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Phylogenetic comparison of methanogen diversity in different wetland soils

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Aspects of archaeal diversity in peat soil samples from climatically and geographically distinct wetlands (subarctic: West Siberia Bog, Russia; temperate: Akaiyachi Mire, Japan; subtropical: Okefenokee Swamp, USA) were studied by molecular phylogenetic techniques. DNA was extracted directly from the soil samples and 16S rRNA genes were amplified by polymerase chain reaction. Partial sequences of the amplified 16S rDNAs (total 426 clones) were compared with known sequences from GenBank and the Ribosome Database Project (RDP). Peat-derived sequences were mostly related to Euryarchaeota, principally methanogens. Sets of sequences (operational taxonomic unit; OTU) were created for each wetland (21 OTUs for West Siberia; 22 OTUs for Akaiyachi; 33 OTUs for Okefenokee). The majority of the OTUs clustered in and showed low similarities to the *Methanosarcinales* family (West Siberia) or the *Methanomicrobiales* family (Akaiyachi and Okefenokee). In terms of the Shannon-Weaver diversity index, the archaeal community diversity in Okefenokee Swamp was greater than that of the other wetlands.

Key Words——*Archaea*; *Methanosarcina*; peat

Introduction

Although wetlands account for only about 5% of the terrestrial surface, they play a disproportionately important role in elemental cycling and biogeochemistry (Mitsch and Gosselink, 2000; Schlesinger, 1997). In addition to studies of N, P, S, and various metals, considerable attention has been directed towards the role of wetlands in methane cycling, since natural and agricultural wetlands contribute approximately half of the contemporary global methane emissions to the atmosphere (Cicerone and Oremland, 1988). However, in spite of extensive analyses of methane production, oxidation, and emission, much remains to be learned about the microbiology of methane transformations and the diversity of organisms that are responsible for methane cycling.

A number of studies have used molecular approaches to address various aspects of methanogen and methanotroph diversity in wetlands, including rice paddies and various peatlands (Chin et al., 1999; Dedysh et al., 1998; Edwards et al., 1998; Grosskopf et al., 1998; Hales et al., 1996; Jensen et al., 2000; Kudo et al., 1997; Munson et al., 1997; Murrell et al., 1998; Nercessian et al., 1999; Ritchie et al., 1998). Most of these studies have documented a major role for the "group II" methanotrophs, identified novel methanotrophs, and documented the presence in peats of novel methanogens that are closely related to

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species currently in culture (Calhoun and King, 1997; King, 1994). Nonetheless, relatively little is known about the determinants of methanotroph or methanogen diversity within a given system, or the biogeography of either group among systems.

Because of the fundamental importance of methanogens in the dynamics of methane cycling, we compared their diversities in 3 low-pH wetlands that vary substantially in history and climate. The final goal of our study is to determine if a major limiting factor, such as pH, constrains diversity among systems, or if other factors (e.g., temperature regimes) contribute to distinct population structures for systems that are geographically isolated.

Here we report archaeal (especially methanogenic bacteria) population structures in acidic wetland peats from ecosystems with substantially different climatic regimes and histories. Population structures were compared by using polymerase chain reaction (PCR) amplification of microbial community DNA with primers specific for archaeal 16S rRNA genes. Clone libraries of PCR amplicons from each peat were constructed for partial sequencing and phylogenetic analysis.

Materials and Methods

Sampling sites and sample collection.

