 5 min at 94°C, followed by 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. Final reaction concentrations were: 0.05 U μL^{-1} *Taq* polymerase (Promega, Madison, WI, USA), 1.0x PCR buffer supplied with the enzyme, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 μg μL^{-1} bovine serum albumin (BSA), both primers at 0.1 μM, and nuclease-free water (Promega). PCR products were verified by running them on a 0.7% agarose gels, staining the gel with SYBR Green I (Cambrex Biosciences, East Rutherford, NJ, USA) and visualizing the products using a Fluor-S™ MultiImager (Bio-Rad, Hercules, CA, USA). Isolate amplicons (~1,450 bp) were then digested in 20 μL reaction volumes using the restriction enzyme *Msp*I (Promega) at 10 U μL^{-1} with 1.0x buffer (supplied with the enzyme), 10 μg μL^{-1} BSA, and nuclease-free water. Restriction digests were carried out in a PTC 100 thermal cycler (MJ Research) held at 37°C for 4.5 h with a final step at 70°C for 15 min to stop the reaction as specified by the manufacturer. Restriction fragments were separated in 2% agarose gels. Gels were stained with ethidium bromide and the RFLP patterns visualized using the Fluor-S™ Multi-Imager (Bio-Rad). For bacterial isolate RFLP fingerprints yielding the same or similar banding patterns using the *Msp*I restriction enzyme, additional aliquots of the 16S rDNA amplicons were digested using the restriction enzymes *Rsa*I and *Hha*I (Promega), according to the manufacturer's protocols, and the resulting RFLP fingerprints digitized as described above.

Sequencing

After ribotype screening, 16S rDNA amplicons of interest were cleaned prior to sequencing using ExoSAP-IT® (USB, Cleveland, OH, USA). Reactions were carried out in a PTC 100 thermal cycler (MJ Research) held at 37°C for 15 min followed by 80°C for 15 min to inactivate the reaction. All sequencing reactions were performed at the Cornell Biotechnology Resource Center in Ithaca, NY, USA, using an Automated 3730X1 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were deposited in the GenBank and assigned the accession numbers EU571115–EU571206.

Data Analysis

A chimera check on the aligned sequences was run using the Ribosomal Database Program II, release 8.1 [10]. Cluster analysis of RFLP fingerprints was carried out with the unweighted pair group method with arithmetic linkage method using band presence/absence in the BioNumerics software (Applied Maths©, Sint-Martens-Latem, Belgium). Resulting binary data were further analyzed using JMP 5.1 (SAS Institute, Cary, NC, USA). Sequence alignment as well as phylogenetic and molecular evolutionary analyses were conducted with MEGA version 3.1 [39], using the Jukes–Cantor neighbor-joining algorithm. Distance matrices were constructed by the DNADIST program in PHYLIP [14]. Relatedness of clone libraries between paired soils and medium types were compared using ∫-LIBSHUFF [35], and rarefaction curves, diversity indices, and lineage through time were evaluated using DOTUR [35].

Results

Soil Physical and Chemical Analyses

Paired Anthrosol and adjacent soil samples from each site differed markedly in soil color, with Anthrosols having a dark, blackish color down to 1 m or deeper, which is due primarily to the presence of BC. Graphitic BC is a portion of total BC (Table 1) and, while it does not serve as a proxy for the highly heterogeneous BC, it is elevated in the Anthrosols [26]. Adjacent soils had a yellowish to deep orange color with a slightly darker surface O horizon. Paired samples listed in Table 1 were chosen based on visual observation of soil horizons, including factors such as rooting depth and bioturbation. Adjacent soils at Hat and LG had a higher percentage of sand and clay than the Anthrosols, which contained from two to over six times more silt than the adjacent soils. Anthrosols generally had a higher pH, higher cation exchange capacity (CEC), and considerably higher total P and Ca contents (the latter to a lesser extent in Acu). Organic C and total N were similar between the paired soil samples from each site. At the Hat and LG sites, C/N ratios in the Anthrosols were significantly higher than those of the adjacent soils (Table 1).

MPN in SEL and R2L

All of the Anthrosols cultured in both SEL and R2L media had MPN estimates within the same order of magnitude, regardless of site or sampling depth, except for the lowest horizons at the Hat and Acu sites, which were below the estimated range of surface soil populations (Fig. 1). Adjacent soils had population estimates within the same order of magnitude as their paired Anthrosols in only three of the seven paired samples (Hat, 0–30 and 40–70 cm; LG, 0–10 cm). At all sites, the estimated number of culturable organisms declined considerably faster with depth in the adjacent soils than in the Anthrosols. In the adjacent soils at

