Microb Ecol (2010) 60:192–205
DOI 10.1007/s00248-010-9689-3

ENVIRONMENTAL MICROBIOLOGY

Amazonian Anthrosols Support Similar Microbial Communities that Differ Distinctly from Those Extant in Adjacent, Unmodified Soils of the Same Mineralogy

Julie M. Grossman · Brendan E. O'Neill · Siu Mui Tsai · Biqing Liang · Eduardo Neves · Johannes Lehmann · Janice E. Thies

Received: 4 May 2010 / Accepted: 7 May 2010 / Published online: 24 June 2010

Abstract We compared the microbial community composition in soils from the Brazilian Amazon with two contrasting histories; anthrosols and their adjacent non-anthrosol soils of the same mineralogy. The anthrosols, also known as the Amazonian Dark Earths or terra preta, were managed by the indigenous pre-Colombian Indians between 500 and 8,700 years before present and are characterized by unusually high cation exchange capacity, phosphorus (P), and calcium (Ca) contents, and soil carbon pools that contain a high proportion of incompletely combusted biomass as biochar or black carbon (BC). We sampled paired anthrosol and unmodified soils from four locations in the Manaus, Brazil, region that differed in their current land use and soil type. Community DNA was extracted from sampled soils and characterized by use of denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism. DNA bands of interest from Bacteria and Archaea DGGE gels were cloned and sequenced. In cluster analyses of the DNA fingerprints, microbial communities from the anthrosols grouped together regardless of current land use or soil type and were distinct from those in their respective, paired adjacent soils. For the Archaea, the anthrosol communities diverged from the adjacent soils by over 90%. A greater overall richness was observed for Bacteria sequences as compared with those of the Archaea. Most of the sequences obtained were novel and matched those in databases at less than 98% similarity. Several sequences obtained only from the anthrosols grouped at 93% similarity with the *Verrucomicrobia*, a genus commonly found in rice paddies in the tropics. Sequences closely related to *Proteobacteria* and *Cyanobacteria* sp. were recovered only from adjacent soil samples. Sequences related to *Pseudomonas*, *Acidobacteria*, and *Flexibacter* sp. were recovered from both anthrosols and adjacent soils. The strong similarities among the microbial communities present in the anthrosols for both the Bacteria and Archaea suggests that the microbial community composition in these soils is controlled more strongly by their historical soil management than by soil type or current land use. The anthrosols had consistently higher concentrations of incompletely combusted organic black carbon material (BC), higher soil pH, and higher concentrations of P and Ca compared to their respective adjacent soils. Such characteristics may help to explain the longevity and distinctiveness of the anthrosols in the Amazonian landscape and guide us in recreating soils with

J. M. Grossman · B. E. O'Neill · B. Liang · J. Lehmann · J. E. Thies (✉)
Department of Crop and Soil Sciences, Cornell University,
722 Bradfield Hall,
Ithaca, NY 14853, USA
e-mail: jet25@cornell.edu

S. M. Tsai
Cellular and Molecular Biology Laboratory,
Centro de Energia Nuclear na Agricultura, Universidade de São Paulo,
Piracicaba, SP, Brazil

E. Neves
Museu de Arqueología e Etnología, Universidad de São Paulo,
São Paulo, SP, Brazil

Present Address:
J. M. Grossman
Department of Soil Science, North Carolina State University,
Raleigh, NC 27695, USA
e-mail: julie_grossman@ncsu.edu

Present Address:
B. Liang
Institute of Earth Sciences, Academia Sinica,
128, Sec. 2, Academia Road, Nangang,
Taipei 11129, Taiwan

sustained high fertility in otherwise nutrient-poor soils in modern times.

Introduction

Soil management practices and land use influence many physical, chemical, and biological properties of soil. For decades, research has focused on soil physical and chemical variables, with less attention paid to how soil management affects microbial communities and associated ecosystem services. Here, we determined the relative effects of soil mineralogy, recent land use, and ancient soil management practices on soil microbial community composition in anthrosols and adjacent unmodified soils in the Brazilian Amazon. These anthrosols, also known as '*Terra Preta de Indio*' or Amazonian Dark Earths, have distinctive physical properties and sustained high fertility. They are characterized by unusually high total carbon (C) contents, high cation exchange capacity (CEC), high phosphorus (P) and calcium (Ca) contents [28], and the presence of both pottery sherds [37] and charred biomass of unknown organic origin, referred to here as black carbon (BC) [30]. It is now generally accepted that these soils were modified between 600 and 8,700 years before present (bp) by indigenous pre-Colombian Indians, likely for agricultural use [37, 43]. Unlike most tropical soils, the anthrosols contain large pools of stable soil organic matter and are highly fertile. Thus, they are sought after for use by local farmers and horticulturalists. The physical characteristics and fertility of the Amazonian anthrosols have been studied extensively in recent years; however, microbial communities extant in these soils and how they compare to adjacent unmodified soils of the same mineralogy have yet to be described.

Preliminary work suggests that, along with their unique chemistry [5], the anthrosols have distinct bacterial, archaeal, and fungal communities that together result in the unique processes, such as high labile C retention, that have been observed in these soils [39, 53]. A survey of one anthrosol site in the Western Amazon showed that the anthrosol harbored similar organisms to the adjacent non-anthropic soil, but their richness was increased by 25% [23].

We characterized the bacterial and archaeal communities in four anthrosols and adjacent unmodified soils under four different current land uses to determine the importance of recent soil management activities relative to ancient soil management practices on the composition of these communities. Although the Amazon region is known for its diversity of flora and fauna, this represents the first published report describing soil microbial diversity in the Brazilian anthrosols across a range of land uses and soil mineralogy and relative to unmodified adjacent soils.

Methods

Site Characteristics and Soil Sampling

Soils were collected from four sites: Lago Grande (LG), Hatahara (HAT), Açutuba (ACU), and Dona Stella (DS), located within the Amazon basin near Manaus, Brazil (3°8′ S, 59°52′ W, 40–50 m above sea level). Anthrosols from these sites have been dated to span from about 600 to 8,700 years bp (Table 1; modified from [37]; E.G. Neves, unpublished data, 2005). The natural vegetation is tropical lowland rain forest. The anthrosols sampled from LG, HAT, and ACU developed on oxisols, whereas the DS anthrosol developed on a spodosol. At the time of sampling, the LG site supported a mature secondary forest, the HAT site was under unimproved pasture, the ACU site was under grass peripheral to a working subsistence farm and the DS site was under natural campinarana vegetation. Indicator species for each site are given in Table 1. The anthrosol at DS, the oldest site, bore lithic remains, whereas the other three sites contained ceramic artifacts indicative of pre-Colombian occupation. Table 1 provides key characteristics of the anthrosol and adjacent soil samples. Soil sampling sites were also chosen based on maximum color difference between the anthrosols (BC-rich, dark coloration, with artifacts) to adjacent soils (typical pale yellow or white soils without artifacts). Soils from the four sites were sampled using a ring auger. Samples were taken from soil profiles at various depths according to horizons containing distinct depositional features, after removal of the litter layer if present. The depth of sampling in the anthrosols varied due to the anthropogenic origin of the soils. Figure 1 shows sample depths and land use at each site at the time of sampling.

DNA Extraction

Community DNA was extracted from sub-samples of each site soil, two from each anthrosol and two from each adjacent soil. A 0.5 g fresh weight of sample was extracted using a bead-beating method (FastDNA® Spin Kit for soil, Qbiogene, Irvine, CA, USA), modified according to the manufacturer's instructions as follows. Phenol-chloroform and the supernatant from Step 5 of the extraction kit were added to a clean Eppendorf tube and vortex-mixed, then centrifuged for 1 min at 14,000×*g* to separate the organic and chloroform phases. The surface phase was removed and added to a new tube containing binding matrix as described in Step 6 of the kit procedures. The final DNA eluted was diluted to 5 ng DNA μl^{-1} using ultra-pure water and frozen at −20°C. The quality and relative quantity of extracted DNA was checked by agarose gel electrophoresis in 1× Tris–acetate–EDTA (TAE) buffer (1.5% *w*/*v*). The gel was stained with

Table 1 Chemical properties of anthrosols and adjacent soils in the central Amazon (adapted from Liang et al. [31])

Site	Vegetation	Type	Black carbon[a] % of total C	Age Years	pH 1:2.5 KCl	Organic C mg g^{-1}	Total N	C/N	CEC[b] mg kg^{-1}	Total P	Total Ca
HAT	Mixed pasture grasses	Anthrosols	79.4	600–1,000	5.5	22.0	1.0	23	211.3	9,064	17,545
		Adjacent	20.2		3.8	21.8	1.6	14	88.4	273	115
LG	Old secondary forest (*Theobroma grandiflorum*, *Theobroma cacao*, *Manguifera indica* L., *Euterpe oleracea*)	Anthrosols	60.9	900–1,100	4.9	31.5	1.8	18	222.4	526	6,354
		Adjacent	9.3		3.5	17.5	1.3	14	59.2	251	119
ACU	Edge of field peripheral to a subsistence farm	Anthrosols	73.1	2,000–2,300	4.2	15.7	1.0	16	56.3	777	332
		Adjacent	44.3		3.9	15.4	0.8	20	52.0	198	50
DS	Natural campinarana vegetation (*Compsoneura debilis*, *Pithecelobium leucophyllum*, *Dicymbopsis froesii)*	Anthrosols	75.1	6,700–8,700	4.1	16.5	1.1	15	26.7	5,026	40
		Adjacent	10.6		2.6	10.2	0.4	27	80.8	139	165

[a] The aromaticity of organic C in anthrosols and adjacent soils was measured by (CP/MAS) ^{13}C NMR and calculated based on the percentage of aromatic-C to total C [31]

[b] Potential cation exchange capacity

SYBR-Green I (Sigma, St Louis, MO, USA) and the image digitized using a Fluor-S™ Multi-imager (BioRad, Hercules, CA, USA).