West Siberia Bog: Peat samples from West Siberia were collected from the Bakchar Bog at the Plotonikovo field station (lat 56°51′N, long 82°50′E) in the Tomsk Region of Russia during July 1998. The bog (about 450 km^2) is located in the watershed between the rivers Bakchar and Iksa. The predominant vegetation at the collection site is *Carex* spp. and *Sphagnum* spp. Surface and interstitial waters are tannin-rich, with a pH of approximately 4. Peat samples for microbial community analysis were collected at depths of 25 cm below the water table with stainless steel pipes in conjunction with 100-ml glass syringes. Sterilized 8-ml polypropylene tubes (Sarstadt, Germany) were used for storing the samples. The samples were immediately transferred to the tubes at the site. The tubes were slowly filled to overflowing with several volumes of sample water and capped with no headspace. After capping, all tubes were transferred to an AnaeroPack system (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) that was made anoxic by following the manufacturer's instructions and kept in a cooling box. After the samples had been taken to the Plotonikovo laboratory (<2 h), they were stored in liquid nitrogen (−196°C) as soon as possible. Peat samples were transported in liquid nitrogen from the Plotonikovo laboratory to Japan, and then stored at −80°C prior to extraction for DNA analysis.

Akaiyachi Mire: Akaiyachi Mire (lat 37°30′N, long 140°00′E, altitude 525 m) extends north and west of the Akai River near its outlet into the northeastern end of Lake Inawashiro, Japan. The mire is roughly octagonal, measuring ca. 900 m from north to south and ca. 700 m from east to west, and covering an area of 43.6 ha (Yoshioka, 1961). This area has been designated as a natural monument since 1928. The mire is surrounded mainly by rice paddies and partly by fields of buckwheat and stands of pine trees (*Pinus densiflora*). Surface and interstitial waters are tannin-rich and acidic, with a pH around 4.2.

Peat samples were collected near the center of the mire in December 1997 in an area dominated by *Carex* spp. The peat samples with water for microbial community analysis were collected at depths of 30 cm below the water table and stored as described above for the West Siberia Bog samples.

Okefenokee Swamp: The Okefenokee Swamp (lat 30°44′N, long 82°10′W) is the largest acidic wetland in North America, occupying approximately 1,600 km^2 and located primarily in southeastern Georgia, USA. Most of the swamp has been officially protected since 1937, and in 1974 a portion was designated a National Wilderness Area. Surface and interstitial waters are acidified to about pH 4 owing to the presence of fulvic acids and the activity of *Sphagnum* (Bosserman, 1984). Ionic strengths are low (conductivity <80 μmho) (Auble, 1984; Bosserman, 1984), with both water and major nutrient inputs derived primarily from precipitation (Rykiel, 1984). Water levels fluctuate substantially annually and interannually, with extensive drought and fire occurring periodically. Vegetation within the flooded areas of the swamp consists of numerous grasses, sedges and other emergent macrophytes (e.g., *Pontederia*, *Nymphaea*, *Orontium*, *Sagittaria*, *Cyperus*, *Carex*) and woody genera including *Pinus* and *Taxodium.* Details of the swamp, including aspects of its microbiology and biogeochemistry, are given elsewhere (Cohen et al., 1984).

For this study, peat samples were collected with aluminum core tubes near Cooter Lake during April 1998. At the time of collection the peat surface was submerged approximately 50 cm. After the core samples

had been taken to the laboratory (<4 h), they were cut with a knife into several depth intervals (depths of 0–2 cm, 2–5 cm, 5–10 cm, and 10–15 cm). All sub-core samples were immediately packed into an AnaeroPack system and immediately frozen (−20°C). For transport to Japan, all samples were packed in a cooler containing dry ice. On arrival, samples were stored at −80°C prior to extraction for DNA analysis.

DNA extraction and sequence analysis. DNA was extracted and purified directly from all peat samples with a Fast DNA kit for soil (BIO 101, Inc., Vista, CA, USA), which utilizes physical extraction with glass beads. The procedure was conducted according to the manufacturer's instructions. Nucleic acids were precipitated from the solution by using 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol, incubation on ice, and centrifugation for 30 min at 12,000×*g*. Precipitated nucleic acids were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

Archaeal diversities for each wetland were analyzed by comparing partial sequences of 16S rRNA genes obtained by PCR. The 16S rRNA genes were amplified by PCR with primer sets specific for *Archaea* (915F, 5′-AGGAATTGGCGGGGGA-3′; 1492R, 5′-TGACTGACTGAGGYTACCTTGTTACGACTT-3′) (Amann et al., 1995; Borneman et al., 1996). Another primer set, 1A (forward) (5′-TCYGKTTGATCCYGSCRGAG-3′) and 1100A (reverse) (5′-TGGGTCTCGCTCGTTG-3′) (Munson et al., 1997), was also used for the West Siberia peat samples to compare the sensitivities of the primers for assessing archaeal community structure.

