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Novel Group within the Kingdom *Crenarchaeota* from Boreal Forest Soil

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We report here the results of phylogenetic analysis of archaeal 16S rRNA gene sequences amplified by PCR with *Archaea*-specific primers with mixed-population DNA extracted directly from forest soil used as a template. Nucleotide signature and phylogenetic analyses show that the sequences obtained belong to the domain *Archaea* and form a new cluster. Its phylogenetic position suggests that sequences are from a previously undescribed terrestrial group within the kingdom *Crenarchaeota*.

Microbial diversity in soil has been studied by analysis of 16S rRNA gene sequences (15) and in many cases new groups within the *Bacteria* and *Eucarya* have been found (5, 12). However, there have been only a few reports about the diversity of *Archaea* in nonextreme environments such as soil. Three studies that relied on PCR analysis have shown *Archaea* to be present in soil from a soybean field, blanket bog peat, and pasture soil (4, 11, 18).

In this paper, we report the presence of a previously undescribed terrestrial group of low-temperature *Archaea* organisms in intact boreal forest soil found by using *Archaea*-specific primers in PCR. The phylogenetic analysis of the 900-nucleotide-long 16S rRNA gene sequences revealed that they form a new cluster within the kingdom *Crenarchaeota* and are only distantly related to hitherto described crenarchaeal 16S rRNA gene sequences.

Materials and Methods. Samples taken with a 72-mm-diameter soil corer from the humus layer were obtained from a forest in Northern Finland (65° 15′ N, 28° 50′ E) with podzol on moraine soil type. Twenty humus cores were collected and combined to form one bulk sample (16). The experimental area was covered by a mixed forest of Norway spruce with some 200- to 300-year-old Scots pines. Crude DNA was isolated from the soil sample with proteinase K, cetyltrimethylammonium bromide, and chloroform and then applied to Wizard DNA Clean-Up System Minicolumns (Promega) with isopropanol purification (17). The purified DNA was used as a template for PCR with a pair of *Archaea*-specific primers in order to amplify an ~900-bp-long 16S rRNA gene region between positions 7 and 927 (*Escherichia coli* numbering). The following oligonucleotide primer sequences were used: forward, 5′-TTCCGGTTGATCCTGCCGGA-3′, taken from Giovannoni et al. (10) (archaebacterial probe); and reverse, 5′-CCCGCCAATTCCTTTAAGTTTC-3′, designed by us by alignment of 10 sequences of different archaea from the EMBL database. PCR was carried out under the following reaction conditions: 94°C for 4 min and 40 cycles of 94°C for 1 min, 55°C for 1 min, and 73°C for 3 min. The negative control (water instead of DNA) showed no amplification. The PCR product was run in agarose gel electrophoresis. DNA of the appropriate size was extracted from the gel and cloned into pGEM-T vector plasmid (Promega). Clones carrying ~900-bp inserts were first identified by gel electrophoresis, and then the archaeal origin of the inserts was checked in Southern blot hybridization with an *Archaea*-specific probe (corresponding region of 16S rRNA from *Halobacterium salinarum* DSM 668 without primers). The inserts of 74 of the clones obtained were divided into groups according to *Ava*II, *Msp*I, and *Rsa*I restriction patterns. Representatives from nine different groups were chosen for the sequence analysis, which was done with an Auto Read 1000 sequencing kit and the A.L.F. DNA sequencer (Pharmacia LKB).

Phylogenetic analysis. The programs CHECK_CHIMERA, RANK_SIMILARITY, and SUGGEST_TREE from the Ribosomal Database Project (RDP) (13) were applied to the sequences studied in order to detect possible chimeric artifacts, to find the most similar sequences from the RDP database, and to place Finnish forest soil type B (FFSB) sequences on an existing phylogenetic RDP tree. Sequences were manually aligned with 16S rRNA sequences retrieved from the EMBL and RDP databases based on their primary and secondary structures with Gelassembler sequencing editor (Wisconsin Package, version 8.1) (9a). Sites of uncertain alignment were excluded from the analysis. Three methods of phylogenetic analysis from PHYLIP package version 3.5c (distributed by the author, J. Felsenstein [7]) (neighbor-joining with distances calculated with Jukes and Cantor correction, parsimony, and maximum likelihood) were used. The bootstrap method (8) (PHYLIP package) was used in order to check the topology perturbation in the neighbor-joining and parsimony methods. Different phylogenetic trees were made to find out the relationship between FFSB clones, *Crenarchaeota*, and *Euryarchaeota*.