PCR and Denaturing Gradient Gel Electrophoresis

Community DNA extracted from each duplicate soil sample was amplified using nested PCR (Table 2) of the 16S rRNA V3 region for Bacteria and Archaea in individual reactions in an MJ Research PTC 200 thermal cycler (BioRad, Hercules, CA, USA). Concentrations of reactants in initial and nested PCRs for Bacteria (50 µl final volume) and initial PCRs for Archaea (25 µl final volume) were as follows: 1× PCR buffer (Promega, Madison, WI, USA), 3.25 mM $MgCl_2$, 200 µM dNTPs (in equal concentrations, Promega), 0.5 µM forward and reverse primers (Integrated DNA Technologies, IDT, Coralville, IA, USA), 0.1 µg μl^{-1} bovine serum albumin (BSA, Promega), 1.0 U *Taq* polymerase (Promega) in ultra-pure water (Fluka/Sigma-Aldrich, Switzerland), and 5 ng of template DNA. In the nested PCR reaction for Archaea, primer concentration was reduced to 0.2 µM, the *Taq* polymerase was increased to 1.5 U, and water adjusted accordingly to make 50 µl reactions. After amplification, the PCR products were verified by running the amplicons on a 1.5% agarose gel stained with SYBR-Green I (Sigma). To confirm repeatability, each PCR reaction was performed at least three times.

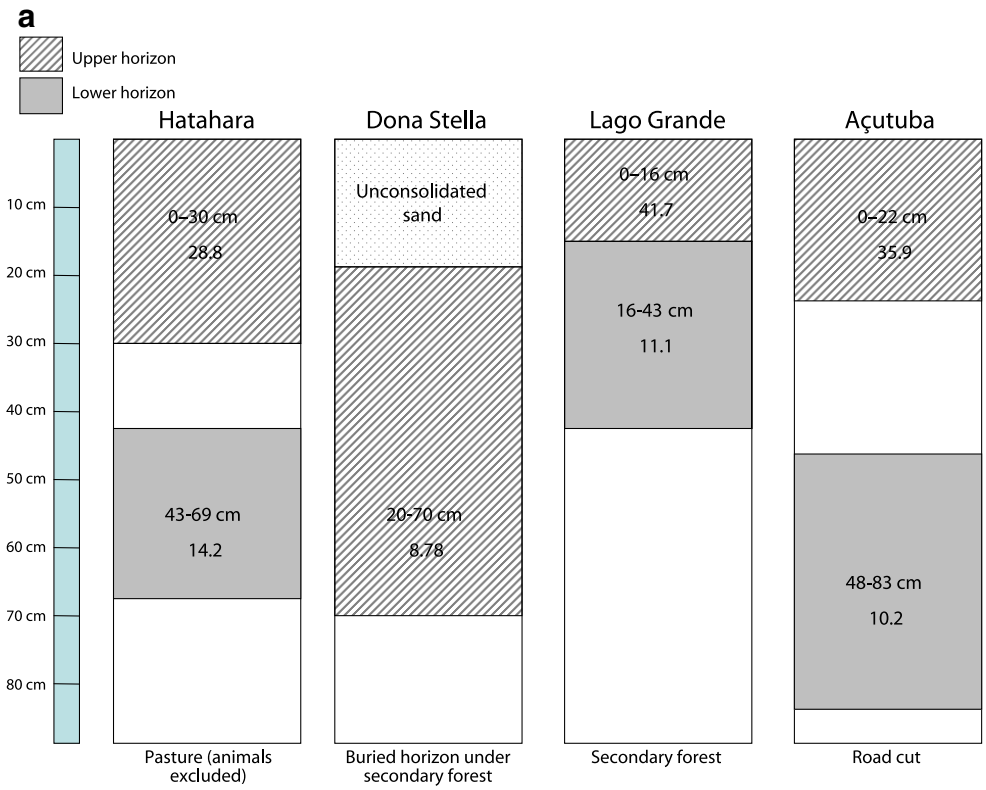
For denaturing gradient gel electrophoresis (DGGE) analysis, equal volumes of amplified sample DNA were loaded onto an 8% polyacrylamide gel, with denaturant gradients of 40–55% for Bacteria and 47–57% for Archaea (with 7 M urea and formamide representing 100% denaturant) and run at 60°C, 75 V for 14 h in a BioRad DCode System (BioRad) and then stained with SYBR Green I (Sigma). Gels were digitized using a Fluor-S™ Multi-imager (BioRad). Quantity One 4.2 software (BioRad) was used to detect resulting bands in the DGGE gels.

DGGE Data Analysis

DGGE fingerprint profiles were analyzed using BioNumerics software (version 1.5, Applied Maths, Kortrijk, Belgium) with gel images normalized using identified internal reference bands (bands present in all lanes). Similarities between profiles were calculated using Pearson's coefficient and an average linkage (unpaired group method with arithmetic means; UPGMA) dendrogram was derived for Bacteria and Archaea DGGE profiles separately.

Cloning and Sequencing

DNA in bands that were either unique to the anthrosols or common to all samples was excised using wide orifice pipette tips filled with 30 µl phosphate buffer, expelled into 100 µl tubes, and left to elute overnight at 20°C. To check for contamination from neighboring bands, a 2 µl aliquot of eluted DNA was re-amplified using the primers and conditions described above, but without the GC clamp on the forward primer. Effort was made to extract one to two bands from each sample in the analysis; however, close proximity of bands to each other made it difficult to assure



* Dona Stella anthrosol was found buried below unconsolidated sand identified as possibly being a defensive mound structure.

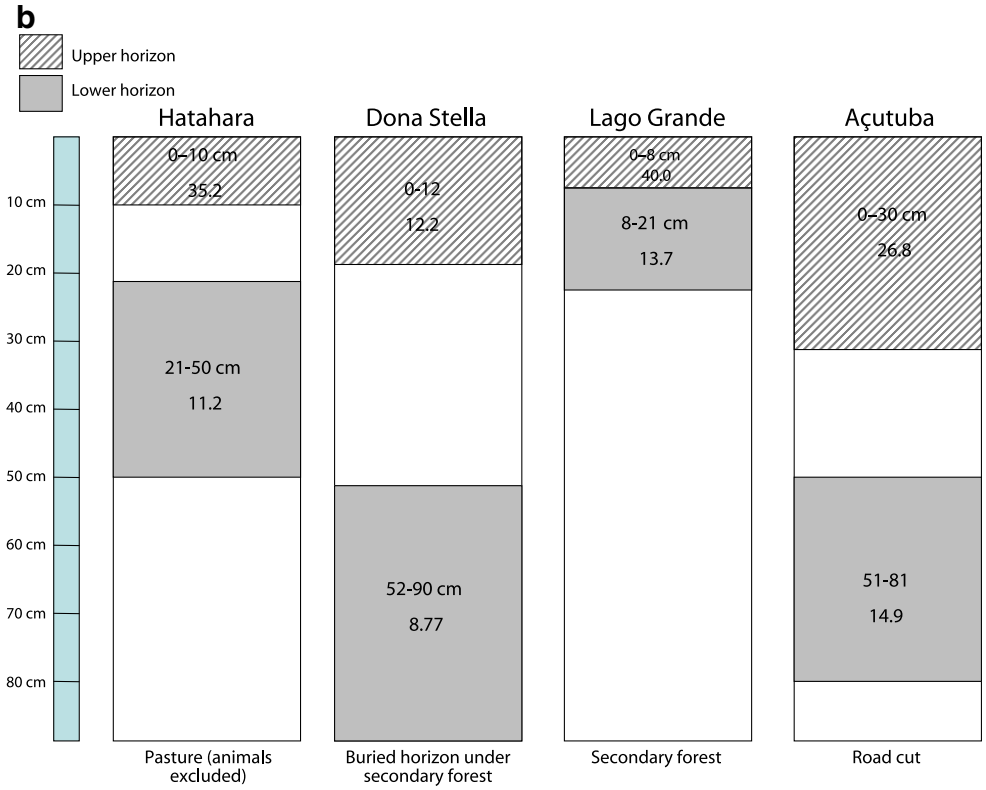


Figure 1 **a** Depth of anthrosol sampling, current land use, and total extractable DNA (ng μl^{-1} extract solution). **b** Depth of sampling of the adjacent soils, current land use, and total extractable DNA (ng μl^{-1} extract solution)