PCR was carried out in 20-μl reaction volumes with a Perkin-Elmer GeneAmp PCR System 9700 (Perkin-Elmer, Foster City, CA, USA) as follows: 4 min of denaturation at 94°C, followed by 35 cycles each with 1 min of denaturation at 94°C, 1 min at 50°C (annealing), and 1.5 min at 72°C (extension), with a final 7-min extension step at 72°C after cycling was complete. PCR amplicons were loaded onto 1.0% SeaKem agarose (Whittaker Bioproducts, Rockland, ME, USA) gels and electrophoresed in a TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2.5 mM EDTA, pH 8.3); DNA bands in the gel were visualized with UV transillumination after staining with ethidium bromide. Band sizes were calibrated by using a pHY DNA Marker (TaKaRa Shuzo, Ohtsu, Japan). Bands of the expected size (about 580 bp) were excised and purified by gel extraction. Agarose gel electrophoresis was performed with 2% (w/v) agarose gels of 1× Tris-acetate-EDTA buffer (TAE; 10 mM Tris, 1 mM EDTA, pH 8) that contained 2 μg ml^{-1} ethidium bromide. The band of PCR products was identified in the gel under 365-nm UV irradiation and cut into small pieces with a razor blade. The gel pieces were inserted into the upper cartridge of a SUPREC-01 tube (TaKaRa Shuzo) and the tube was frozen at −80°C for 30 min. The tube was centrifuged at 4°C, 14,000×*g* for 10 min. Two-hundred microliters of TE buffer was then added to the gel pieces in the cartridge. The tube was again centrifuged at 4°C, 14,000×*g* for 10 min and the cartridge was discarded. DNA in the supernatant was precipitated with ethanol.

To construct clone libraries, purified PCR amplicons were cloned into the pCR2.1 vector and *E. coli* INVaF′ by using an Original TA Cloning Kit (Invitrogen, Leek, The Netherlands) according to procedures recommended by the manufacturer. Plasmid DNAs for sequencing were prepared from overnight cultures of clones containing 16S rDNA with a Miniprep Spin Kit (Quiagen, Crawley, UK). Purified plasmid DNA was sequenced with an ABI model 377 XL automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) and a Big Dye Terminator cycle-sequencing kit (Applied Biosystems).

We obtained partial sequences from 426 clones (West Siberia, 161 clones; Akaiyachi, 134 clones; Okefenokee, 131 clones). All sequences were compared with similar sequences of reference organisms through a BLAST search (Altschul et al., 1997). Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) with the CLUSTAL W software package (Thompson et al., 1994). We also constructed phylogenetic trees by using the maximum-likelihood method with the MOLPHY (version 2.3b3) software package (Adachi and Hasegawa, 1996). The maximum-likelihood distance matrix was calculated with NucML in the MOLPHY software package. The main branch pattern of maximum-likelihood trees were essentially the same as those of neighbor-joining trees.

Diversity analysis. Clones that had similar (<4% dissimilarity) small subunit ribosomal DNA (SSU rDNA) sequences were assumed to belong to the same operational taxonomic unit (OTU) and were used for further phylogenetic analysis as an OTU. The OTUs were designated WSB-1 to WSB-21 for those from the West Siberia Bog, AM-1 to AM-22 for those

Table 1. Comparison of physicochemical properties of sampling sites.