Phylogenetic relationships. In our work, we obtained nine archaeal 16S rRNA gene sequences (FFSB1 to -7, -10, and -11) by PCR with *Archaea*-specific primers and DNA extracted from a forest soil sample as a template. Comparison of the FFSB sequences with sequences in the EMBL database by using the FASTA program showed only 75 to 79% identity with the most homologous *Archaea* 16S rRNA gene sequences. The three methods of phylogenetic analysis used consistently placed eight of the FFSB sequences (1 to 5 and 7, 10, and 11) into one group, which was distantly related to all of the other known crenarchaeotal sequences. This group was situated on the same lineage as the planktonic clade (low-temperature marine *Archaea* group I [6] together with other environmental sequences [2, 3, 9, 14]) between the latter and the rest of the

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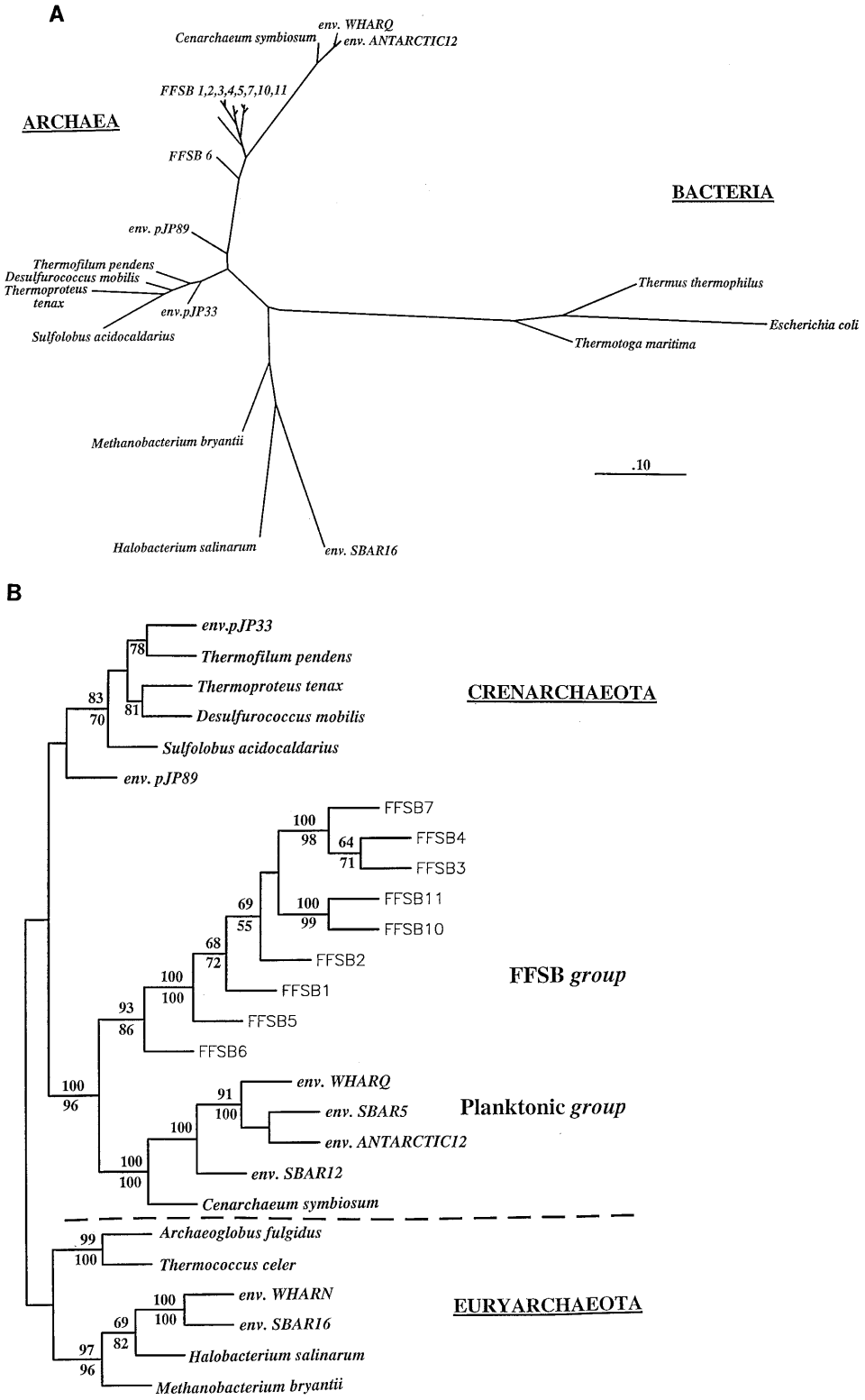