Table 2 PCR conditions and primer sets

Reaction	PCR conditions	Primers
Bacteria—initial	Denaturing for 5 min at 94°C, followed by 35 cycles of: denaturing at 94°C for 30 s, annealing at 57°C for 45 s, extension at 72°C for 45 s; final extension at 72°C for 10 min	338f [25] 1378r [21]
Bacteria—nested	As above	338f with GC clamp [25] 518r [35]
Archaea—initial	As above	46f and 1110r [40]
Archaea—nested	Denaturing for 5 min at 94°C; 4 cycles of denaturing at 94°C for 30 s, annealing at 56°C for 45 s with temperatures decreasing by 0.5°C in each cycle, and extension at 72°C for 45 s; then 29 cycles of denaturing at 94°C for 30 s, annealing at 53.5°C for 45 s, and extension at 72°C for 45 s; followed by a final extension at 72°C for 10 min (touchdown program)	340f with a GC clamp and 519r [40]
T-RFLP	Denaturing for 5 min at 94°C, 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	Fluorescently labeled 27f (50-[6FAM] AGA GTT TGA TCC TGG CTC AG-30) and unlabeled 1492r (50-GGT TAC CTT GTT ACG ACT T-30) (IDT) [34]

that the DNA extracted was from a single band. Following amplification, the PCR products were rerun on a DGGE gel adjacent to the original samples from which they were excised to confirm their band positions relative to the parent bands. In some cases, bands were re-excised, re-amplified, and run on a DGGE gel two to three more times to assure no adjacent band DNA remained. In a few cases, contaminating DNA from adjacent bands could not be separated from that of the band of interest. These samples were removed from the analysis. PCR products from the remaining bands of interest were cleaned using a QIAquick Spin PCR Purification Kit (Qiagen, Valencia, CA, USA) and cloned using a pGEM-T Easy Vector System I with *Escherichia coli* JM109 competent cells according to manufacturer's instructions (Promega). After ligation and transformation, *E. coli* clones derived from each band excised were grown individually to check for the presence of the insert.

For bands excised from the Bacteria denaturing gradient gel, approximately five to 15 successful clones from each experiment (plate) were randomly selected for further analysis. A total of 77 clones were re-amplified using primers T7 and SP6 as per Table 2 for Bacteria—initial. Clones containing inserts of the expected size were analyzed further by restriction fragment length polymorphism (RFLP) analysis. Clones that yielded no product or a product of unexpected size were not analyzed further; this included clones derived from bands 1–3 (see Fig. 2). From Archaea, nine clones were amplified and successfully cloned from seven total bands excised.

PCR products from clones were screened by RFLP analysis to select representative clones for sequencing. PCR products were digested for 4 h at 37°C in 30 µl reactions containing 1.0 U MspI (New England Biolabs, Ipswich, MA, USA), 1× buffer, and 15 µl of amplified sample DNA made to volume in ultra-pure water (Fluka/Sigma-Aldrich). The digested products were separated by gel electrophoresis in a 3.5% agarose gel in 1× Tris–borate–EDTA buffer run for 5 h at 50 V. The RFLP patterns were compared visually. A total of 33 Bacteria and 10 Archaea representative clones were submitted for sequencing at the Life Sciences Resource Center at Cornell University, Ithaca, NY. Phylogenetic and molecular analyses were conducted using MEGA version 3.0 [24].

Clones from Bacteria bands 4, 5, 6, 7, and 9 (Fig. 2) and Archaea bands 1, 2, 3, 4, and 7 (Fig. 5) were assigned GenBank accession numbers EU683687-EU683717 and HM167478-HM167487, respectively (Table 3).

Terminal Restriction Fragment Length Polymorphism Analysis

Sample DNA was amplified by PCR using the parameters given in Table 2. Final concentrations of reactants in each of two, 50 µl reactions prepared for each sample were: 0.05 U μl^{-1} Taq polymerase (Applied Biosystems, Foster City, CA), 1× PCR buffer supplied with the enzyme, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 mg ml^{-1} BSA, both primers at 0.1 mM, nuclease-free water (Promega) and 5 µl of DNA template. The amplicons from replicate 50 µl reactions for each sample were pooled and the DNA quantified against a calf thymus DNA standard curve, with both made visible using an ethidium bromide (EtBr) solution using a BioRad Fluor-S™ MultiImager and the accompanying Quantity One™ software. The amplified DNA was lyophilized and then re-suspended in nuclease-free water

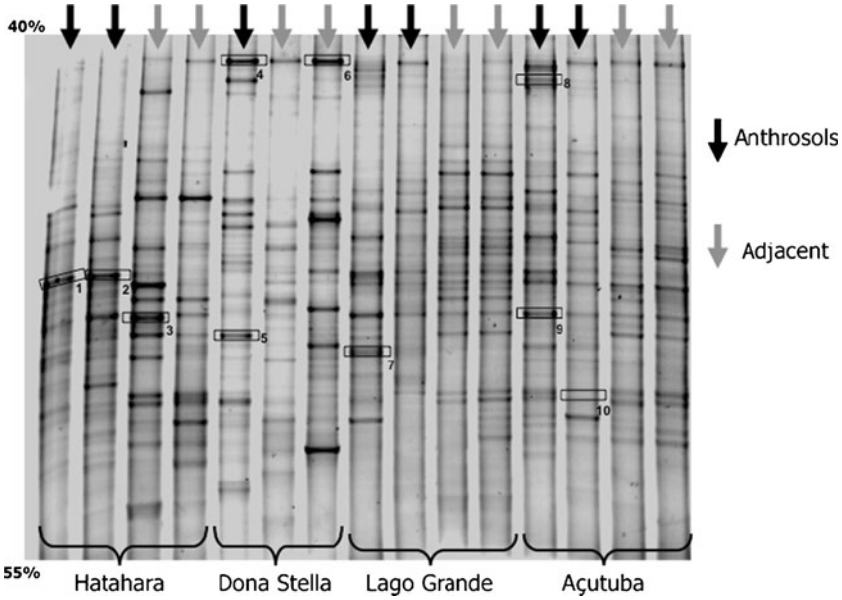


Figure 2 16S rRNA gene (V3 region) DGGE profiles of community DNA amplified using nested Bacteria primer sets 338f and 1378r followed by GC-338f and 518r

(Fluka/Sigma-Aldrich). to a concentration of approximately 20 ng μl^{-1}. The restriction enzymes Hha1 (Promega) and Sau96I (New England Biolabs, Ipswich, MA) were used to digest amplified sample DNA. Two separate restriction enzyme digests were prepared per sample that contained 1.0 µl of enzyme (either Hha1 or Sau96 I), 3.0 µl of the respective 10× buffer, 0.3 µl of 10 mg ml^{-1} BSA, 10.7 µl nuclease-free water and 15 µl of amplified sample DNA. Restriction digests were carried out in an MJ Research PTC 100 thermal cycler held at 37° C for 4.5 h with a final step of 70°C for 15 min to stop the reaction. Complete digestion of the DNA was verified by inspecting digested products run on a 1.5% agarose gel stained with EtBr and visualized using a Fluor-S™ MultiImager (BioRad).

Digested DNA was purified using a PERFORMA® DTR Edge Plate (Edge BioSystems, Gaithersburg, MD, USA) and lyophilized for a final time. DNA was re-suspended in a 10 µl mixture containing 9.85 µl of formamide and 0.15 µl of Liz 500 size standard (Applied Biosystems). Terminal fragment size analysis was performed using a 3730X1 ABI electrophoretic capillary sequencer (Applied Biosystems) in conjunction with the Genemapper Software (Applied Biosystems) at Cornell University's Life Sciences Resource Center, Ithaca, NY.

The terminal-RFLP (T-RFLP) data were analyzed by the Additive Main Effects with Multiplicative Interaction (AMMI) model using MATMODEL™ software (Microcomputer Power, Ithaca, NY, USA) [16]. The AMMI model (also known as a doubly centered PCA) combines the additive elements of ANOVA with the multiplicative elements of PCA. See Gauch [15] and Culman et al. [9] for further details and explanation of the AMMI model.