	West Siberia Bog	Akaiyachi Mire	Okefenokee Swamp
Latitude, longitude	56°51′N, 82°50′E	37°30′N, 140°0′E	30°44′N, 82°10′W
Climatic zone	subarctic	temperate	subtropical
Trophic level	oligo-mesotrophic	oligo-mesotrophic	oligo-mesotrophic
Soil temperature	below 15°C	between 0 and 25°C	above 15°C
pH	3.0–4.5	4.0–5.0	3.8–4.5
DOC conc.	30–45 mgC L^{-1}	20–60 mgC L^{-1}	40–100 mgC L^{-1}

from Akaiyachi Mire, and OS-1 to OS-33 for those from Okefenokee Swamp. The Shannon-Weaver diversity index (H') was calculated from the number of clones analyzed (N) and the number of clones with identical OTUs (ni) to give the equitability index:

$$H' = \left| \sum \frac{ni}{N} \ln \frac{ni}{N} \right|.$$

Nucleotide sequence accession numbers. Clones obtained from DNA extracted from the peat samples from each wetland were named WSB-1 to WSB-21, AM-1 to AM-22, and OS-1 to OS-33. The sequences were deposited in the EMBL database under accession numbers AB055983 to AB056058.

Results and Discussion

Although the 3 sampling sites (West Siberia, Akaiyachi, and Okefenokee) have different developmental histories and occur in different climatic zones, they share some basic physicochemical properties (Table 1). Each of the wetlands is classified as acidic, oligo-mesotrophic, and consists of peats with high concentrations of organic matter (Bosserman, 1984; Dedysh et al., 1998; Mitsch and Gosselink, 2000; Yoshioka, 1961). Extensive stands of *Sphagnum* sp. contribute to acidification, along with elevated concentrations of humic and fulvic acids. At all sites, levels of dissolved salts are typically low and are derived primarily from precipitation (Bosserman, 1984; Dedysh et al., 1998; Rykiel, 1984).

For the analysis of archaeal community structure in natural environments, several primer sets specific for *Archaea* have been used—e.g., 1A and 1100A (Munson et al., 1997), from position 109 to 934 of *E. coli* 16S rDNA (Chin et al., 1999; Grosskopf et al., 1998), 21f and 958r (Cytryn et al., 2000), and 1100F and 1400R (Kudo et al., 1997). In this study, we used 2 primer sets for the West Siberia peat samples, 1A and 1100A and 915F and 1492R (Amann et al., 1995). We obtained 73 sequences of more than 500 bp each with the 1A and 1100A primer set and 75 sequences of more than 500 bp with the 915F and 1492R primer set. Phylogenetic trees from each of the primer sets consisted of similar cluster structures and component ratios. However, clone diversity was apparently higher with the primer set of 915F and 1492R (18 OTUs) than with the 1A and 1100A primer set (15 OTUs). Consequently, we used the former set for comparisons among sites in this study.

We obtained and sequenced 426 clones from the 3 wetland peats—161 clones from West Siberia, 134 from Akaiyachi, and 131 from Okefenokee. None of the clones was identified as *Eukaryota* or *Eubacteria*, and none was a chimeric artifact.

We identified 21 OTUs from the 161 West Siberia Bog clones. Sequences from these OTUs plus selected archaeal sequences from GenBank were used to generate a phylogenetic tree based on the neighbor-joining method (Fig. 1). The OTU sequences were grouped into 10 clusters, most of which were classified as *Euryarchaeota* (especially methanogens). However, some of the clones were related to *Crenarchaeota* sequences, which include extreme thermophiles or sulfur-dependent archaea (Woese et al., 1990). About 90% (149 clones) of the 161 clones analyzed belonged to a monophyletic group within the *Methanosarcinales*, but these sequences have relatively low identities to the sequences of cultured *Methanosarcinales*. Methanogen OTUs accounted for 57% of all the OTUs.