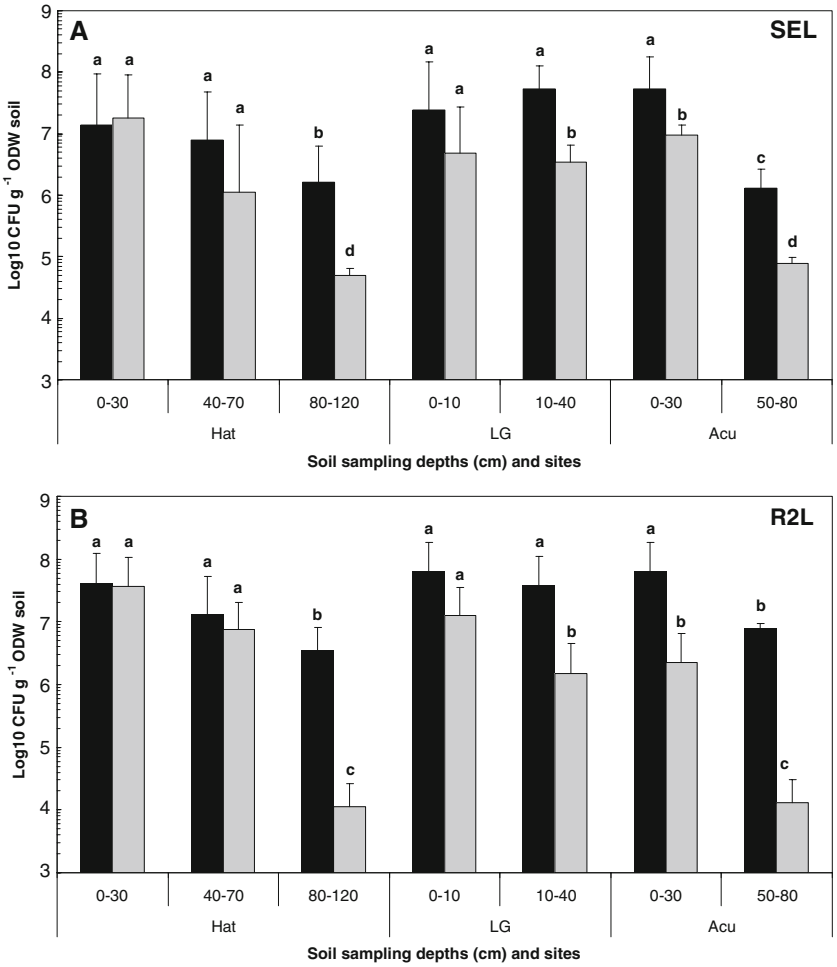


Figure 1 MPN (log_{10}) of bacteria CFU per gram ODW soil by site, depth, and soil (*black bar* Anthrosol, *gray bar* adjacent soil) in **a** SEL and **b** R2L. MPNs were calculated using the MPNES software [46]. *Letters over each bar* indicate standard errors (95% confidence interval) within the same or different range

the Hat and Acu sites, population estimates for the deepest depths were more than two orders of magnitude lower than surface populations.

Selective Medium Nutrients

The pH of soil extract media ranged from 7.02 to 7.36 (data not shown) for all samples and was not considered a major variable in these experiments. The ion concentration of salts in the commercial R2A medium was lower than most elements extracted from the soil samples used to make the soil extract media (Fig. 2). Exceptions to this were concentrations of potassium (K) and P in adjacent soil extracts. Nitrogen (N) concentrations were lower in SEA compared to R2A (from the contribution of N salts alone) by an average of 24.7%, with SEA from the Anthrosols generally more N-deficient than SEA from adjacent soils. Nitrate nitrogen concentrations were lower in all SEA media by more than an order of magnitude compared to NO_3^-–N provided in the salts added to R2A. Between the paired soil extracts, the Anthrosols generally had lower NH_4^+–N and NO_3^-–N concentrations than the adjacent soils; however, N concentrations in adjacent soil samples decreased more rapidly with soil depth.

Isolate Growth and Screening

Nearly all bacterial colonies formed on R2A plates developed during the first week of incubation for all sites, soil types, and depths, while on SEA, colonies continued to form over weeks to months (Fig. 3). Appearance of new isolate colonies by soil type, depth, site, and medium type and all interactions across time was fastest on R2A in week 1 for the Anthrosols sampled at shallow depths ($p=0.0086$) and on R2A for adjacent soil surface and subsurface samples ($p=0.0086$ and $p=0.0071$, respectively). Colony development on SEA from adjacent soils occurred over a longer period, with a greater proportion of colonies forming 2 weeks or more after inoculation. As with the MPN from liquid cultures, total colony-forming units (CFU) per gram of soil dropped off faster at deeper depths in the adjacent soils than in the Anthrosols on both R2A and SEA (data not shown). All bacterial isolates grouped into 86 different