Results

Extractable DNA and Soil Characteristics

Total extractable DNA varied by soil depth. Samples taken from the upper soil horizons contained almost threefold higher concentrations of DNA than those from the lower horizons (Fig. 1a, b), with a mean of 31.5 versus 11.6 ng DNA μL^{-1}, respectively. Results of Liang et al. [30] showed that each pair of anthrosol and adjacent soil were of similar mineralogy; however, the anthrosols had significantly higher total P (three to 33 times) and Ca (seven to 153 times, except −0.8 times in DS), base saturation (two to four times, except 0.7 times in ACU), and pH values compared to their paired adjacent soils. These and additional soil characteristics are presented in Table 1.

Microbial Community Composition—Bacteria

Bacterial community composition was strongly affected by the historical land use practices (anthrosols vs. adjacent soils). DGGE profiles of bacterial amplicons were extremely complex (Fig. 2). Anthrosol and adjacent samples that originated from the same site differed from one another dramatically, except at the DS site. Considerable changes in community composition were observed from site to site, and between anthrosol and adjacent soils within each site (Fig. 2). Bands 1 and 2 were common in the anthrosols. Bands 4, 6, and 10 were present in all samples. Bands 3 and 9 were common to all samples, except those from DS. Band 8 was also commonly found, however, it was noticeably stronger in the upper horizon samples from the ACU, DS, and LG sites.

Our most striking finding was that the anthrosol DGGE fingerprints were very similar to each other regardless of

Table 3 GenBank accession numbers

Band4Eub2A	EU683687
Band4Eub2B	EU683688
Band4Eub2D	EU683689
Band4Eub2F	EU683690
Band4Eub3C	EU683691
Band5Eub3D	EU683692
Band5Eub3E	EU683693
Band5Eub3F	EU683694
Band5Eub3G	EU683695
Band5Eub3H	EU683696
Band5Eub4A	EU683697
Band5Eub4B	EU683698
Band5Eub4C	EU683699
Band5Eub4E	EU683700
Band5Eub4F	EU683701
Band5Eub4G	EU683702
Band5Eub4H	EU683703
Band6Eub5C	EU683704
Band6Eub6C	EU683705
Band6Eub6E	EU683706
Band7Eub7A	EU683707
Band4Eub1B	EU683708
Band4Eub1A	EU683709
Band7Eub2A	EU683710
Band6Eub1D	EU683711
Band5Eub1F	EU683712
Band6Eub1G	EU683713
Band7Eub1H	EU683714
Band9Eub2F	EU683715
Band9Eub3B	EU683716
Band7Eub2B	EU683717
Band1Arch1D	HM167478
Band1Arch1C	HM167479
Band2Arch3E	HM167480
Band4Arch7A	HM167481
Band2Arch4B	HM167482
Band3Arch5D	HM167483
Band3Arch6D	HM167484
Band3Arch5A	HM167485
Band7Arch11F	HM167486
Band1Arch1E	HM167487

site (UPGMA dendrogram, Fig. 3), while at the same time diverged considerably from their respective adjacent soils. Among the anthrosols, ACU and LG were the most similar (40% similarity) with the upper horizon samples being 60% similar to each other. In contrast, these anthrosols had only a 20% similarity to their respective adjacent soils (Fig. 3). When comparing anthrosols to adjacent soils from the HAT, ACU, and LG sites, less than 20% of their bands were shared, demonstrating a tremendous difference in community composition between these two historical land uses. DGGE fingerprints from soils sampled from the DS site (the only spodosol) were only distantly related to any of the other samples (10% similarity).

Microbial Community Composition—Archaea

Differences between Archaea communities found in the anthrosols and adjacent soils were striking, with these soils differing in their banding patterns by over 90% (UPGMA dendrogram, Fig. 4). In general, fewer operational taxonomic units (OTUs) were resolved in the DGGE profiles of the Archaea 16S rRNA genes compared to those of the Bacteria. The Archaea DGGE profile (Fig. 5) had distinct bands that were: (1) unique to the anthrosols (bands 5 and 3), (2) common to all soils, but strongest in the anthrosols (bands 4 and 7), and (3) unique to adjacent soils (band 1). Anthrosols from three sites, ACU, LG, and DS, had over half (58%) of their bands in common. Adjacent soils from these same sites also clustered tightly together, sharing 64% of their bands. The upper horizon sample from DS, the only spodosol, again did not cluster with either the other anthrosols or adjacent soils.

RFLP Analysis of Clones

A total of 77 Bacteria clones excised from the DGGE bands were screened by RFLP. To determine how well these clones captured the diversity of genotypes within a selected band, the cumulative number of distinct RFLP patterns was plotted as a function of the number of clones screened (Fig. 6). This technique is analogous to generating a rarefaction curve to roughly estimate species richness from species abundance data [54].

Cloning and Sequencing: Identification of Organisms

Cloned and sequenced bands from Bacteria and Archaea DGGE gels identified organisms with a high level of similarity to those contained within the GenBank database (Figs. 7 and 8). Two clones from within a Bacteria profile band found only in anthrosols (band 7) was found to closely match the sequence of *Verrucomicrobia* sp. Organisms common to both anthrosols and adjacent soils included species of *Acidobacteria*, *Psuedomonas*, and γ *Proteobacteria*.

T-RFLP Data Analysis

AMMI analysis of the bacterial community T-RFLP fingerprints supported the DGGE analysis and again illustrated that the anthrosol communities were distinctly different from those in the adjacent soils (Fig. 9). The community in DS soils once again diverged strongly from those at the other sites.

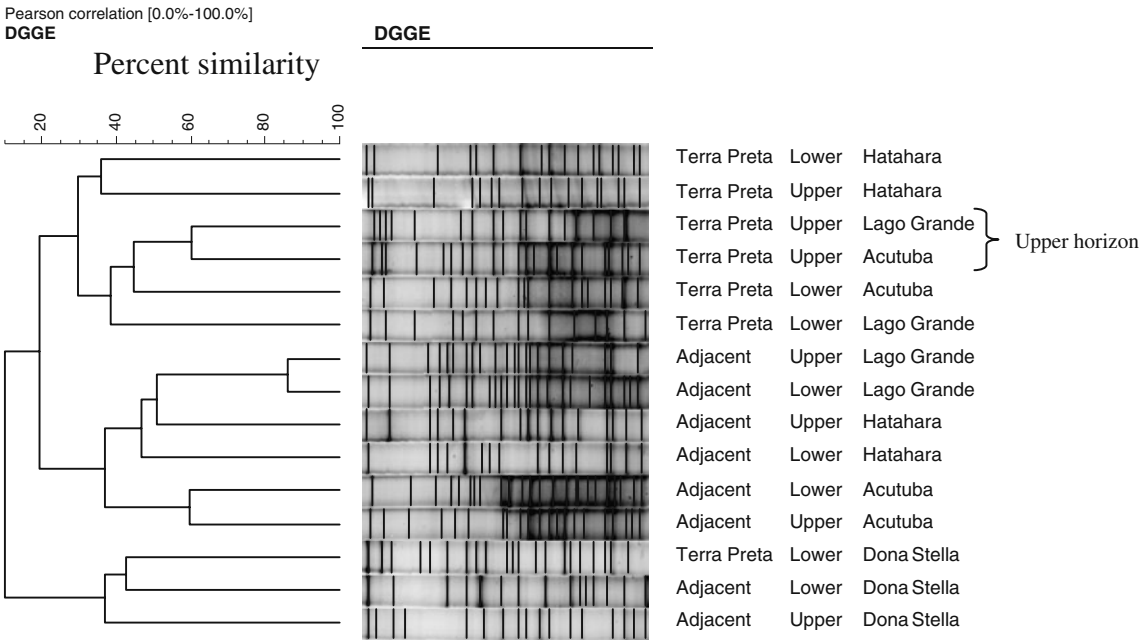


Figure 3 Cluster analysis based on DGGE profiles of the soil Bacteria community 16S rRNA gene (V3 region) in the anthrosol and adjacent soils. The dendrogram was generated using UPGMA. Band *marking lines* are shown for clarity of band location. Original image is shown in Fig. 2

Discussion

Of the four anthrosols, three (ACU, LG, and HAT) were more similar to each other than to any of the adjacent soils, despite similarities in current land use and soil mineralogy for each site pair. Soils at these sites were formed on oxisols, whereas, DS was formed on a spodosol and clustered with its adjacent soil, well removed from samples from the other