The phylogenetic tree of the Akaiyachi Mire included 22 OTUs that were grouped into 7 clusters, most of which were similar to the *Euryarchaeota* and some to *Crenarchaeota*, as were found for the West Siberia samples (Fig. 2). Of the 134 clones analyzed, 99

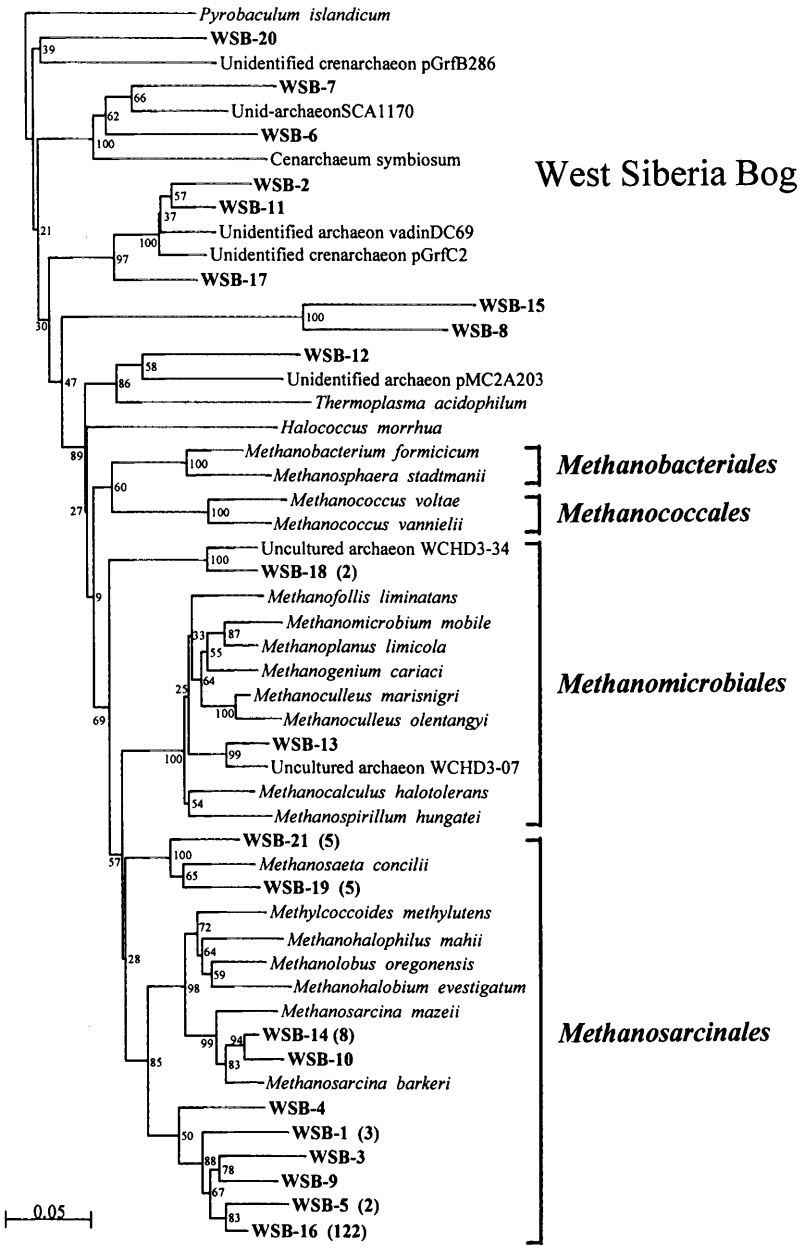

Fig. 1. Phylogenetic tree based on partial sequences of 16S rRNA genes obtained from the West Siberia Bog by PCR with universal archaeal primer sets.

The tree was constructed using neighbor-joining method with the CLUSTAL W software package. Numbers in parentheses show number of clones in each OTU. Numbers at branching points show results from bootstrap analysis. The median number of changes per sequence position is shown on the scale bar.

clones belonged to a monophyletic group within the *Methanomicrobiales*, but again, these sequences have relatively low sequence identities to known *Methanomicrobiales.* Methanogen OTUs accounted for 55% of all OTUs.