FIG. 1. (A) Phylogenetic tree inferred by maximum-likelihood analysis based on alignment of 746-bp-long 16S rRNA sequences, showing the phylogenetic affiliation of novel FFSB clones. The scale bar represents 0.1 change per nucleotide position. (B) Neighbor-joining analysis showing the phylogenetic relationship between the new FFSB group, the planktonic group, and the rest of the members of the kingdom *Crenarchaeota*. The percentage of 100 bootstrap resamplings that supported topology in neighbor-joining analysis (above line) and parsimony (below line) is indicated. No values are given for groups with bootstrap values less than 50%. *Thermotoga maritima* and *Thermus thermophilus* were used as outgroups. Bacterial genera are abbreviated as follows: env. (environmental isolate) WHARQ, Woods Hole bacterioplankton DNA clone WHARQ, AC M88079; env. ANTARCTIC12, strain ANTARCTIC12, AC U11043; env. SBAR5, Santa Barbara Channel bacterioplankton DNA clone SBAR5, AC M88075; env. pJP89, Mud Volcano area of Yellowstone National Park ("Black Pool") hot spring DNA clone pJP89, AC L25305; env. pJP33, Mud Volcano area of Yellowstone National Park ("Black Pool") hot spring DNA clone pJP33, AC L25300; env. SBAR16, Santa Barbara Channel bacterioplankton DNA clone SBAR16, AC M88077; env. WHARN, Woods Hole bacterioplankton DNA clone WHARN, AC M88078; and env. SBAR12, Santa Barbara Channel bacterioplankton DNA clone SBAR12, AC M88076. (Note that this tree does not show phylogenetic distances.)

TABLE 1. Intradomain nucleotide signature analysis for FFSB sequences[a]

Position(s)[b]	Nucleotide signature				
	Crenarchaeota	*Euryarchaeota*	pJP89	Group I	FFSB
27–556	C-G	G-C	Cren	Cren	Cren
28–555	C-G	G-Y[c]	Cren	Cren	Cren
30–553	G-C	Y-R[d]	**Eury**	Cren	Cren
34–550	C-G	U-G	Eury	Eury	Eury (Eucar)
289–311	G-C	C-G	Eury	Eury	Eury (Eucar)
501–544	C-G	R-Y	Eury	Eury	Eury
503–542	G-C	C-G	Cren	Cren	Cren (Eucar)
504–541	G-Y	Y-R	**Eury**	**Eury**	A-U (Eucar)
513–538	U-A	C-G	**G-C**	Cren	Cren (Eucar)
518	U	C	Cren	Cren	Cren
658–747	G-C	Y-R	Cren	Cren	Cren
692	C	U	Eury	Eury	Eury (Eucar)

[a] Intradomain nucleotide signature analysis (19) of the 16S rDNA (genes coding for rRNA) sequences studied together with pJP89 (the most homologous sequence from *Crenarchaeota* [data from RDP obtained with the RANK_SIMILARITY program]) and group I (planktonic group). Differences between FFSB and pJP89 and group I are indicated in boldface and sequence signatures for *Crenarchaeota*, *Euryarchaeota*, and *Eucarya* are indicated as Cren, Eury, and Eucar, respectively.

[b] *E. coli* numbering.

[c] Y, pyrimidine.

[d] R, purine.

Crenarchaeota (Fig. 1A). Different outgroups (*Bacteria* and *Eucarya*) did not change the phylogenetic location of FFSB sequences in the trees. Bootstrap analysis (Fig. 1B) supported the inferred topology (100% in neighbor-joining, 96% in parsimony) and confirmed that the evolutionary distances are long enough to consider the FFSB clade as a separate group, distinct from other crenarchaeotal and planktonic sequences. Phylogenetic placement of the FFSB sequences on an existing RDP tree gave the same separation of the FFSB group as those from the PHYLIP package (data not shown). One of the nine clones, FFSB6, was placed as an individual branch. The phylogenetic distance between FFSB6 and the most homologous available archaeal sequence, pJP89 (a crenarchaeotal clone from a hot spring [2]), was the same as that between FFSB6 and the planktonic group (Fig. 1A). Therefore, we suspect that FFSB6 is a representative of one more branch inside the kingdom *Crenarchaeota.*

Diagnostic signature and feature analysis. Thorough analysis of the diagnostic signatures and features revealed that all the FFSB clones belong to the archaeal domain. Sixty-six of the 68 relevant positions that distinguish *Archaea* from *Bacteria* and *Eucarya* (19, 20) show features common to *Archaea*. The intradomain nucleotide signature comparison (19) of FFSB sequences shown in Table 1 supported phylogenetic placement of the FFSB sequences between other *Crenarchaeota* and the planktonic group.