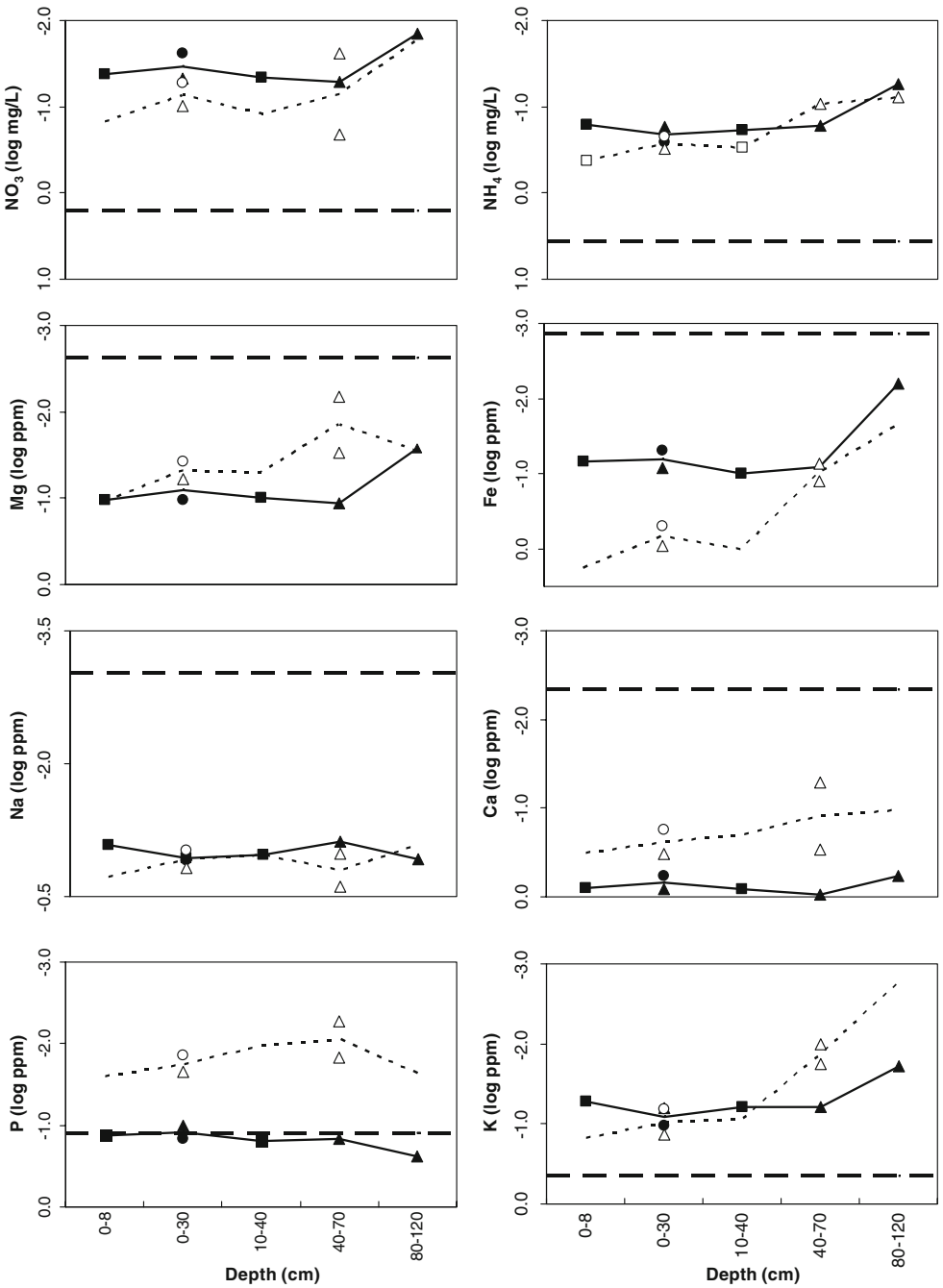


Figure 2 Comparison of nutrient concentrations in plating media. *Bold dashed lines* represent concentrations in R2A medium. Depths (in centimeters) correspond to different sampling depths as in Fig. 1 (*squares* from site LG, *circles* from Acu, *triangles* from Hat). *Open symbols with dotted lines* are from adjacent soils and *filled symbols with solid lines* are from Anthrosols

ribotypes from which at least one representative was selected for sequencing.

Isolate Sequencing

The Anthrosol sequences were broadly distributed across many taxa of the bacteria, while over 80% of the sequences recovered from adjacent soil isolates grouped to *Bacillus* or *Paenibacillus* (Fig. 4a). Comparison of isolates selected by use of the different media showed that, a broader distribution of bacteria, based on ribotype, was recovered on SEA, while most isolates (65%) taken from R2A plates were *Bacillus* species.