Clones from the Okefenokee Swamp fell into 33 OTUs that were grouped into 10 clusters, primarily of the *Euryarchaeota* and *Crenarchaeota* as before (Fig. 3). Of the 131 clones analyzed, 36 belonged to a polyphyletic group within the *Crenarchaeota*, and 31 clones belonged to a monophyletic group within the *Methanomicrobiales.* All of the Okefenokee sequences show low identities to known *Archaea.* Methanogen OTUs accounted for 45% of all OTUs.

The ratio of the number of OTUs in each methanogen family to the total number of OTUs in dif-

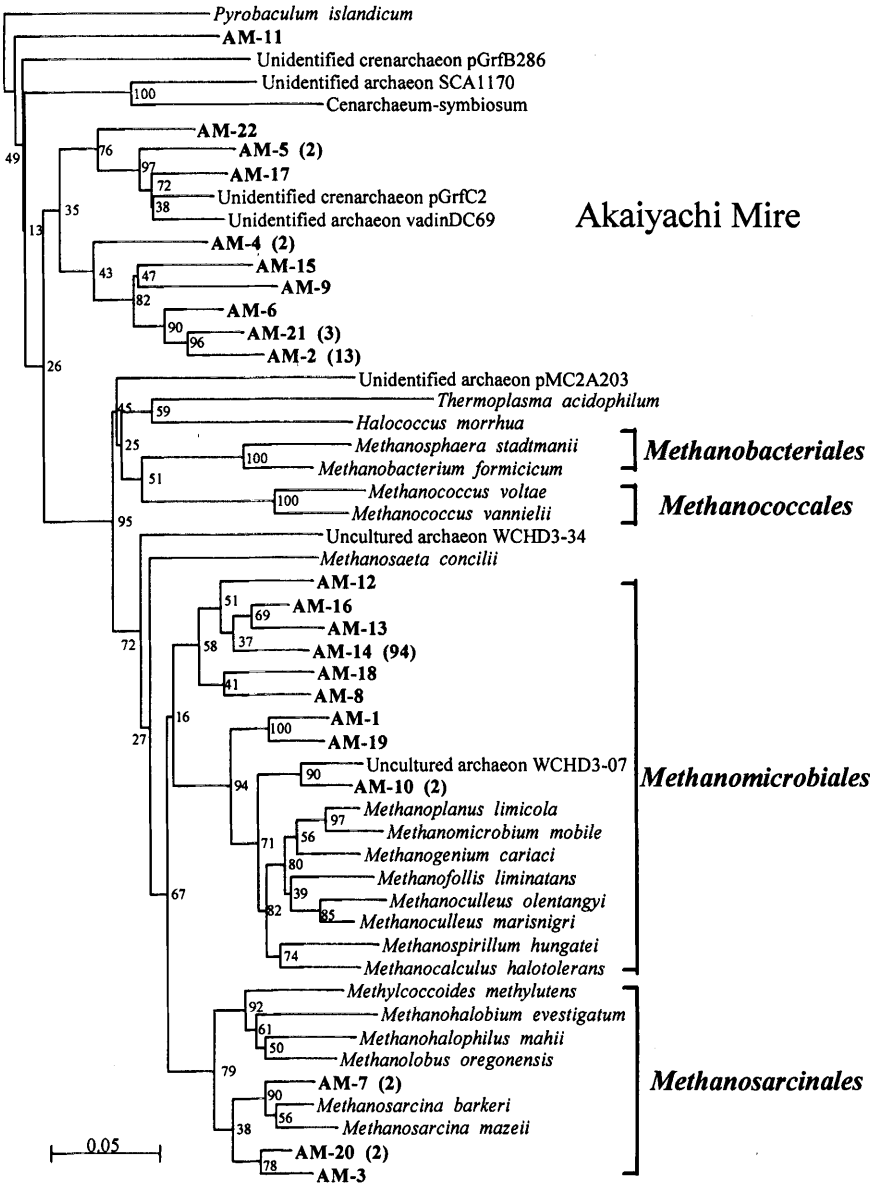

Fig. 2. Phylogenetic tree based on partial sequences of 16S rRNA genes obtained from the Akaiyachi Mire by PCR with universal archaeal primer sets.