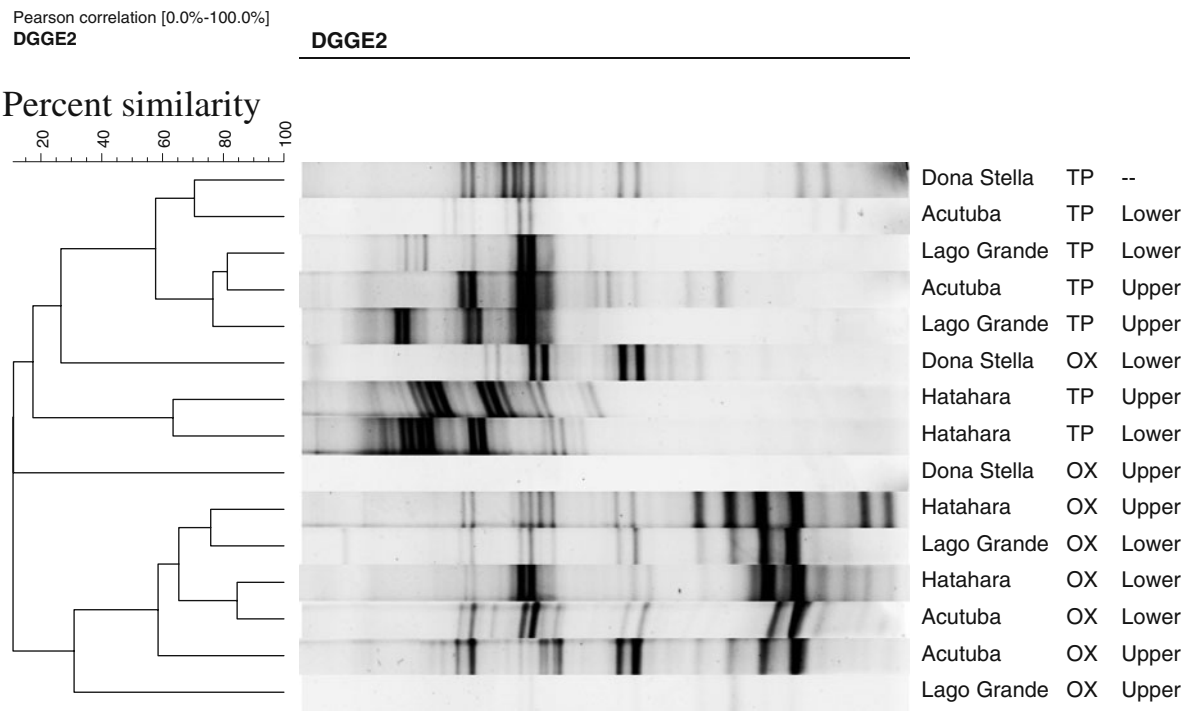


Figure 4 Cluster analysis based on DGGE profiles of the soil Archaea community 16S rRNA gene (V3 region) in the anthrosol and adjacent soils. The dendrogram was generated using UPGMA. Band *marking lines* showing relatedness are not used as in Fig. 3 due to skew in the data image and clarity of the original bands

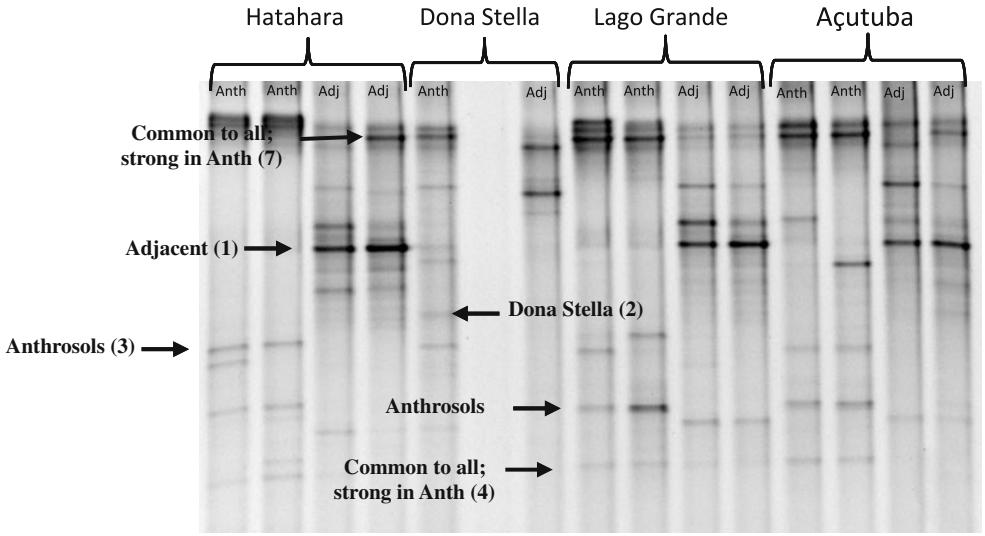


Figure 5 16S rRNA gene (V3 region) DGGE profiles of soil community DNA amplified using nested Archaea primer sets 46f and 1110r followed by GC-340f and 519r

sites. The soil type (spodosol), age of formation (~8,700 years bp) and its buried location all contribute to DS being unlike the other anthrosols. This soil also had a lower CEC.

The similarity between the anthrosols was most striking between the LG and ACU sites. This was unexpected as these sites had distinctly different vegetation cover (Table 1) and the composition of organic C inputs is known to influence the soil microbial community strongly [2, 4, 6, 7, 41]. Land conversion from tropical forest to agricultural use, such as is the case for the ACU site, has been found in many studies to lead to fundamental changes in the size, activity, and composition of soil microbial communities [8, 20, 32, 38]. Given this, we expected to find that the two land uses represented by ACU and LG would have led to substantially different microbial communities. Our finding of similar soil microbial communities between these two anthrosols is intriguing in that it suggests other factors are more strongly at play in stimulating the development of these assemblages of organisms.

With the primers used in this study, the overall microbial community composition at the sites sampled contained a less diverse Archaea community as compared to that of the Bacteria. The finding of reduced complexity in the Archaea community in both anthrosol and adjacent soils is similar to that of Borneman and Triplett [3] who found that out of 100 sequences derived from soils from Eastern Amazonia in Brazil, only two were classified as Archaea. Typically,

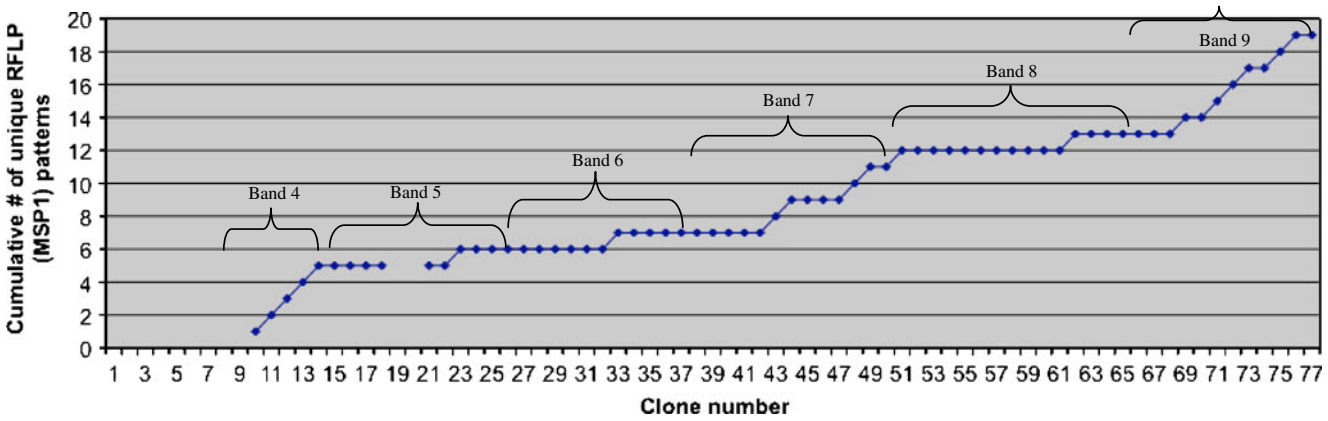


Figure 6 Rarefaction curve based on cloned Bacteria sequences generated from DNA excised from DGGE gels derived from the anthrosol samples. The clone number refers to the sequential number of clones analyzed using RFLP. *Bands 1–3* and *band 10* are not represented due to problems in cloning reactions

