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Molecular Microbial Diversity in Soils from Eastern Amazonia: Evidence for Unusual Microorganisms and Microbial Population Shifts Associated with Deforestation

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Although the Amazon Basin is well known for its diversity of flora and fauna, this report represents the first description of the microbial diversity in Amazonian soils involving a culture-independent approach. Among the 100 sequences of genes coding for small-subunit rRNA obtained by PCR amplification with universal small-subunit rRNA primers, 98 were bacterial and 2 were archaeal. No duplicate sequences were found, and none of the sequences had been previously described. Eighteen percent of the bacterial sequences could not be classified in any known bacterial kingdom. Two sequences may represent a unique branch between the vast majority of bacteria and the deeply branching, predominantly thermophilic bacteria. Five sequences formed a clade that may represent a novel group within the class *Proteobacteria*. In addition, rRNA intergenic spacer analysis was used to show significant microbial population differences between a mature forest soil and an adjacent pasture soil.

The Amazon region contains the largest body of fresh water and the largest rain forest in the world. It is also well known for its enormous diversity of flora and fauna, including 15,000 documented animal species, 8,000 of which were new to biology when discovered (30). At least 40% of the world's freshwater fish and 25% of the world's bird species reside in Amazonia. Over 5,000 tree species have been described in the region, 235 of which were found in one hectare in central Amazonia (32).

Perhaps the best way to illustrate the vast biodiversity of the Amazon region is to review the work of T. L. Erwin. Erwin examined beetle species in the tree canopies of four forests in central Amazonia near Manaus, Brazil (11). He found that 83% of the beetle species were unique to one of the forests, 14% of the species were found in two of the four forests, and only 1% were found in all four forests. In an upland Amazonian forest near Tambopata, Peru, Erwin examined the beetle species in the tree canopy of two forests 50 m apart over three seasons. These two nearby forests shared only 8.7% of all of the species identified (12). These results illustrate the basis for Erwin's estimate that, rather than there being 1.5 million species on earth, there are at least 30 million species of insects alone (10).

Even with all of the work that has been done on biodiversity in the Amazon to date, clearly much more work is needed to understand the enormous genetic complexity of this region. This is even more true of microbial life in Amazonia. A broad study of bacterial carbon metabolism in the Amazon River, which included a determination of the number of bacteria per liter of Amazon River water, was recently published (5). In addition, a monumental compilation of soil carbon levels in the Amazon region of Brazil has been completed (25).

However, very few studies have examined specific soil microbial populations in the Amazon region. Manfio and Goodfellow (24) and Linhares (22) described populations of *Streptomyces* and actinomycetes, respectively, from the Amazon rain forest. These studies used culture-based isolation techniques, which have been shown to isolate only 1% of the total soil microbial community (3, 13, 18, 34, 40, 41). Fortunately, descriptions of these uncultured microorganisms can now be obtained by using analyses of the small-subunit rRNA genes (SSU rDNA) (1, 29, 44).

In this work, we examine the microbial diversity found in two eastern Amazonian soils by analysis of 100 SSU rDNA sequences. We also illustrate the impact of deforestation on the soil microbial community by analysis of an intergenic spacer region between the small- and large-subunit rRNA genes.

MATERIALS AND METHODS

Site description and soil collection. On 15 February 1996, soil samples were obtained from a mature forest and an active pasture at the Fazenda Vitoria (Victory Ranch), located near the town of Paragominas, Para State, Brazil (2°59′S, 47°31′W) (26, 27). This area represents a typical region of logging and deforestation in eastern Amazonia. The pasture was established in 1969 and planted with *Brachiaria humidicola*. Soil samples were obtained by removing the leaf litter and collecting the top 10 cm of soil. The samples were immediately frozen in liquid nitrogen (−196°C) and then stored at −70°C after their arrival at our laboratory in Madison, Wis.

SSU rDNA library construction. DNA was extracted from soil, and SSU rDNA clone libraries were constructed essentially as described previously (8). Exceptions included a further purification of the soil DNA by agarose gel isolation of DNA larger than 10 kb with β-Agarase 1 (New England Biolabs). SSU rRNA genes were amplified by 30 cycles of PCR using the universal SSU rDNA PCR primers 530F (**TGACTGACTGA**GTGCCAGCMGCCGCGG) and 1492R (**TGACTGACTGA**GAGCTCTACCTTGTTACGMYTT). Each primer contained an additional sequence (in boldface type) that has the stop codon, TGA, in all three reading frames. This addition reduces the possibility of producing false blue colonies when screening the clones by α-complementation. SSU rDNA clones were screened for orientation by digestion with *Sac*I (underlined sequence of the 1492R primer). Determining orientation enabled the direct comparison of clones because we were able to sequence the same ~500-base region of the SSU rDNA molecule.