Rarefaction curves for the 16S rRNA sequences recovered showed the Anthrosols to have a significantly higher number of unique species among the 30 isolates sampled for each site soil compared to the adjacent soils (Fig. 4b) ($p=0.05$). No such divergence was observed when the number of unique sequences recovered from R2A and SEA media were compared. Comparison of these libraries based on soil and medium type was carried out using ∫-LIBSHUFF to measure genetic distance in library coverage. In this analysis, a

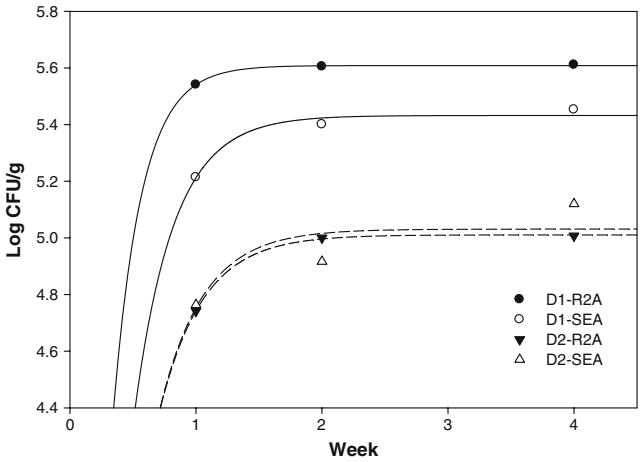


Figure 3 Growth curves based on colony appearance during incubation at 30°C on R2A and SEA grouped into weekly observations, with means across three sites for upper (*D1*) and lower (*D2*) soil depths fitted with exponential growth curves

library, C_X, is analyzed for singleton sequences across the evolutionary distance contained within the 16S rRNA gene and the change in coverage, C_{XY}, is compared to a paired library. The reciprocal comparison is then made using the other library, C_Y, and the coverage change C_{YX}. p values derived by comparing the libraries from Anthrosol isolates and adjacent soil isolates were significantly different across the same genetic distance, showing distinct compositions of isolates from each soil (Fig. 4c). In comparing isolates recovered using different media, the low p value for C_{YX} (0.0158) and higher p value for C_{XY} (0.2554) indicated that the samples from R2A were a subset of those isolated on SEA [36], in spite of the latter having fewer isolates. Finally, in lineage through time plots (Fig. 4d), phylogenies as a function of time were analyzed to compare divergent lineages between the libraries [4]. The greater concavity observed in the lineage from adjacent soil isolates indicated an abundance of closely related species, as suggested by comparing the libraries derived from adjacent soils cultured on the R2A medium. A comparatively greater convex curve, as observed for isolates recovered from SEA and from the Anthrosols, indicated a higher degree of divergent lineages.

Pairwise comparisons of isolates based on soil and medium type showed that libraries derived from the Anthrosols did not differ from each other whether they were cultured on SEA or R2A, while adjacent soil libraries differed significantly from each other based on medium type (Table 2). Richness estimates calculated using Chao1 indicated that the isolates from SEA prepared from adjacent soil had significantly lower richness, but taken with SEA isolates from the Anthrosols, soil extract media recovered a broader range of bacterial families than did the use of R2A to culture isolates from either soil (Table 2).

Sequencing and RFLP results suggest that, a broader range of taxa, identified to the family level using RDP II Classifier [10], was cultured on Anthrosol SEA than on SEA from adjacent soils. Sequences obtained from unique RFLP types showed 19 bacterial families among the isolates examined, and of these, 15 were represented in isolates from the Anthrosols compared to only eight families represented in the isolates from the adjacent soils, with four families occurring in both soil types. Furthermore, of the isolates initially cultured on SEA, representatives of 16 families were observed in 49 sequenced 16S rRNA gene amplicons from Anthrosols and only ten families were represented within the 48 sequenced isolates taken initially from adjacent soil SEA. There was also a clear pattern in the types of organisms predominating in each soil type. All 14 sequences that clustered with the *Actinobacteria* came from Anthrosols and 11 out of the 14 unique sequences of *Proteobacteria* obtained originated from the Anthrosols (Fig. 3). Of 35 unique RFLP types found only from adjacent soil isolates, sequencing revealed 30 belonged to the *Firmicutes*. Furthermore, sequences from 15 families were derived from subsurface soil samples and, of these, adjacent soils contained representatives from only four families, whereas Anthrosols contained representatives from 13 families.

Discussion

Paired Anthrosols and adjacent soils used in this study were formed from the same parent material and experienced the same prevailing environmental conditions. However, culturing, isolating, ribotyping, and sequencing revealed that the Anthrosols had higher populations of culturable bacteria to greater soil depths and contained much higher species richness than the adjacent soils. Out of all distinct RFLP types generated, both unique and those occurring more than once among the isolates, 53% were derived from Anthrosol isolates, 33% were from adjacent soil isolates, and 14% shared isolates from both soil types (data not shown). Given that environmental conditions were controlled for between soil types, using soil extract medium as the selective agent revealed greater family-level diversity among isolates, especially in the Anthrosols, than did a standard minimal medium (R2A). For six out of seven paired soils across three sites and multiple depths, the ratio of bacterial families represented to the number of isolates recovered was greater in the Anthrosols than in the adjacent soils. Ten bacterial families were represented in the isolates recovered on R2A, while 15 bacterial families were recovered on SEA, despite its lower nutrient contents, slower growth rates suggesting less available C, and greater difficulty isolating and subculturing from the SEA media.