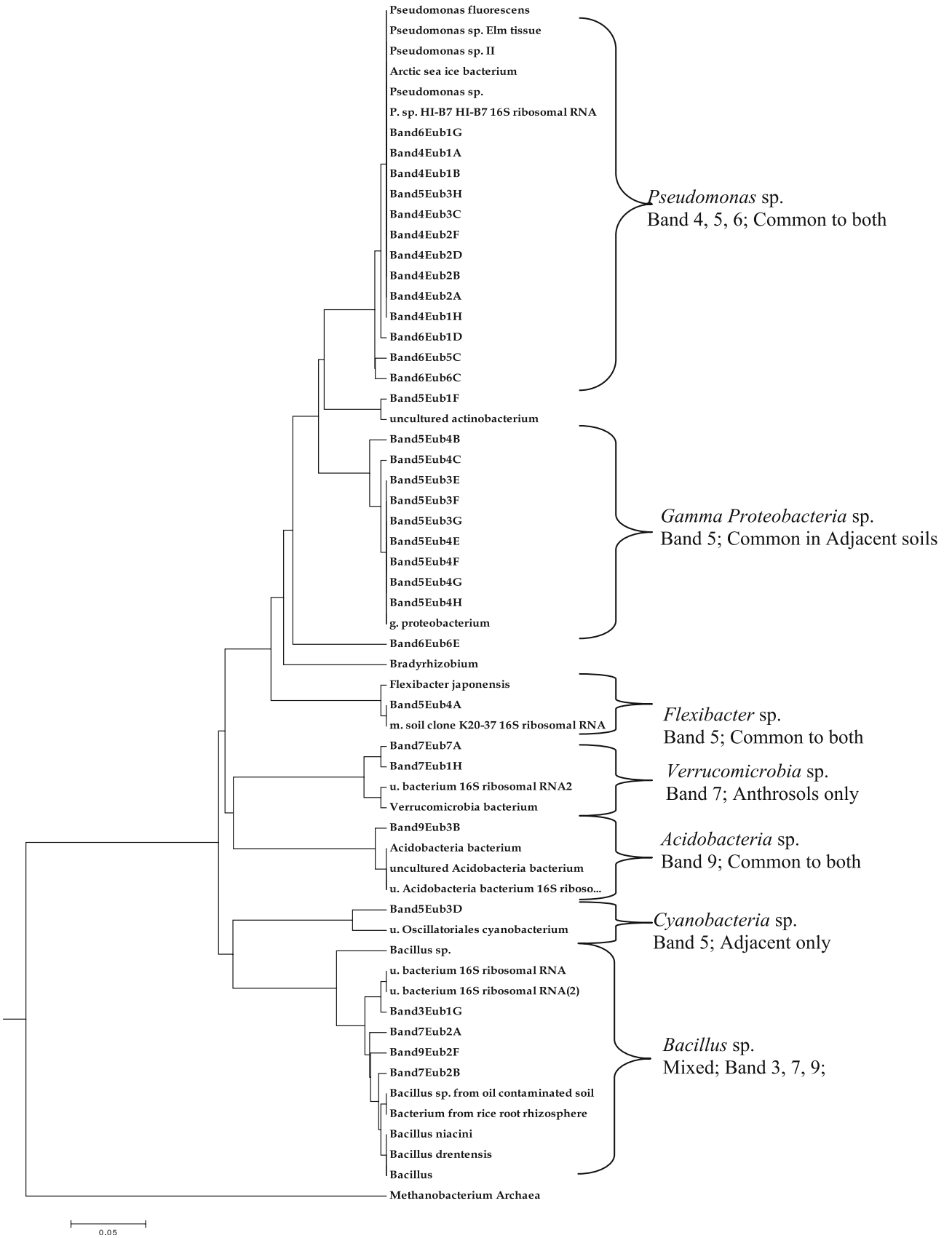


Figure 7 Anthrosol and adjacent soils GenBank sequence matches from DNA excised, cloned, and sequenced from a DGGE profile of the Bacteria 16S rRNA gene (V3 region) bands

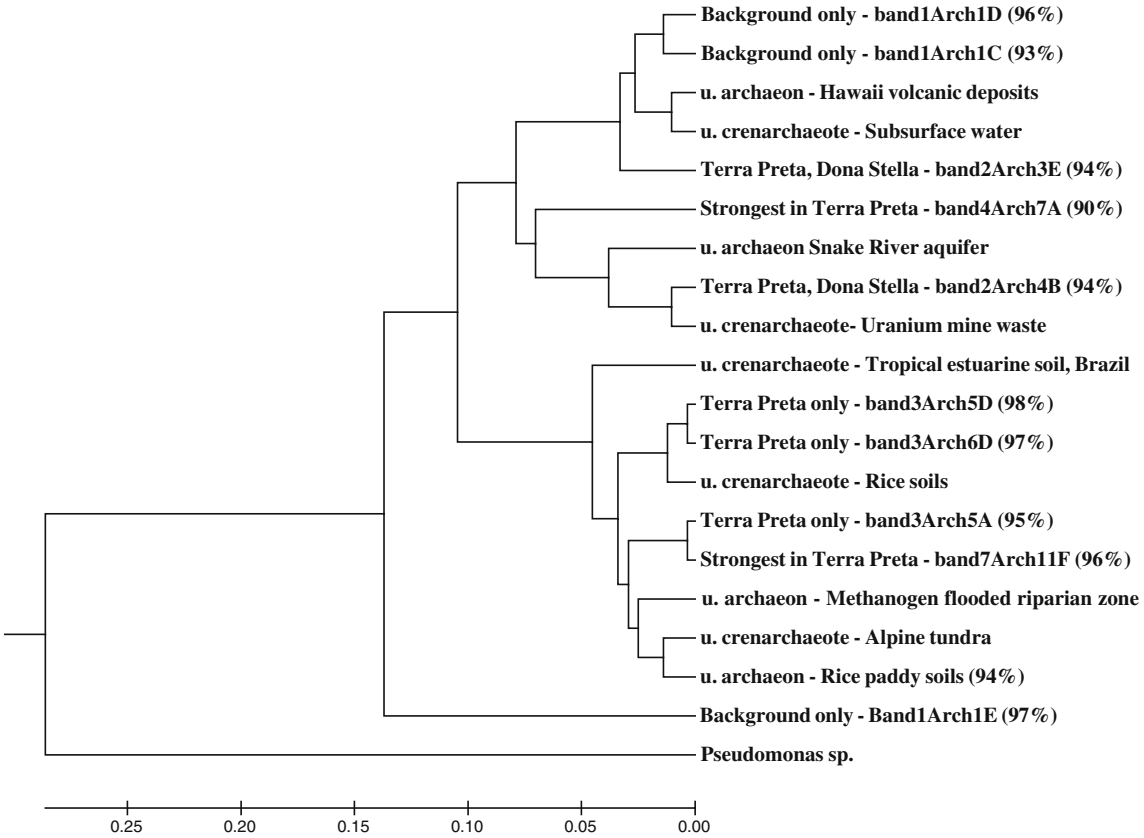


Figure 8 Anthrosol and adjacent soils GenBank sequence matches from DNA excised, cloned, and sequenced from a DGGE profile of Archaea 16S rRNA gene (V3 region) bands

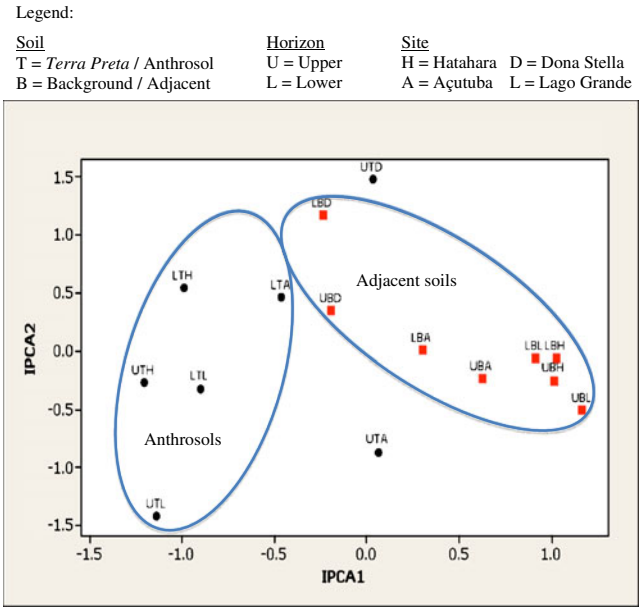


Figure 9 AMMI analysis of bacterial community T-RFLP fingerprints of the 16S rRNA genes

Archaea represent an average of 10% of the total phylotypes in a given environmental sample [46].

Tight clustering of some of the upper and lower soil horizon samples between anthrosol sites suggests that Bacteria communities are comparable by depth between the different anthrosols. The similarity between community compositions was especially prominent in the upper horizons of the ACU and LG sites, which had almost 60% of their bands in common (Fig. 3). The buried DS anthrosols exhibited characteristics similar to those of the lower horizons of sampled adjacent soils in both diversity of OTUs and community composition. Communities of both Bacteria and Archaea are known to change in total quantity and diversity by soil depth as organisms become specialized to accommodate the typically C- and nutrient-poor conditions found in deeper soil horizons [14, 42]. Anthrosols are fundamentally distinct from most tropical soils in that they often have dark brown or black A horizons that may extend 1 m or more deep [12]. Despite these deeper A horizons, the anthrosols in our study did not contain more extractable DNA at depth when compared to adjacent soils (Fig. 1a, b), as might be expected. However,

most probable number (MPN) counts of Bacteria in liquid R2 cultures have been shown in other studies to be significantly higher in these anthrosols as compared to their adjacent soil samples [39].