The P36b sequence was obtained by PCR amplification using primers designed to hybridize to P36a, P36F (GGTGGATTAGATAGTTACTC) and P36R (GGCCTTTAAGGTATCTCC), and by using the amplification product from the universal PCR (described above) as the template. The 100-μl PCR mixtures contained the following ingredients at the indicated final concentrations or total amounts: 1 μl of template DNA, 50 mM Tris (pH 8.3), 500 μg of bovine serum albumin per ml, 2.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates

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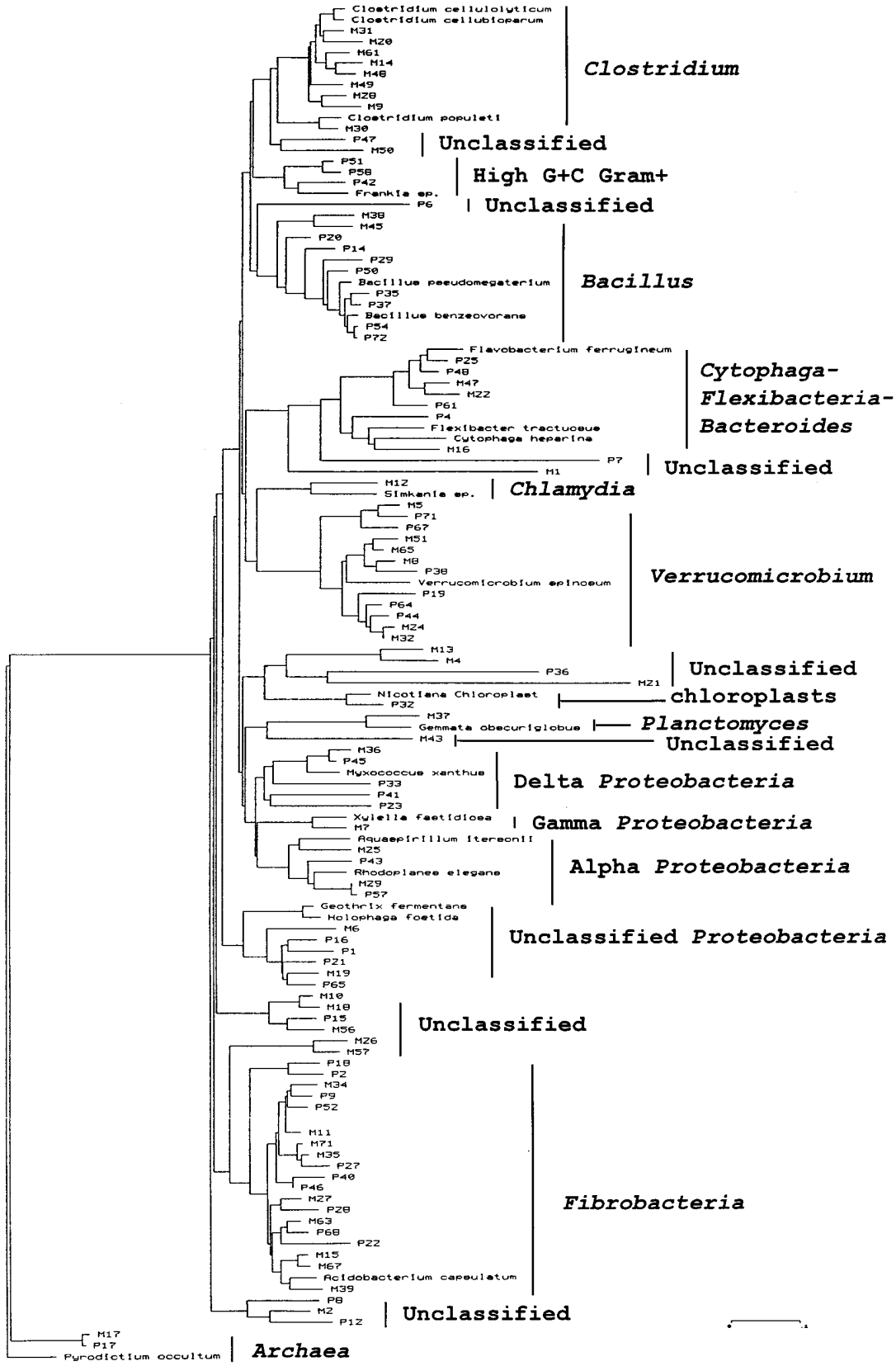

FIG. 1. Phylogenetic analysis of 100 SSU rDNA sequences from Amazonian forest and pasture soils. Soil clones are represented by either a P (reformed pasture) or an M (mature forest), followed by a clone number. Names of the major taxa are shown in boldface type. Approximately 500 bases of each clone were sequenced, from position 531 to position 1031 (*E. coli* positions). The number of changes per sequence position, at the median rate, is shown on the scale bar. Soil analysis was done by the Soil Testing Laboratory of the University of Wisconsin—Madison as described by Schulte et al. (37).

TABLE 1. Summary of the phylogenetic assignments of 100 Amazonian forest and pasture soil SSU rDNA clones (obtained from the tree analysis [Fig. 1])

Major taxon	Phylogenetic assignment of 50 clones each from:	
	Mature forest	Pasture
Archaea	1	1
Crenarchaeota	1	1
Bacteria	49	49
Chloroplasts	0	1
Planctomyces and relatives	8	6
Chlamydia	1	0
Planctomyces	1	0
Verrucomicrobium	6	6
Clostridium and relatives	11	8
Bacillus	2	8
Clostridium	9	0
High G+C gram positive	0	3
Cytophaga-Flexibacteria-Bacteroides	3	4
Fibrobacterium	9	10
Proteobacteria	6	10
α subdivision	2	2
β subdivision	1	0
δ subdivision	1	4
ζ subdivision	2	4
Unclassified	12[a]	7
Eucarya	0	0

[a] One unclassified (Fig. 1) clone, M21, was determined to be related to a mitochondrial SSU rDNA sequence.

(dNTPs), 400 nM (each) primer, and 5 U of *Taq* polymerase. The reagents were combined and heated to 94°C for 1 min. Forty cycles of PCR were performed with an Air Thermo-Cycler (Idaho Technologies) at 94°C for 0 s, 60°C for 10 s, and 72°C for 20 s, followed by elongation at 72°C for 3 min.