Conclusion. As a result of the phylogenetic and diagnostic feature analyses, we conclude that the FFSB sequences studied are distinct from all sequences within the kingdom *Crenarchaeota*. We think that FFSB clones represent an undescribed, independent group of terrestrial low-temperature *Crenarchaeota*. This novel group (or two groups if we consider FFSB6 as another branch) was first discovered in boreal forest soil. Thus, we have shown that members of the soil *Archaea* are represented not only by the methanogens, which belong to *Euryarchaeota*, but also by crenarchaeotal species. At this point, we know nothing about their metabolism or other properties, nor can we say how prevalent they are in the soil because of differences in amplification efficiency (PCR) as well as possible differences in the efficiency of extraction of DNA from them. One way to assess their abundance is to screen specific oligonucleotide probes with respect to their hybridization efficiency against RNA in the natural communities by dot blot and in situ hybridizations (1).

Nucleotide sequence accession number. Sequences from this study have been deposited in the EMBL database and have been assigned accession numbers x96688 to x96696.

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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. **Barns, S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace.** 1994. Remarkable archaeal diversity detected in Yellowstone National Park hot spring environment. Proc. Natl. Acad. Sci. USA **91:**1609–1613.
3. **Barns, S. M., C. F. Delwiche, J. D. Palmer, and N. R. Pace.** 1996. Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc. Natl. Acad. Sci. USA **93:**9188–9193.
4. **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.
5. **Bruce, K. D., W. D. Hiorns, J. L. Hobman, A. M. Osborn, P. Strike, and D. A. Ritchie.** 1992. Amplification of DNA from native populations of soil bacteria by using the polymerase chain reaction. Appl. Environ. Microbiol. **58:**3413–3416.
6. **DeLong, E. F.** 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA **89:**5685–5689.
7. **Felsenstein, J.** 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle.
8. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
9. **Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1992. Novel major archaebacterial group from marine plankton. Nature (London) **356:**148–149.

9a. **Genetics Computer Group.** 1994. Program manual for the Wisconsin Package, version 8.1, September 1994. Genetics Computer Group, Madison, Wis.

10. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligonucleotide probes for identification of single microbial cells. J. Bacteriol. **170:**720–726.
11. **Hales, B., C. Edwards, D. A. Ritchie, G. Hall, R. W. Pickup, and J. R. Saunders.** 1996. Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. Appl. Environ. Microbiol. **62:**668–675.
12. **Liesack, W., and E. Stackebrandt.** 1992. Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriol. **174:**5072–5078.
13. **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.
14. **McInerney, J. O., M. Wilkinson, J. W. Patching, T. M. Embley, and R. Powell.** 1995. Recovery and phylogenetic analysis of novel archaeal rRNA sequences from a deep-sea deposit feeder. Appl. Environ. Microbiol. **61:** 1646–1648.
15. **Pace, N. R., D. A. Stahl, D. J. Lane, and G. J. Olsen.** 1986. The analysis of natural microbial population by ribosomal RNA sequences. Adv. Microb. Ecol. **9:**1–55.
16. **Pietikäinen, J., and H. Fritze.** 1995. Clear-cutting and prescribed burning in coniferous forest: comparison of effects on soil fungal and total microbial biomass, respiration activity and nitrification. Soil Biol. Biochem. **27:**101–109.
17. **Saano, A., E. Tas, S. Piippola, K. Lindström, and J. D. van Elsas.** 1995. Extraction and analysis of microbial DNA from soil, p. 49–67. *In* J. T. Trevors and J. D. van Elsas (ed.), Nucleic acids in the environment: methods and application. Springer-Verlag, Berlin, Germany.
18. **Ueda, T., Y. Suga, N. Yahiro, and T. Matsuguchi.** 1995. Molecular phylogenetic analysis of a soil microbial community in soybean field. Eur. J. Soil Sci. **46:**415–421.
19. **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.
20. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.