Organisms Identified through Cloning and Sequencing

The tight clustering observed in our dendrograms indicates that the anthrosols contain many shared OTUs between sites. Bands of interest from denaturing gradient gels were difficult to extract individually given their proximity to neighboring bands. Thus, selected bands were chosen both for their uniqueness and for their ability to be extracted independently from other nearby bands. Sequencing of clones exhibiting unique RFLP patterns, totaling 33 clones from common and unique bands from the Bacteria profiles and 10 clones from the Archaea profiles, showed that both the anthrosols and adjacent soils contain organisms that are taxonomically distinct from those found in sequence databases, with most Bacteria sequences matching database sequences at less than 98% similarity.

One band excised from the LG anthrosol, and also found at ACU, was of particular interest in that it matched at 93% similarity to the *Verrucomicrobia*. Although fairly well characterized in freshwater, *Verrucomicrobia* from soil is less described, and is an increasingly important phylum of Bacteria with very few cultivated representatives [5, 48]. Although *Verrucomicrobia* are present in many different ecosystems, in tropical environments they are especially common in paddy rice soil [51]. The ecological role of the *Verrucomicrobia* remains unknown. Two of our clones found only in the anthrosols grouped with this phylum, and at an especially low level of sequence similarity to those present in the database (93%).

Clones of Archaea were found to be of Crenarchaeota descent. Four anthrosol clones sequenced from the Archaea denaturing gradient gel profile were unique and clustered with crenarchaeotal database accessions derived from rice paddy soils (Fig. 9), although no match was exact (94–98% similarity). Archaea were once thought to be synonymous with extreme environments, however, more recent data suggest they are present in most environmental samples [46]. In 2006, it was revealed that archaeal ammonia oxidizers ('nitrifiers') are far more abundant in soils than their well-known bacterial counterparts [29], suggesting that Archaea may wield significant control over this key process in the global nitrogen cycle. Complementing this finding, DeLuca et al. have shown that BC produced after a forest wildfire increased gross and net nitrification rates [10, 11]. This finding raises questions about specific roles and identities of ammonia oxidizing Crenarchaeota in BC-rich soils such as the studied anthrosols. In particular, it is possible that the increase in soil nitrate observed by DeLuca and by others [27] in areas of increased BC concentrations may be related to specific subgroups of Archaea nitrifiers. Archaea bands from DS, a buried anthrosol and the oldest of the samples, were also found to be only distantly related to other Archaea in the database (94%). These differences in the DS microbial populations may be related to the buried condition of the soils, leading to reduced rates of oxygen and moisture, or else related to the fact that Dona Stella soils are thousands of years older than the anthrosols from the other sites.

This study significantly augments previous work done to identify soil organisms common in the soils of Brazil and other tropical regions [7]. We found that clones common in both anthrosols and adjacent soils matched at 97% to *Acidobacteria* sp., a genus that has been shown to be widely represented in both anthrosol and adjacent soils in a separate study in the western Amazon [23]. In this and our study, clones of the *Gamma* subdivision of the *Proteobacteria* were found to be common in adjacent soils. They were not detected in our anthrosols except in the DS soil. Borneman and Triplett [3] found that of the *Gamma Proteobacteria* clones identified in Amazonia (5% of total identified sequences, five clones), four were found in pasture soil and only one was derived from a mature tropical forest. Combined, these findings suggest either that *Gamma Proteobacteria* may have a competitive advantage over other soil bacteria in terms of ability to survive and reproduce in more degraded or lower fertility soils, such as the adjacent soils in this study, or that they are more readily cloned from environmental samples. Our study presents further evidence to support the idea that tropical soils serve as a habitat for a wide variety of novel microorganisms [3, 23].

Proposed Mechanisms

Observed differences in microbial community composition between anthrosols and adjacent soils, even within the same location, are no doubt related to the many edaphic differences between the two soil histories. The exact origin of these soils is still unknown and the heterogeneity of anthrosol types identified throughout the Amazon region points to no one source of added materials. It is likely that household waste production, burial activities, and agricultural production all contributed in some way to the formation of the anthrosols [37] and would explain the significantly higher P, Ca, and pH values (Table 1.) observed in anthrosols. Soil pH is known to be one of the primary predictive drivers of soil bacterial community diversity and richness [13], and supports the observed differences between the anthrosols and adjacent soils with divergent pH values in this study.

Anthropic modifications to the anthrosol parent material included additions of black carbon [18, 30, 31, 33]. Black

carbon forms as the result of the incomplete combustion of organic materials [26]. The estimated BC content of the sites was shown by Liang [31] to be 293% (HAT), 555% (LG), 65% (ACU), and 608% (DS) higher in the anthrosols than in their respective adjacent soils. Charred organic material indirectly alters soil biogeochemical cycling and microbial processes [22, 55] with mechanisms described as its high sorption properties and increased cation exchange capacity [31]. Microbes in anthrosols may colonize BC utilizing the porous physical nature of particles to protect themselves from soil predators [44], while residual bio-oils and materials adhered to the BC particle surface may also directly support microbial life [52], effectively selecting for organisms that are able to utilize nutrients adsorbed to its surface. High concentrations of BC have been shown to increase nitrogen mineralization potential, biological nitrogen fixation, and nitrate availability [10, 11, 47, 56].

At temperatures above 200°C, thermal decomposition of wood char occurs and decreases the ability of microbes to directly mineralize BC materials [1]. The long residence time and recalcitrance of BC [31] may suggest that it is not itself being used as a source of cell C or energy by microbial populations; but that a slow chemical oxidation and mineralization of soil BC is responsible for much of the loss of BC mass over time that has been observed in some studies [45]. Due to its resistance to microbial decomposition [18, 26, 31, 50], direct surface oxidation of BC may be more chemical than microbial in nature [49]. However, co-metabolism of BC with the addition of glucose has been reported, with authors concluding that BC in soils may promote growth of microorganisms and the decomposition of associated labile C compounds [19].

We have determined that in the case of anthrosols in the Brazilian Amazon, historical land use, ostensibly by the indigenous indios 600–8,700 years bp, has a greater influence over soil microbial community composition than recent soil management practices. We found strong similarities between the microbial communities present in anthrosols for both Bacteria and Archaea rDNA amplicons, despite site differences in current land uses. Edaphic trends across all sites, including higher P, Ca, pH, and BC concentrations in anthrosols as compared to the adjacent soils, suggest that historical land use sufficiently altered soil properties and determined the assemblages of soil Bacteria and Archaea populations observed in the present day. Microbial communities in the anthrosols at various depths shared common OTUs demonstrated by the clustering of some upper and lower horizon samples between sites. Major differences in community diversity were observed between anthrosols and adjacent soils, even within the same site. Among the novel Bacteria we identified in the anthrosols were *Verrucomicrobia*, a division commonly found in rice paddies in the tropics. We also found that our clones of Archaea from the anthrosols clustered with GeneBank entries obtained from rice paddy soils. While a range of soil properties may contribute to the positive agricultural effects observed in tropical anthrosols, enthusiasm and interest is building around the idea of using BC as a soil amendment [17], specifically by replicating the sustained soil fertility observed in anthrosols even under conditions of extreme weathering. To take full advantage of anthrosols' purported positive attributes, it is critical to first understand the ways in which anthrosol properties influence soil microbial populations and the biogeochemical cycling processes they control.