SSU rDNA clone sequencing and analyses. Approximately 500 bases (position 531 to position 1031, *Escherichia coli* positions) of 50 pasture and 50 forest clones were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer). For selected clones, designated in the figure legends, the entire region from position 531 to position 1492 (*E. coli* positions) was sequenced. Sequence identities were determined with only alignable positions. The phylogenetic tree analysis of the 100 SSU rDNA sequences (Fig. 1) was done by aligning the sequences with PILEUP (Genetics Computer Group, Madison, Wis.), calculating a pairwise Jukes-Cantor distance matrix (19), and performing a neighbor-joining analysis (35). Maximum-likelihood tree analyses (15) were done with fastDNAml 1.0 (28) and empirical base frequencies. CHECK_CHIMERA (http://rdp.life.uiuc.edu) analysis of the 100 SSU rDNA clones used in this study suggests that none of the sequences were artifacts created by PCR amplification (23).

rRNA intergenic spacer analysis (RISA). Representative soil samples from the mature forest and pasture were obtained by mixing four replicate samples, collected from locales 10 m apart. DNA was extracted from these soil samples as described above. The intergenic spacer region between the SSU and large-subunit rRNA genes was amplified in 200-μl PCR mixtures at the following final concentrations or total amounts: 500 ng of soil DNA, 50 mM Tris (pH 8.3), 500 μg of bovine serum albumin per ml, 2.5 mM $MgCl_2$, 250 μM dNTPs, 400 nM (each) primer, and 10 U of *Taq* polymerase. The PCR primers were 1406F (TGYACACACCGCCCGT) (universal rRNA small subunit) and 23SR (GGGTTBCCCCATTCRG) (bacterial 23S rRNA large subunit). All reagents were combined and heated at 94°C for 1 min. Twenty-five cycles of PCR were performed with an Air Thermo-Cycler (Idaho Technologies) at 94°C for 15 s, 56°C for 15 s, and 72°C for 30 s, followed by elongation at 72°C for 1 min. The PCR products were precipitated with ethanol, resuspended in 18 μl of Tris-EDTA, and denatured by addition of 10.8 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF) and heating at 80°C for 10 min. The DNA was loaded on a prerun (45 min) 5% polyacrylamide–Tris-borate-EDTA gel (1.5 mm thick, 36 cm long) containing 7 M urea and electrophoresed at 75 W for 6 h. The polyacrylamide was cross-linked at a 30:1 ratio of acrylamide to bisacrylamide. Gels were silver stained with SILVER SEQUENCE DNA staining reagents (Promega) or by the protocol described by Bassam et al. (4).

Nucleotide sequence accession numbers. Clones M1 to M71 were assigned GenBank accession numbers U68589 to U68637; clones P1-P35, P36a, and P37-P72 were assigned accession numbers U68638 to U68688; and P36b was assigned accession number U86604.

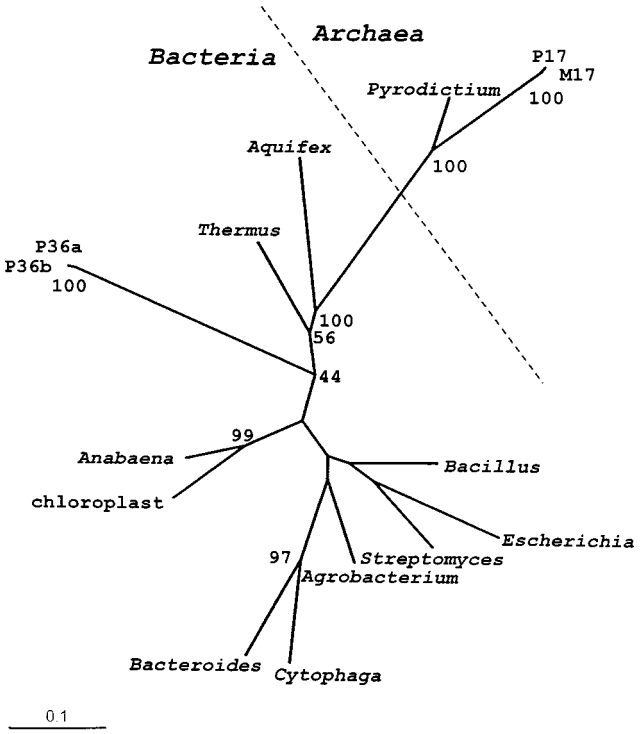

FIG. 2. A maximum-likelihood analysis of soil SSU rDNA clones (P17, M17, P36a, and P36b) using 808 alignable nucleotide positions. Numbers at the nodes represent the percentages of 500 bootstrap replications that support the branching pattern radiating away from the node (14). Bootstrap percentages less than 44% are not shown. The number of changes per sequence position, at the median rate, is shown on the scale bar. Accession numbers for the reference sequences are as follows: D14505, K00637, L10067, M21087, M58768, M58781, M83548, X56806, X59559, U00006, and X60514.

RESULTS

Microbial diversity. The microbial diversity found in Amazonian forest and pasture soils is illustrated by a phylogenetic tree analysis of 100 SSU rDNA sequences (Fig. 1 and Table 1). Ninety-eight percent of the clones were of bacterial origin. Seventy-eight percent were from the bacterial phyla *Planctomyces* and relatives, *Clostridium* and relatives, high-G+C gram positive, *Cytophaga-Flexibacter-Bacteroides*, *Fibrobacterium*, and *Proteobacteria*. Two percent of the clones were from the archaeal kingdom *Crenarchaeota*, while none were from the kingdom *Eucarya*.