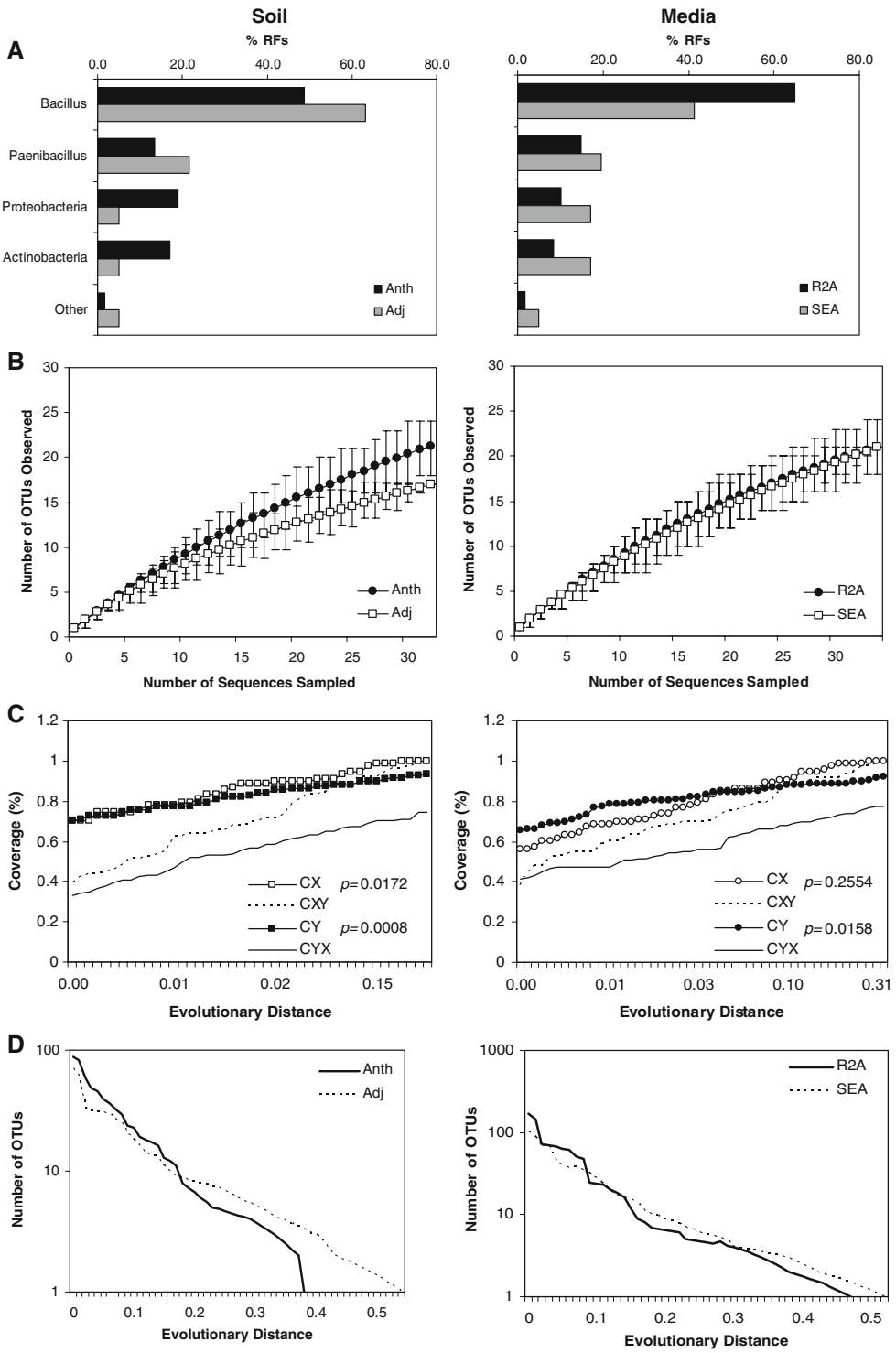


Figure 4 Comparison of soil and media by **a** distribution of RF types among major soil bacteria types, **b** rarefaction curves with 95% confidence interval error bars, **c** ∫-LIB-SHUFF analysis, and **d** lineage through time plot for 16S rRNA gene libraries

R2A is a standard minimal medium designed to cultivate an array of slow-growing, heterotrophic organisms from many environments [31], but it does not reflect the nutrient balances constraining microbial growth in many soils [33]. In this study, colonies formed more rapidly on R2A medium for all soil samples (Fig. 3), suggesting that it favored growth of a distinct group of copiotrophic organisms [15]. Of the colonies isolated on R2A, 38% grew exclusively on this medium and not on the SEA derived from the same sample. Of these, 84% grouped with the *Bacillaceae* and *Paenibacillaceae*, which likely reflects recovered spores rather than actively metabolizing cells. Of the isolates from SEA, 19% grew only on their respective SEA and not on R2A. Of these, less than half were in the

Table 2 Pairwise comparisons of RF types across all sites from two soil types, isolated on two media, using ∫-LIBSHUFF, Chao1 richness estimate, and number of bacterial families per number of isolates