References

1. Baldock JA, Smernik RJ (2002) Chemical composition and bioavailability of thermally altered *Pinus resinosa* (Red pine) wood. Org Geochem 33:1093–1109
2. Borga P, Nilsson M, Tunlid A (1994) Bacterial communities in peat in relation to botanical composition as revealed by phospholipid fatty acid analysis. Soil Biol Biochem 26:841–848
3. Borneman J, Triplett E (1997) Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl Environ Microbiol 63:2647–2653
4. Bossio DA, Scow KM, Gunapala N, Graham KJ (1998) Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microb Ecol 36:1–12
5. Buckley DH, Schmidt TM (2001) Environmental factors influencing the distribution of rRNA from Verrucomicrobia in soil. FEMS Microbiol Ecol 35:105–112
6. Carney K, Matson P (2005) Plant communities, soil microorganisms, and soil carbon cycling: does altering the world belowground matter to ecosystem functioning? Ecosystems 8:928–940
7. Carney KM, Matson PA (2006) The influence of tropical plant diversity and composition on soil microbial communities. Microb Ecol 52:226–238
8. Cleveland CC, Townsend AR, Schmidt SK, Constance BC (2003) Soil microbial dynamics and biogeochemistry in tropical forests and pastures, southwestern Costa Rica. Ecol Appl 13:314–326
9. Culman SW, Duxbury JM, Lauren JG, Thies JE (2006) Microbial community response to soil solarization in Nepal's rice-wheat cropping system. Soil Biol Biochem 38:3359–3371
10. DeLuca TH, MacKenzie MD, Gundale MJ, Holben WE (2006) Wildfire-produced charcoal directly influences nitrogen cycling in ponderosa pine forests. Soil Sci Soc Am J 70:448–453
11. DeLuca TH, Sala A (2006) Frequent fire alters nitrogen transformations in ponderosa pine stands of the inland northwest. Ecology 87:2511–2522
12. Eden MJ, Bray W, Herrera L, McEwan C (1984) Terra preta soils and their archaeological context in the caqueta basin of southeast Colombia. Am Antiquity 49:125–140
13. Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. Proc Natl Acad Sci U S A 103:626–631
14. Fierer N, Schimel JP, Holden PA (2003) Variations in microbial community composition through two soil depth profiles. Soil Biol Biochem 35:167–176
15. Gauch H Jr (1992) Statistical analysis of regional yield trials: AMMI analysis of factorial designs. Elsevier Science Publishers, Amsterdam
16. Gauch H Jr, Furnas R (1991) Statistical analysis of yield trials with MATMODEL. Agron J 83:916–920

17. Glaser B (2007) Prehistorically modified soils of central Amazonia: a model for sustainable agriculture in the twenty-first century. Philos Trans R Soc B-Biol Sci 362:187–196
18. Glaser B, Guggenberger G, Haumaier L, Zech W (2001) Persistence of soil organic matter in archaeological soils (Terra Preta) of the Brazilian Amazon region. In: Rees B, Ball B, Campbell C, Watson C (eds) Sustainable management of soil organic matter. CAB International, Wallingford, pp 190–194
19. Hamer U, Marschner B, Brodowski S, Amelung W (2004) Interactive priming of black carbon and glucose mineralisation. Organ Geochem 35:823–830
20. Henrot J, Robertson GP (1994) Vegetation removal in two soils of the humid tropics—effect on microbial biomass. Soil Biol Biochem 26:111–116
21. Heuer H, Krsek M, Baker P, Smalla K, Wellington EMH (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63:3233–3241
22. Ishii T, Kadoya K (1994) Effects of charcoal as a soil conditioner on citrus growth and vesicular arbuscular mycorrhizal development. J Jpn Soc Hortic Sci 63:529–535
23. Kim JS, Sparovek G, Longo RM, De Melo WJ, Crowley D (2007) Bacterial diversity of terra preta and pristine forest soil from the Western Amazon. Soil Biol Biochem 39:684–690
24. Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163
25. Lane DJ (1991) 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. Wiley, New York, pp 115–117
26. Lehmann J (2007) Bio-energy in the black. Front Ecol Environ 5:381–387
27. Lehmann J, da Silva JP, Steiner C, Nehls T, Zech W, Glaser B (2003) Nutrient availability and leaching in an archaeological Anthrosol and a Ferralsol of the Central Amazon basin: fertilizer, manure and charcoal amendments. Plant Soil 249:343–357
28. Lehmann J, Kern D, German L, McCann J, Van Coimbra MG, Moreira A (2003) Soil fertility and production potential. In: Lehmann J, Kern D, Glaser B, Woods WI (eds) Amazonian dark earths: origin, properties and management. Kluwer Academic Publishers, Boston, pp 105–124
29. Leininger S et al (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442:806–809
30. Liang B et al (2006) Black Carbon increases cation exchange capacity in soils. Soil Sci Soc Am J 70:1719–1730
31. Liang B et al (2008) Stability of biomass-derived black carbon in soils. Geochim Cosmochim Acta 72:6069–6078
32. Luizao RCC, Bonde TA, Rosswall T (1992) Seasonal variation of soil microbial biomass—the effects of clearfelling a tropical rainforest and establishment of pasture in the central amazon. Soil Biol Biochem 24:805–813
33. Marris E (2006) Putting the carbon back: black is the new green. Nature 442:624–626
34. Moeseneder MM, Arrieta JM, Muyzer G, Winter C, Herndl GJ (1999) Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl Environ Microbiol 65:3518–3525
35. Muyzer G, Dewaal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S ribosomal rna. Appl Environ Microbiol 59:695–700
36. Neill BE (2007) Microbial communities in Amazonian dark earth soils analyzed by culture-based and molecular approaches. In: Crop and Soil Sciences vol. M.S. Cornell University, Ithaca, NY, p 76
37. Neves EG, Petersen JB, Bartone RN, Augusto Da Silva C (2003) Historical and socio-cultural origins of Amazonian dark earths. In: Lehmann J, Kern D, Glaser B, Woods WI (eds) Amazonian dark earths: origin, properties, management. Kluwer Academic Publishers, London, pp 29–50
38. Nusslein K, Tiedje JM (1999) Soil bacterial community shift correlated with change from forest to pasture vegetation in a tropical soil. Appl Environ Microbiol 65:3622–3626
39. O'Neill B, Grossman JM, Tsai MT, Gomes JE, Lehmann J, Peterson J, Neves E, Thies JE (2009) Bacterial community composition in Brazilian anthrosols and adjacent soils characterized using culturing and molecular identification. Microb Ecol 58:23–35
40. Ovreas L, Forney L, Daae FL, Torsvik V (1997) Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol 63:3367–3373
41. Paterson E, Gebbing T, Abel C, Sim A, Telfer G (2007) Rhizodeposition shapes rhizosphere microbial community structure in organic soil. New Phytol 173:600–610
42. Pesaro M, Widmer F (2002) Identification of novel Crenarchaeota and Euryarchaeota clusters associated with different depth layers of a forest soil. FEMS Microbiol Ecol 42:89–98
43. Petersen JB (2001) Gift from the past: terra preta and prehistroic amerindian occupation in Amazonia. In: McEwan C (ed) Unknown Amazonia. British Museum, London, pp 86–105
44. Pietikainen J, Kiikkila O, Fritze H (2000) Charcoal as a habitat for microbes and its effect on the microbial community of the underlying humus. Oikos 89:231–242
45. Preston CM, Schmidt MWI (2006) Black (pyrogenic) carbon: a synthesis of current knowledge and uncertainties with special consideration of boreal regions. Biogeosciences 3:397–420
46. Robertson CE, Harris JK, Spear JR, Pace NR (2005) Phylogenetic diversity and ecology of environmental Archaea. Cur Opin Microbiol 8:638–642
47. Rondon MA, Lehmann J, Ramirez J, Hurtado M (2007) Biological nitrogen fixation by common beans (*Phaseolus vulgaris* L.) increases with bio-char additions. Biol Fertil Soils 43:699–708
48. Sangwan P, Chen XL, Hugenholtz P, Janssen PH (2004) *Chthoniobacter flavus* gen. nov., sp. nov., the first pure-culture representative of subdivision two, spartobacteria classis nov., of the phylum *Verrucomicrobia*. Appl Environ Microbiol 70:5875–5881
49. Schmidt MWI, Kogel-Knabner I (2002) Organic matter in particle-size fractions from A and B horizons of a Haplic Alisol. Eur J Soil Sci 53:383–391
50. Schmidt MWI, Skjemstad JO, Jager C (2002) Carbon isotope geochemistry and nanomorphology of soil black carbon: black chernozemic soils in central Europe originate from ancient biomass burning. Global Biogeochem Cycles 16:70.71–70.78
51. Tanahashi T, Murase J, Matsuya K, Hayashi M, Kimura M, Asakawa S (2005) Bacterial communities responsible for the decomposition of rice straw compost in a Japanese rice paddy field estimated by DGGE analysis of amplified 16S rDNA and 16S rRNA fragments. Soil Sci Plant Nutr 51:351–360
52. Thies JE, Rillig MC (2008) Characteristics of biochar: biological properties. In: Lehmann J, Joseph S (eds) Biochar for environmental management. Earthscan, Dunstan House, London
53. Thies JE, Suzuki K (2003) Amazonian dark earths: biological measurements. In: Lehmann J, Kern D, Glaser B, Woods WI (eds) Amazonian dark earths: origin, properties and management. Kluwer Academic Publishers, Norwell, pp 287–332
54. Tipper JC (1979) Rarefaction and rarefiction—use and abuse of a method in paleoecology. Paleobiology 5:423–434
55. Warnock DD, Lehmann J, Kuyper TW, Rillig MC (2007) Mycorrhizal responses to biochar in soil—concepts and mechanisms. Plant Soil 300:9–20
56. Yamato M, Okimori Y, Wibowo IF, Anshori S, Ogawa M (2006) Effects of the application of charred bark of *Acacia mangium* on the yield of maize, cowpea and peanut, and soil chemical properties in South Sumatra, Indonesia. Soil Sci Plant Nutr 52:489–495