Novel SSU rDNA sequences. Our analysis of the Amazonian soils found numerous SSU rDNA clones that were only distantly related to known sequences. A maximum-likelihood analysis of two of these clones, P36a and P36b, suggests that they may represent an evolutionary intermediate between the vast majority of bacteria and the deeply branching, predominantly thermophilic bacteria (Fig. 2). Their bifurcating stem-loop structure at helix 35 (Fig. 3) supports a bacterial classification, since both archaea and eucarya possess an unbranched stem-loop structure at this location (16, 46).

However, other characteristics of these sequences suggest that they are phylogenetically distant from most bacteria. P36a has a 15-base deletion in helix 41 and a 10-base insertion in helix 35 (Fig. 3) compared with *E. coli*. In addition, P36a and P36b deviate from bacteria at 4 of 34 (11.8%) and 4 of 28 (14.3%) bacterial signature nucleotides, respectively (Fig. 3).

Other notable SSU rDNA sequences included five clones obtained from the forest and pasture soils. A maximum-like-

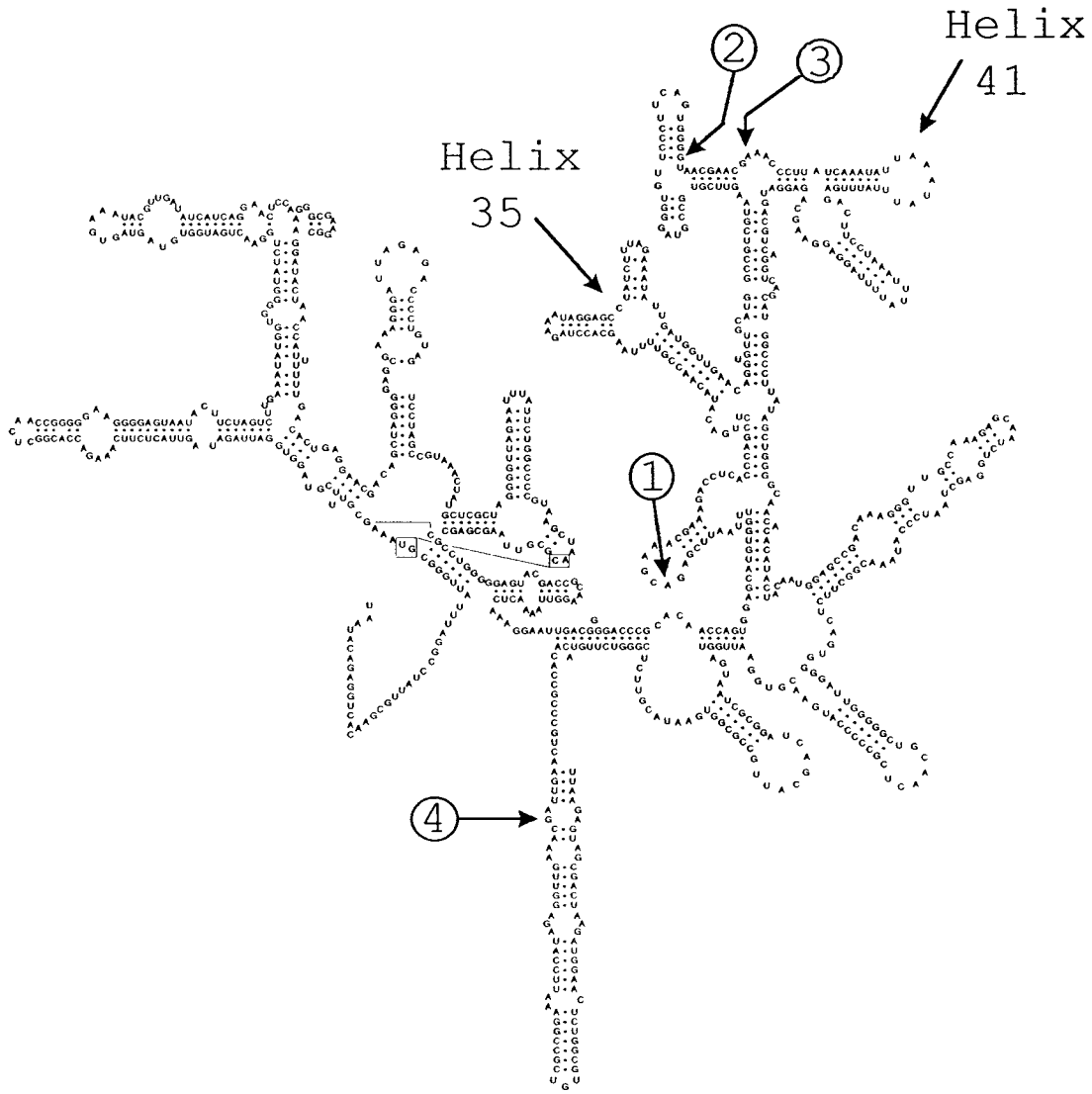

FIG. 3. Secondary structure of the partial (943 bases) SSU rDNA clone P36a, from the pasture soil. The numbered arrows indicate signature nucleotides that deviate from the bacterial domain. The nucleotides at arrow numbers two through four are archaeal or eucaryotic signatures, whereas the A residue at arrow one is an unclassified domain signature. The structure was illustrated with CARD (http://rrna.uia.ac.be).

lihood analysis of these clones suggests that they are related to *Holophaga foetida*, *Geothrix fermentans*, and *Acidobacterium capsulatum* (Fig. 4).