Soil	Medium	*p* value, using ∫-LIBSHUFF				Estimated richness Chao1 (0.05 evolutionary distance)	Bacterial families/isolates
		Adj		Anth			
		SEA	R2A	SEA	R2A		
Adj	SEA	–	0.0000[a]	0.1500	0.3932	12[a]	6/28
	R2A	0.0001[a]	–	0.0010[a]	0.0184[a]	24	5/51
Anth	SEA	0.1116	0.0000[a]	–	0.9827	31	12/45
	R2A	0.0053[a]	0.0001[a]	0.4907	–	34	9/66

[a] Significant difference from other values for respective assay

Bacillaceae and *Paenibacillaceae*. Slower bacterial growth on SEA and its selectivity (Fig. 3) may be due to differences in C availability, substrate quality, and the lack of key nutrients, particularly P for adjacent soils and N for both soil types (Fig. 2), and this should reflect constraints to microbial growth in situ. Adjacent soil SEA micronutrients and N content decreased significantly with soil depth and so did bacterial populations in R2L (Fig. 1a) and on R2A (Fig. 3), while higher nutrient contents at deeper depths in the Anthrosols corresponded with higher numbers of culturable bacteria on all media (Fig. 1). These results suggest that differences in the diversity of bacteria recovered from the different media are not simply due to the lower nutrient contents in the soil extract media.

Analysis of PCR-RFLP ribotypes and phylogeny of 16S rRNA gene amplicons showed distinct patterns in library composition based on soil type and the medium on which colonies were initially isolated (Fig. 4). Ribotypes of isolates from the Anthrosols were more evenly distributed between bacterial groups than isolates from the adjacent soils and, similarly, SEA media captured a broader distribution of bacteria than R2A (Fig. 4a). Based on 16S rRNA gene sequences, isolate libraries from the different soils differed significantly both for species rarity and coverage (Fig. 4b, c), with a clear phylogenetic divergence between isolate libraries from each soil type (Fig. 4d). These trends were less pronounced when libraries were compared according to medium type, suggesting that selection bias based on medium was not as strong as the underlying differences based on soil type. Pairwise comparisons of soil and medium type across all sites showed a significant difference between bacteria selected by R2A versus SEA for adjacent soils, but no similar divergence by medium for the Anthrosols (Table 2). SEA captured greater family-level diversity, while R2A increased estimated richness by strongly favoring growth of unique *Firmicutes* species, particularly for the adjacent soils (Fig. 5; Table 2).

The higher culturable bacterial diversity in the Anthrosols based on ribotype patterns and family-level sequence classification and the higher estimated populations over a greater soil depth suggest ecosystem-level differences in these soil microbial communities. Most *Proteobacteria* isolates and all sequenced *Actinobacteria* isolates were recovered from the Anthrosols and, of these, most were initially isolated on SEA from samples taken from the lower horizons (Fig. 5). As a soil subsystem, the Anthrosols, with higher mineral nutrient contents as well as BC at lower depths (Figs. 1a, b and 2), have an extended area for root growth and thus increased rhizosphere interaction with microbes, leading to a larger volume for physical and chemical interactions, compared to the shallow zone of decomposition and nutrient reabsorption in many tropical soils [43]. The abundance of gram-positive *Firmicutes* recovered from surface soils, especially in adjacent soils on R2A (Figs. 4a and 5), may represent a more ephemeral ecology of organisms adapted to rapid growth when nutrients become available [17] and spore formation when nutrients are scarce or moisture is limited. A greater diversity of *Actinobacteria* in the BC-rich Anthrosols suggests that these organisms may play an important role in nutrient dynamics in these soils. The presence of BC has been shown to greatly increase soil CEC [26]. BC particles also have an extremely high surface area, a highly aromatic, recalcitrant structure, and their surfaces undergo decomposition and oxidation over time [25]. *Actinobacteria* are physiologically adapted to degrade carbon-rich, recalcitrant materials [1, 27] and their branching morphology may allow them to permeate the porous structure of BC thus increasing surface nutrient exchange.

The high number of unique sequences in isolates recovered on Anthrosol SEA media may represent organisms distinct to this soil type, as opposed to R2A, which may select for isolates that co-occur in both soil types or are functionally redundant. Based on similarity to database matches and bootstrap values in constructing multiple trees [12], the screened isolates yielded five new putative bacterial families. Three of the five putative new families were recovered from the adjacent soil isolates, two of