The tree was constructed using neighbor-joining method with the CLUSTAL W software package. Numbers in parentheses show number of clones in each OTU. Numbers at branching points show results from bootstrap analysis. The median number of changes per sequence position is shown on the scale bar.

ferent wetland soils is summarized in Table 2. It is noteworthy that the *Methanosarcinales* clones were dominant in the West Siberia sample, while the *Methanomicrobiales* clones were dominant in Akaiyachi. There are very few papers reporting DNA sequencing data of methanogens in peatlands (Hales et al., 1996; Nercessian et al., 1999). Nercessian et al. reported the sequences of *mcrA* that encode the α-subunit of methyl coenzyme M reductase in methanogens. Because Hales et al. reported sequences of 16S rDNA fragments amplified with the 1Af and 1100Ar primer set instead of the 915F and 1492R primer set we used, it is not reasonable to compare our 16S rDNA sequences with those obtained by Hales et al. Nevertheless, the results of their analysis of 16S rDNA from peatlands in the UK revealed 2 predominant species, one of which was related to *Methanosarcina* spp. In our study, too, the dominant OTU group (WSB-16) in West Siberia was classified into the family *Methanosarcinales*. Further studies will be needed to

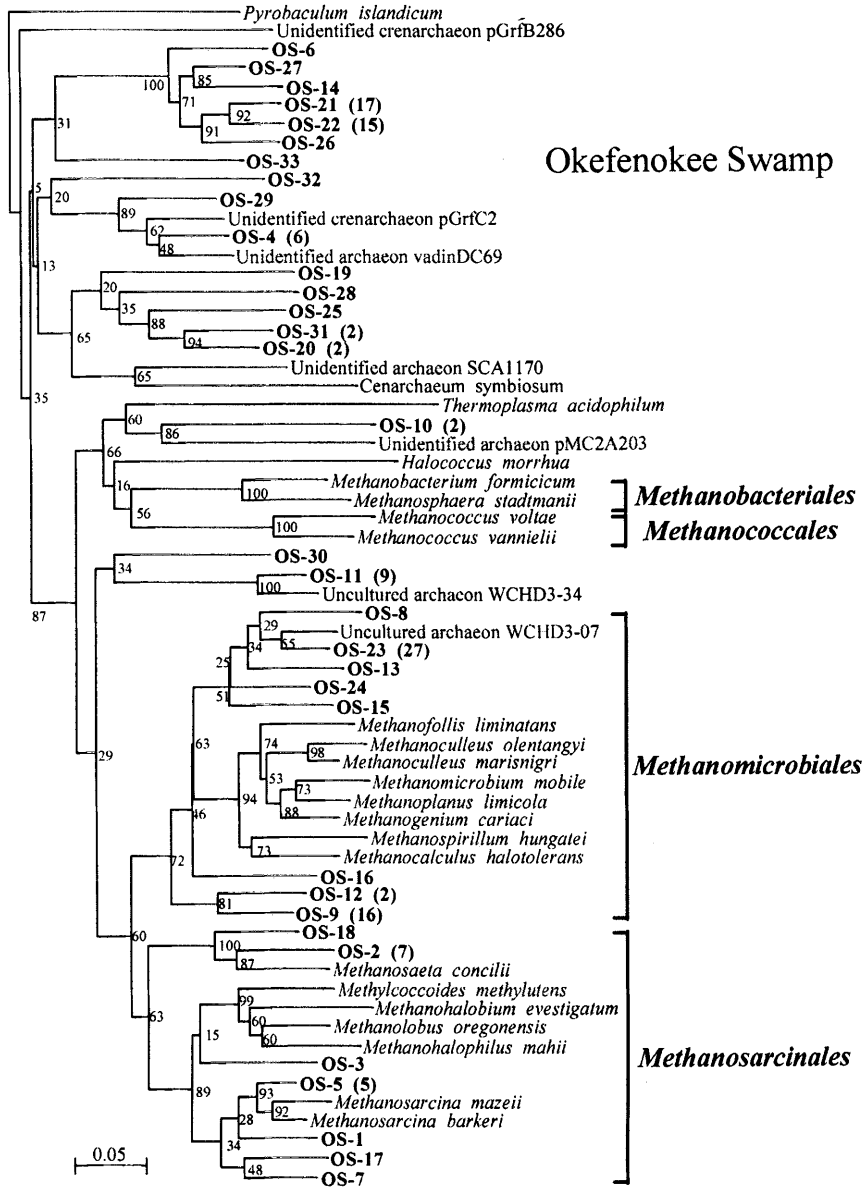

Fig. 3. Phylogenetic tree based on partial sequences of 16S rRNA genes obtained from the Okefenokee Swamp by PCR with universal archaeal primer sets.

The tree was constructed using neighbor-joining method with the CLUSTAL W software package. Numbers in parentheses show number of clones in each OTU. Numbers at branching points show results from bootstrap analysis. The median number of changes per sequence position is shown on the scale bar.

Table 2. The ratios of OTUs in each methanogen family to the total number of OTUs in different wetland soils.

	Methanobacteriales	*Methanococcales*	*Methanomicrobiales*	*Methanosarcinales*	Others
West Siberia Bog	0% (none/21)	0% (none/21)	9.5% (2/21)	47.6% (10/21)	42.9% (9/21)
Akaiyachi Mire	0% (none/22)	0% (none/22)	40.9% (9/22)	13.6% (3/22)	45.5% (10/22)
Okefenokee Swamp	0% (none/33)	0% (none/33)	24.2% (8/33)	21.2% (7/33)	54.5% (18/33)

conclude whether our clones are identical to theirs and whether this finding is common in peatlands located in high latitudes.