RISA. RISA was performed on DNA extracted from a mature forest and an adjacent pasture soil (Fig. 5). This procedure produced hundreds of discrete RISA bands, which ranged in size from approximately 400 bases to 1,400 bases. The different banding patterns between the pasture soil DNA (lanes 2 and 3) and the mature forest soil DNA (lanes 4 and 5) suggest that each soil contains unique microbial populations.

DISCUSSION

Microbial diversity. The microbial diversity found in the mature forest and pasture soils from eastern Amazonia is immense. This assertion is supported by the facts that none of the 100 SSU rDNA clones that we sequenced were duplicates and none had been described previously. Most of these sequences were bacterial. The presence of two archaeal sequences was unexpected because only one partial archaeal SSU rDNA sequence had been reported from soil, in analyses of SSU rDNA clones obtained by PCR amplification with universal SSU rDNA primers (8, 42). This low ratio of archaeal to bacterial sequences suggests that soil may be an inhospitable environment for archaea, in contrast to the marine environment, where archaea can constitute up to 34% of the prokaryotic population (9).

Our Amazonian archaeal clone sequences are most similar to 10 crenarchaeotal sequences isolated from a Wisconsin soil by Bintrim et al. (6) (Fig. 6). However, they are significantly different from the crenarcheotal and euryarcheotal sequences found in the humus layer of a Finnish forest soil (20, 36) (Fig. 6). The discovery of these new Finnish sequences suggests a greater phylogenetic diversity of terrestrial archaea than was previously thought, consisting of both *Crenarchaeota* and *Euryarchaeota*.

Novel SSU rDNA sequences. Nineteen of these Amazonian SSU rDNA clone sequences are distantly related to known

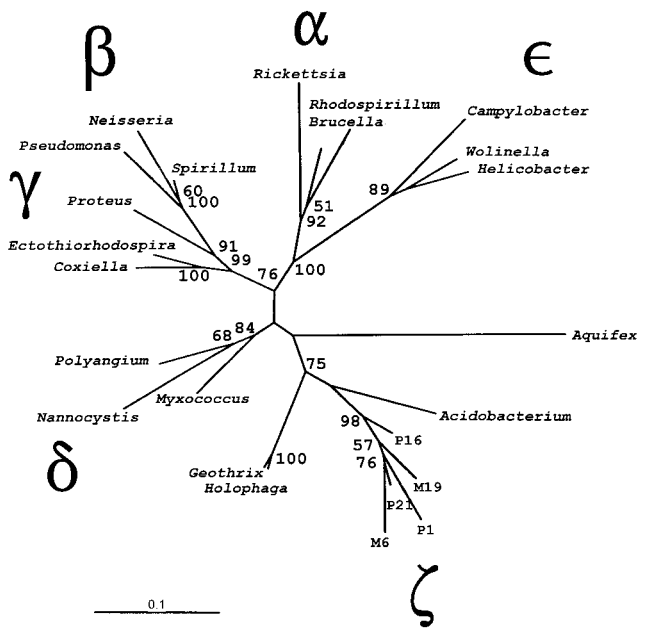

FIG. 4. A maximum-likelihood analysis of soil SSU rDNA clones (M6, M19, P1, P16, and P21) using 850 alignable nucleotide positions. Numbers at the nodes represent the percentages of 100 bootstrap replications that support the branching pattern radiating away from the node (14). Bootstrap percentages less than 50% are not shown. The number of changes per sequence position, at the median rate, is shown on the scale bar. Accession numbers for the reference sequences are as follows: D26171, D30778, L04315, M11224, M34114, M34131, M83548, M59151, M88159, M94279, M94282, U41563, U55820, X07652, X13695, X77215, Y11500, Z22776, and Z25741.

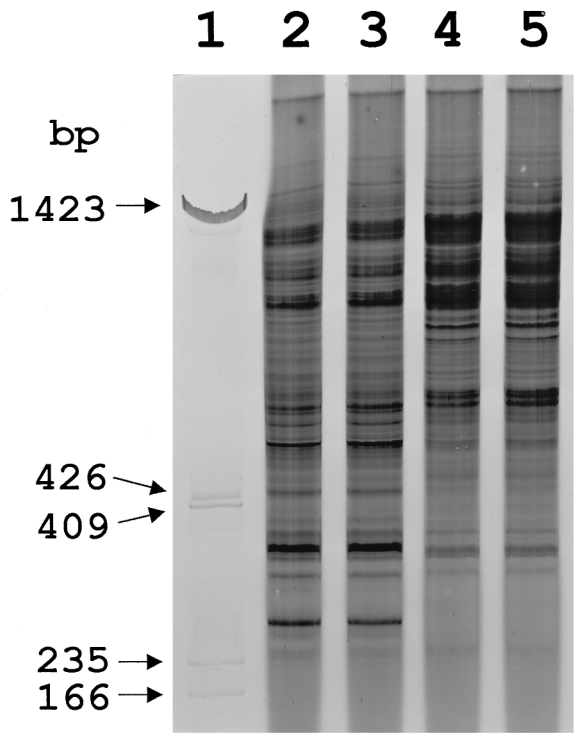

FIG. 5. RISA from Amazonian forest and pasture soils. The intergenic spacer region was PCR amplified and resolved on a denaturing 5% polyacrylamide gel. Lanes: 1, molecular weight markers obtained by *Dde*I restriction digestion of pGEM3Zf(+); 2 and 3, replicate RISA experiments using pasture soil DNA as the template; 4 and 5, replicate RISA experiments using mature forest soil DNA as the template.

sequences (Table 1). Considering only alignable positions, an analysis of these unclassified sequences shows that 17 have between 77 and 85% sequence identity to their closest cultured relatives. Thus, many of these sequences may represent new bacterial kingdoms, based on an interkingdom identity range of 70 to 85% (46). None of the sequences showed 97% or greater homology to the SSU rRNA sequence of any classified organism or any uncultured organism in the databases. Thus, given the bacterial species definition of Stackebrandt and Goebel (38), none of the sequences that we obtained are derived from any known microbial species.