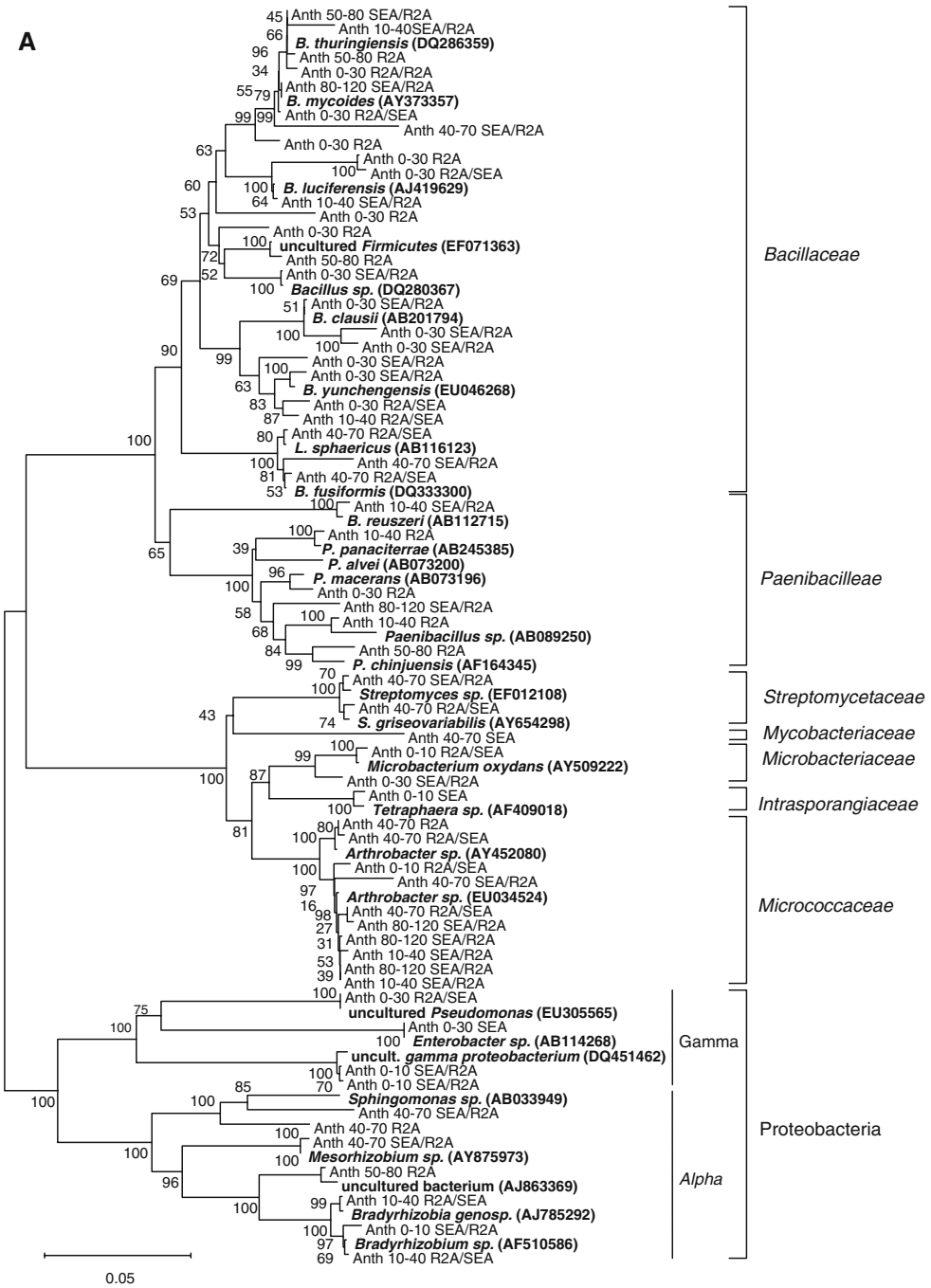
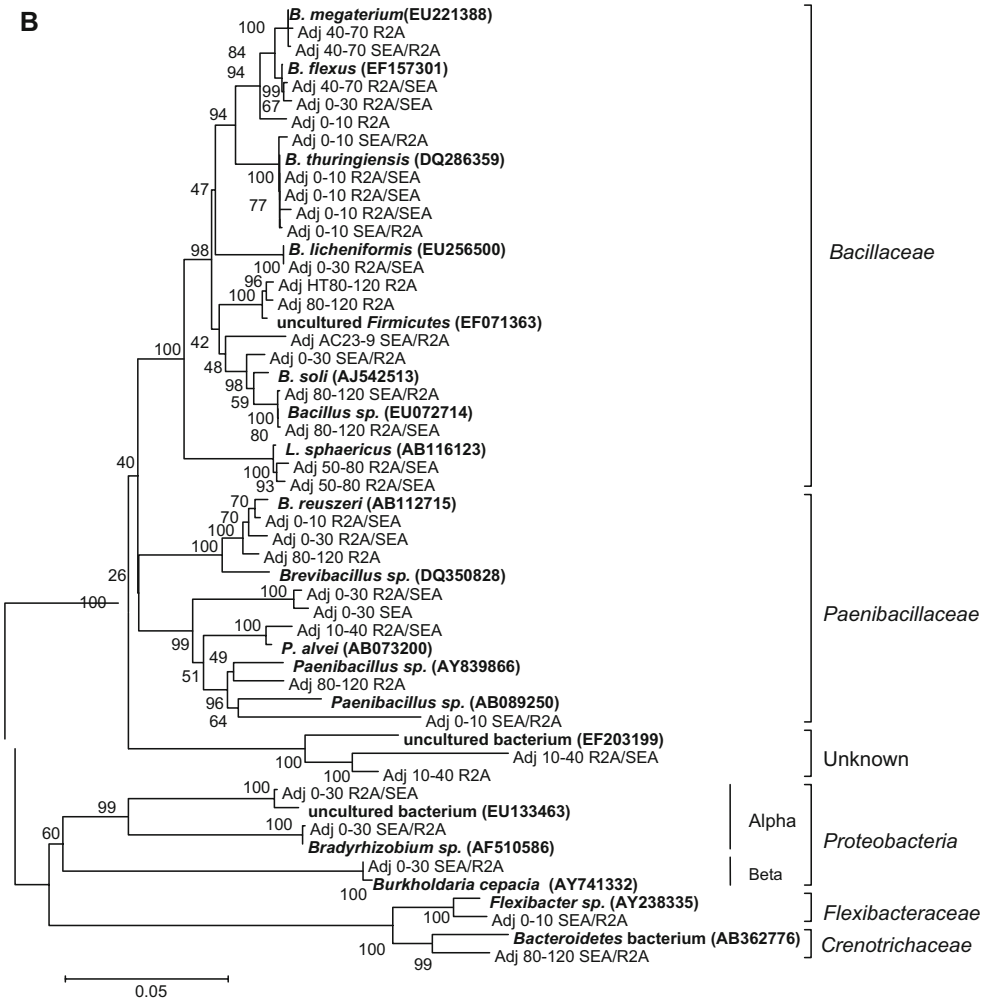