Diversity indices (equitability) were calculated from the numbers of clones per OTU and the total number of clones analyzed (West Siberia Bog, n=161; Akaiyachi Mire, n=134; Okefenokee Swamp, n=131). These indices were 1.20, 1.38, and 2.76 for West Siberia, Akaiyachi, and Okefenokee, respectively. Reasons for the apparently higher diversity of the Okefenokee archaeal 16S rDNA sequences are unclear. Recently, Fey and Conrad (2000) reported that temperature played a role in the archaeal composition of an Italian rice paddy. In particular, methanogen diversity appeared to increase with increasing temperature. Temperature may play a comparable role for *Archaea* among wetlands distributed across latitudinal gradients. Since the diversity of various microorganisms tends to increase with decreasing latitude (Rosenzweig, 1995), the role of latitude and temperature on microbial diversity will be an interesting consideration.

Naturally, the extraction method and PCR protocol used in this study bias the results toward the most abundant populations, since minor components (1% or less) are usually not proportionally represented in the final PCR product or detected in clones. Analysis of minor populations is important for assessing true patterns of diversity, since these populations affect measures of diversity such as equitability (Pielou, 1975). Nonetheless, a more limited analysis of the most abundant populations can reveal the extent to which those populations that most likely have the greatest impact on specific processes (e.g., methanogenesis) differ among systems.

At present, it is not apparent to what extent, if any, methanogen population structure affects the dynamics of methanogenesis in wetlands. However, the differences described here provide a basis for future studies linking population structure, physiological ecology, and in situ rates of methanogenesis to key edaphic parameters (e.g., temperature, pH, organic inputs), and both short- and long-term disturbances (e.g., climate change, hydrologic variability, acid deposition). Aside from being of value in predicting future trends in wetland methanogenesis, such studies will provide a context for understanding the relationship between microbial diversity and biogeochemical cycles.

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