The most unusual of these sequences are the pasture soil DNA sequences P36a and P36b. Based on their phylogenetic distance from sequences of known bacteria, these sequences appear to have a fast evolutionary clock as described by Woese (46). Indeed, the number of nucleotide substitutions between the P36 sequences and those of their closest relative, *Thermus fiji*, is slightly greater than the number of substitutions between the sequence of the archaeon *Pyrodictium occultum* and that of the bacterium *Aquifex pyrophilus* (Fig. 2). The extent of their sequence divergence from most bacteria can also be noted in their 11.8 to 14.3% deviance from the bacterial signature nucleotide consensus. With these domain-specific signature nucleotides, described previously by Woese and colleagues (45, 46), and assuming that each of these positions is independent of any other, the probabilities that any bacterial sequence would diverge at 11.8 and 14.3% of that domain's signature nucleotides are 0.0004 and 0.0002, respectively.

Our phylogenetic tree analysis (Fig. 2) suggests that the P36 sequences may represent an evolutionary intermediate between the clades containing thermophilic bacteria and those containing only nonthermophilic bacteria. However, such a classification must be considered tentative, because of the low (44%) bootstrap support.

Five other clones obtained from the forest and pasture soils formed a clade strongly supported by bootstrap replications (Fig. 4). Their sequences have homology to the SSU rDNA sequences of *Holophaga foetida*, *Geothrix fermentans*, *Acidobacterium capsulatum*, and some members of the δ-*Proteobacteria*. Previous reports have suggested that *Holophaga foetida* belongs to an unclassified group of *Proteobacteria* and that *Acidobacterium capsulatum* may represent a new bacterial division (17, 21). Our analysis of these sequences suggests that they share a common lineage, which may represent a new subdivision of the *Proteobacteria*, the ζ-*Proteobacteria*.

One of our Amazonian clones, M12, was closely related to the human-pathogenic genus *Chlamydia* (Fig. 1). Chlamydiae are difficult organisms to study, as they cannot be grown in culture media (39). Our results in this study and those in a previous report (8) suggest that chlamydiae may be more common in nature than was previously thought.

Possible effects of deforestation on soil microbial communities. Deforestation of tropical forests alters many soil properties. Previous studies have shown that conversion to pasture causes an increase in pH, NH_4^+, and soil bulk density as well as a decrease in NO_3^- and soil porosity (31, 33). The data in Table 2 show that our Amazonian soils also have undergone a similar transformation. In this study, we were interested in determining whether the soil microbial community is affected by the soil changes associated with deforestation.

Comparison of the SSU rDNA clones obtained from the mature forest soils and pasture soils suggests differences between the two sites (Fig. 1 and Table 1). A greater number of

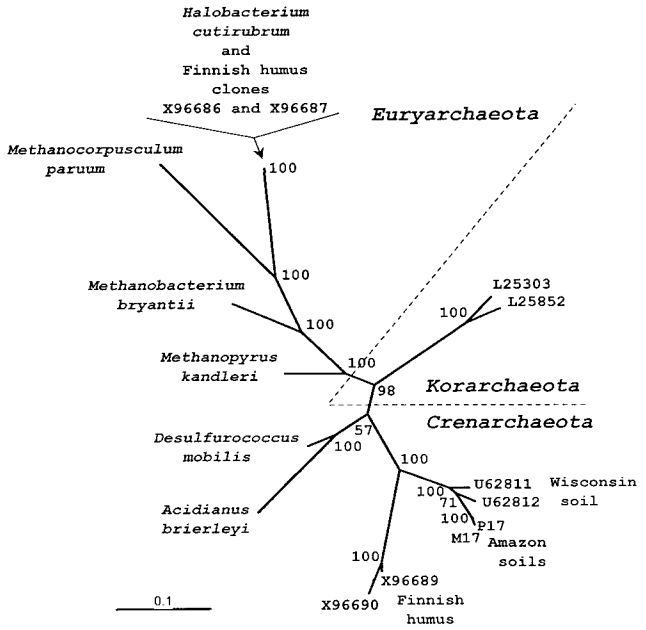

FIG. 6. A maximum-likelihood analysis of soil archaeal SSU rDNA clones (M17 and P17) using 1,266 alignable nucleotide positions. Numbers at the nodes represent the percentages of 100 bootstrap replications that support the branching pattern radiating away from the node (14). The number of changes per sequence position, at the median rate, is shown on the scale bar. Accession numbers for the reference sequences are as follows: K02971, M36474, M59124, M59147, M59932, and U38359.

unclassified organisms are found in the forest than in the pasture. Also, 18% of the forest clone sequences are related to the clostridria, while none of the pasture clone sequences are similar to sequences of that genus. Conversely, four times more sequences found in the pasture clones were related to the *Bacillus* sequences than those found in the forest, and high-G+C gram-positive-like sequences were found only in the pasture clones.