Figure 5 Phylogenetic tree for isolate 16S rRNA gene sequences from **a** Anthrosol (*Anth*) and **b** adjacent (*Adj*) soils. Each isolate entry lists the soil type, the depth range (in centimeters) of soil sample where it was found, followed by the medium (SEA or R2A) on which it was initially isolated, and the alternate medium if the isolate could be cultured after reciprocal plating. Reference sequences are in *bold* with GenBank accession numbers in *parentheses*

which were recovered on R2A and clustered within the *Bacilliales* and one that clustered with the *Bacteriodetes*, which was initially recovered on SEA. The two other putative families cluster within the α-*Proteobacteriales* and both of these were represented in isolates recovered from the Anthrosols cultured on R2A. A possible 30 new species were recovered, mostly on SEA media, although verifying this will require sequencing of housekeeping genes [38].

Other culture-independent studies on Amazonian soil microbial ecology have attributed variation in bacterial diversity to plant communities and land use change [5, 47], while differences in bacterial species richness between Anthrosols and their adjacent soils have been demonstrated under similar aboveground management and ecological conditions [22]. In this study, isolates that grew on R2A were similar between the soil types, but isolates that grew on SEA diverged based on soil type, suggesting that

Figure 5 (continued)



underlying edaphic factors are affecting microbial community composition. Both soils were derived from the same parent material, but modification of the Anthrosols sometime between 600 and 2,300 years BP, that included incorporation of BC [29], led to greater silt content and reduced sand percentages in these soils (Table 1). Furthermore, in spite of the high leaching potential, BC increased CEC in these Anthrosols [26] and resulted in increased pH and higher concentrations of P and Ca, compared to the adjacent soils (Fig. 2; Table 1). Also, due to its large surface area and microporous structure, BC itself may serve as an ideal habitat for microbes [30, 40]. Together with differences in soil texture and the resulting effects on soil moisture, the distinct spatial heterogeneity in the Anthrosols may alter the microbial community composition [49]. The relative abundance of nutrients and the high C/N ratio (Table 1) indicate that N dynamics in the Anthrosols will likely differ significantly from those in adjacent soils. Indeed, BC introduced into soil has been shown to increase nitrification and biological nitrogen fixation [13, 32]. The presence of BC and resultant edaphic effects likely change the factors driving competition in the Anthrosol microbial communities [11] and the growth of *Proteobacteria* and *Actinobacteria* on SEA may indicate that these groups are physiologically active and important to the functioning of the Anthrosol soil community.

Using environmentally derived culturing media, we recovered a high number of genetically distinct organisms and showed clear differences in community composition between the Anthrosols and their adjacent soils. Bacterial populations in the Anthrosols were significantly higher and more diverse, in spite of the same prevailing climate and parent material between the paired samples, particularly in the subsurface samples. Adjacent soil microbial communities contained an abundance of *r*-selected bacteria, characteristic of rapid C mineralization rates, while the Anthrosols were rich in *K*-selected bacteria typical for more stable environments, with slower rates of C turnover [16]. The presence of recalcitrant BC in the Anthrosols may create chemical conditions that foster stable populations of unique

microbial communities and enhance soil fertility [9, 30, 44]. Under conditions of intense weathering, which rapidly deplete fertility in most tropical soils, the chemical composition of these Anthrosols and their distinct microbial populations may in part explain their sustained soil fertility, even centuries after their formation. Replicating the soil biogeochemical conditions and associated stable microbial populations found in the Anthrosols may help to improve agronomic outcomes in tropical soils.

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