However, because of the immense and uncharacterized diversity of soil microorganisms, as well as the significant compositional heterogeneity of soil, a sequence analysis of 100 SSU rDNA clones is insufficient to describe changes in the microbial community that occur with deforestation. Consequently, we developed a simple method that allows a rapid examination of the microbial community structure in soil. This method utilizes the size heterogeneity associated with RISA to differentiate a wide variety of bacterial phyla in soil.

The differences between the microbial communities inhabiting the forest soil and the adjacent pasture soil are illustrated by different RISA banding patterns obtained from each site (Fig. 5). Each soil type shows numerous bands that are unique to that environment. These differences are presumably the result of the different soil properties associated with the conversion to pasture.

TABLE 2. Soil chemical and physical parameters

Parameter	Mature forest	Pasture
pH	5	6
% Organic matter (loss of wt on ignition)	8	11
P (ppm)	6	5
K (ppm)	40	155
Total N (ppm)	2,313	3,112
NH_4-N	17	57
NO_3-N	35	14
% Sand	40	68
% Silt	31	18
% Clay	29	14

The phylogenetic identification of the organisms represented by the RISA bands can be obtained by excising the bands, extracting and cloning the DNA, and then sequencing the SSU rRNA region (approximately 138 bases) of the RISA molecule. This region contains the hypervariable helix 49 (43), which has been previously shown to provide useful phylogenetic identifications (7). Alternatively, a larger portion of the SSU rDNA molecule could be obtained to provide more phylogenetic information. This could be accomplished by PCR amplification of soil DNA using a primer designed to hybridize the sequence of interest and a universal rRNA primer.

This report, as well as others, provides several lines of evidence which suggest that tropical soils and soils from other regions serve as habitats for novel microorganisms. First, the work of Torsvik, Atlas, and colleagues (2, 40) shows that thousands of different genomes exist in a small soil sample. These results are supported by the enormous number of RISA bands shown in Fig. 5. Second, the vast majority of soil microorganisms cannot be cultivated by current techniques and therefore have remained unclassified (3, 13, 18, 34, 40, 41). Third, the molecular phylogenetic surveys of soil have all shown immense and previously undescribed diversity (8, 42, and this work). Finally, the phylogenetic analysis of PCR-amplified SSU rDNA genes from Amazonian soils provides evidence for unusual organisms that cannot be classified in any known kingdom of *Bacteria* (Fig. 1 and 2).

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REFERENCES

1. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
2. **Atlas, R. M., A. Horowitz, M. Krichevsky, and A. K. Bej.** 1991. Response of microbial populations to environmental disturbance. Microb. Ecol. **22:**249–256.
3. **Bakken, L. R.** 1985. Separation and purification of bacteria from soil. Appl. Environ. Microbiol. **49:**1482–1487.
4. **Bassam, B. J., G. Caetano-Anolles, and P. M. Gresshoff.** 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. **196:**80–83.
5. **Benner, R., S. Opsahl, G. Chinleo, J. E. Richey, and B. R. Forsberg.** 1995. Bacterial carbon metabolism in the Amazon River system. Limnol. Oceanogr. **40:**1262–1270.
6. **Bintrim, S. B., T. J. Donohue, J. Handelsman, G. P. Roberts, and R. M. Goodman.** 1997. Molecular phylogeny of *Archaea* from soil. Proc. Natl. Acad. Sci. USA **94:**277–282.
7. **Borneman, J., S. Austin, and E. W. Triplett.** Method development to assess microbial diversity in soil. Proceedings of the International Meeting on Biotechnology and Environmental Risk Assessment, in press.
8. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935–1943.
9. **DeLong, E. F., K. Y. Wu, B. B. Prézetin, and R. V. M. Jovine.** 1994. High abundance of Archaea in Antarctic marine picoplankton. Nature **371:**695–697.
10. **Erwin, T. L.** 1982. Tropical forests: their richness in Coleoptera and other arthropod species. Coleopt. Bull. **36:**74–75.
11. **Erwin, T. L.** 1983. Beetles and other arthropods of the tropical forest canopies at Manaus, Brasil, sampled with insecticidal fogging techniques, p.

59–75. *In* S. L. Sutton, T. C. Whitmore, and A. C. Chadwick (ed.), Tropical rain forests: ecology and management. Blackwell Scientific Publications, Oxford, United Kingdom.
12. **Erwin, T. L.** 1988. The tropical forest canopy—the heart of biotic diversity, p. 123–129. *In* E. O. Wilson (ed.), Biodiversity. National Academy Press, Washington, D.C.
13. **Faegri, A., V. L. Torsvik, and J. Gorksoyr.** 1977. Bacterial and fungal activities in soil: separation of bacteria and fungi by a rapid fractionated centrifugation technique. Soil Biol. Biochem. **9:**105–112.
14. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
15. **Felsenstein, J.** 1981. Evolutionary trees form DNA sequences: a maximum likelihood approach. J. Mol. Evol. **17:**368–376.
16. **Gutell, R. R., B. Weiser, C. R. Woese, and H. F. Noller.** 1985. Comparative anatomy of 16S-like ribosomal RNA. Prog. Nucleic Acid Res. Mol. Biol. **32:**155–216.
17. **Hiraishi, A., N. Kishimoto, Y. Kosako, N. Wakao, and T. Tano.** 1995. Phylogenetic position of the menaquinone-containing acidophilic chemo-organotroph *Acidobacterium capsulatum*. FEMS Microbiol. Lett. **132:**91–94.
18. **Hopkins, D. W., S. J. MacNaughton, and A. G. O'Donnell.** 1993. A dispersion and differential centrifugation technique for representatively sampling micro-organisms. Soil Biol. Biochem. **23:**217–225.
19. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York, N.Y.
20. **Jurgens, G., K. Lindstrom, and A. Saano.** 1997. Novel group within the kingdom *Crenarchaeota* from boreal forest soil. Appl. Environ. Microbiol. **63:**803–805.
21. **Liesack, W., F. Bak, J.-U. Kreft, and E. Stackebrandt.** 1994. *Holophaga foetida* gen. nov., sp. nov., a new, homacetogenic bacterium degrading methoxylated aromatic compounds. Arch. Microbiol. **162:**85–90.
22. **Linhares, A. A.** 1995. Estimates of actinomycetes from Brazilian soils using the dispersion and differential centrifugation techniques, abstr. P1-3.1. 7th International Symposium on Microbial Ecology. Winner Graph, São Paulo.
23. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The ribosomal database project. Nucleic Acids Res. **22:**3485–3487.
24. **Manfio, G. P., and M. Goodfellow.** 1995. Diversity of *Streptomycetes* in a tropical rain forest site: comparison of extraction methods, abstr. P2-20.8. 7th International Symposium on Microbial Ecology. Winner Graph, São Paulo.
25. **Moraes, J. L., C. C. Cerri, J. M. Melillo, D. Kicklighter, C. Neill, D. L. Skole, and P. A. Steudler.** 1995. Soil carbon stocks of the Brazilian Amazon Basin. Soil Sci. Soc. Am. J. **59:**244–247.
26. **Nepstad, D. C., C. R. Carvalho, E. A. Davidson, P. H. Jipp, P. A. Lefebvre, G. H. Negreiros, E. D. Silva, T. A. Stone, S. E. Trumbore, and S. Vieira.** 1994. The role of deep roots in the hydrological and carbon cycles of Amazonian forests and pastures. Nature **372:**666–669.
27. **Nepstad, D. C., C. Uhl, and E. A. S. Serrao.** 1991. Recuperation of a degraded Amazonian landscape: forest recovery and agricultural restoration. Ambio **20:**248–255.
28. **Olsen, G. J., H. Matsuda, R. Hagstrom, and R. Overbeek.** 1994. FastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. Comput. Appl. Biosci. **10:**41–48.
29. **Pace, N. R., D. A. Stahl, D. J. Lane, and G. J. Olsen.** 1986. The analysis of natural microbial populations by ribosomal RNA sequences. Adv. Microb. Ecol. **9:**1–55.
30. **Penny, N. D., and J. R. Arias.** 1982. Insects of an Amazon forest, p. 269. Columbia University Press, New York, N.Y.
31. **Piccolo, M. C., C. Neill, and C. C. Cerri.** 1994. Natural abundance of ^{15}N in soils along forest-to-pasture chronosequences in the western Brazilian Amazon Basin. Oecologia **99:**112–117.
32. **Prance, G. T.** 1977. The phytogeographical support for the theory of Pleistocene forest refuges in the Amazon Basin, based on evidence from distribution patterns in *Caryocaraceae*, *Chrysobalanaceae*, *Dichapetalaceae* and *Lecythidaceae*. Acta Amazonica **3:**5–8.
33. **Reiners, W. A., A. F. Bouwman, W. F. J. Parsons, and M. Keller.** 1994. Tropical rain forest conversion to pasture: changes in vegetation and soil properties. Ecol. Appl. **4:**363–377.
34. **Richaume, A., C. Steinberg, L. Jocteur-Monrozier, and G. Faurie.** 1993. Differences between direct and indirect enumeration of soil bacteria: the influence of soil structure and cell location. Soil Biol. Biochem. **25:**641–643.
35. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for constructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
36. **Schleper, C., W. Holben, and H.-P. Klenk.** 1997. Recovery of crenarchaeotal ribosomal DNA sequences from freshwater-lake sediments. Appl. Environ. Microbiol. **63:**321–323.
37. **Schulte, E. E., J. B. Peters, and P. R. Hodgson.** 1987. Wisconsin procedures for soil testing, plant analysis, and feed and forage analysis. Department of Soil Science, University of Wisconsin—Madison, Madison, Wis.
38. **Stackebrandt, E., and B. M. Goebel.** 1994. A place for DNA-DNA reassociation and 16S ribosomal-RNA sequence-analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
39. **Stephens, R. S.** 1992. Challenge of chlamydial research. Infect. Agents Dis. **1:**279–93.
40. **Torsvik, V., J. Goksoyr, and F. L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56:**782–787.
41. **Tsuji, T., Y. Kawasaki, S. Takeshima, T. Sekiya, and S. Tanaka.** 1995. A new fluorescence staining assay for visualizing living microorganisms in soil. Appl. Environ. Microbiol. **61:**3415–3421.
42. **Ueda, T., Y. Suga, and T. Matsuguchi.** 1995. Molecular phylogenetic analysis of a soil microbial community in a soybean field. Eur. J. Soil Sci. **46:**415–421.
43. **Van de Peer, Y., I. Van den Broech, P. De Rijk, and R. De Wachter.** 1994. Database on the structure of small ribosomal subunit RNA. Nucleic Acids Res. **22:**3488–3494.
44. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature **345:**63–65.
45. **Winker, S., and C. R. Woese.** 1991. A definition of the domains *Archaea*, *Bacteria* and *Eucarya* in terms of small subunit ribosomal RNA characteristics. Syst. Appl. Microbiol. **14:**305–310.
46. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.