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Phylogenetic Characterization of Methanogenic Assemblages in Eutrophic and Oligotrophic Areas of the Florida Everglades†

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Agricultural activities have produced well-documented changes in the Florida Everglades, including establishment of a gradient in phosphorus concentrations in Water Conservation Area 2A (WCA-2A) of the northern Everglades. An effect of increased phosphorus concentrations is increased methanogenesis in the eutrophic regions compared to the oligotrophic regions of WCA-2A. The goal of this study was to identify relationships between eutrophication and composition and activity of methanogenic assemblages in WCA-2A soils. Distributions of two genes associated with methanogens were characterized in soils taken from WCA-2A: the archaeal 16S rRNA gene and the methyl coenzyme M reductase gene. The richness of methanogen phylotypes was greater in eutrophic than in oligotrophic sites, and sequences related to previously cultivated and uncultivated methanogens were found. A preferential selection for the order *Methanomicrobiales* was observed in *mcrA* clone libraries, suggesting primer bias for this group. A greater diversity within the *Methanomicrobiales* was observed in *mcrA* clone libraries than in 16S rRNA gene libraries. 16S rRNA phylogenetic analyses revealed a dominance of clones related to *Methanosaeta* spp., an acetoclastic methanogen dominant in environments with low acetate concentrations. A significant number of clones were related to *Methanomicrobiales*, an order characterized by species utilizing hydrogen and formate as methanogenic substrates. No representatives of the orders *Methanobacteriales* and *Methanococcales* were found in any 16S rRNA clone library, although some *Methanobacteriales* were found in *mcrA* libraries. Hydrogenotrophs are the dominant methanogens in WCA-2A, and acetoclastic methanogen genotypes that proliferate in low acetate concentrations outnumber those that typically dominate in higher acetate concentrations.

Methanogenesis is a terminal step and may be a primary regulator of organic matter decomposition in freshwater wetlands. Natural wetlands systems are among the most important sources of methane, emitting approximately 90 $\times 10^6$ metric tons of methane per year, representing 22% of global methane emissions (6).

The Florida Everglades is one of the largest freshwater marshes in North America and historically a low-nutrient freshwater ecosystem; however, regions of this marsh are now eutrophic due to discharge of nutrient-enriched drainage water from the adjacent Everglades Agricultural Area. This nutrient discharge resulted in a gradient in phosphorus concentrations in the northern Everglades, particularly in Water Conservation Area 2A (WCA-2A) (9, 12, 30, 49). Among the most significant changes effected by the addition of phosphorus to the system was a shift from sparse stands of sawgrass (*Cladium* spp.) to dense stands of cattails (*Typha* spp.), thereby increasing the amount of plant material, and hence organic carbon, added to soils of the eutrophic regions (11). Extensive biogeochemical research has shown greater rates of microbial activities, including methanogenesis, in eutrophic zones of WCA-2A compared to more oligotrophic regions of the marsh resulting from the increased phosphorus and carbon put into the system (3, 11, 14, 66). Sulfate reduction rates are also greater in eutrophic zones, but sulfate reduction plays a minor role in anaerobic mineralization of organic matter compared with methanogenesis (3). Other terminal electron acceptors such as O_2, NO_3^-, Fe(III), and Mn(IV) are rapidly depleted in Everglades soils and do not play a significant role in mineralization of organic matter (11, 50). Little is known of the effects of eutrophication on the composition of methanogenic assemblages, although Drake et al. (14) reported an enrichment of total anaerobes and methanogens in the eutrophic zones of WCA-2A compared to more-oligotrophic zones of the adjacent WCA-3A.

Methanogens are a specialized group of anaerobic microorganisms that utilize a narrow range of substrates, including acetate, H_2-CO_2, formate, and methyl compounds as electron donors (18). In freshwater ecosystems, H_2-CO_2, acetate, and formate are the main methanogenic precursors (54). Acetate is the primary precursor in these ecosystems, and approximately 60 to 80% of the methane is produced from acetate (7, 27, 65). Most studies on the ecology of methanogens have been conducted in soils and rhizospheres of rice paddies and anaerobic biodigesters (20, 31, 38), and little is known about methanogens in ecosystems such as freshwater marshes. Most of these studies targeted the 16S rRNA gene and mainly focused on rice paddies (5, 20, 21, 28, 31, 36, 39). An alternative approach to study methanogenic community composition is by characterization of a functional gene such as that encoding methyl coenzyme M reductase (MCR) (58), which catalyzes the last step of methanogenesis. Several studies have been conducted with this functional gene to assess diversity of methanogens in several environments, such as peat bogs (22, 37, 42), marine sediments (1), termite guts (44), landfills (40), and rice paddies

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TABLE 1. Characterization of 0-to-10-cm layer for eutrophic (F1) and oligotrophic (U3) Everglades soils used in the construction of methanogenic clone libraries

Sampling site	Vegetation	Sampling time	Water table (cm)	TP[b] (mg/kg)	TP_i[b] (mg/kg)	TKN[b] (mg/kg)	Extractable TOC[b] (mg/kg)	MBC[b] (mg/kg)
F1	Cattail	Spring 2001	−9.1	1,536 (57)	549 (33)	402 (44)	4,101 (78)	7,638 (544)
		Summer 2001	61.3	1,089 (205)	388 (108)	309 (29)	2,798 (278)	4,043 (1,000)
U3	Sawgrass, slough	Spring 2001	−0.9	277 (19)	69 (10)	343 (120)	2,892 (90)	2,066 (221)
		Summer 2001	62.5	377 (59)	82 (15)	305 (69)	1,741 (376)	2,276 (607)

[a] Values in parentheses represent the standard deviation of triplicate determinations.
[b] Data are expressed per kilogram (dry weight) of soil. TP, total phosphorus; TP_i, total inorganic phosphorus; TKN, total Kjeldahl nitrogen; TOC, total organic carbon; MBC, microbial biomass carbon.

(38, 39, 48), but none have focused on freshwater wetlands. Non-culture-based microbial diversity studies may provide valuable information on the composition and structure of methanogenic assemblages in freshwater ecosystems and of the effects of eutrophication on methanogenic communities. The objectives of this study were to characterize methanogen assemblages using culture-independent approaches targeting the 16S rRNA and *mcr* genes and to utilize this information to infer possible roles of particular constituents of these assemblages in eutrophic and oligotrophic sites of the northern Florida Everglades during two seasons, spring and summer.

MATERIALS AND METHODS

Site characteristics, sampling, and biogeochemical characterization. Studies were conducted on samples taken from WCA-2A, as previously described (3). Samples were collected along the phosphorus gradient from the nutrient-impacted F1 cattail (*Typha* sp.)-dominated sites and the oligotrophic U3 sawgrass (*Cladium* sp.)-dominated sites. Soil cores were sampled to a depth of 30 cm and placed on ice by staff of the South Florida Water Management District and transported to the laboratory within 24 h of the collection, where they were sectioned in 10-cm increments and manually mixed. Subsamples for DNA analysis were taken and stored at −70°C until analysis. Biogeochemical characterization was conducted at the Wetland Biogeochemistry Laboratory (10, 63) and is presented in Table 1. Samples used for clone libraries were collected during April and August 2001. Methanogenesis rates were measured in samples collected during December 2001 and January and May 2002.

Methanogenesis rate measurement. One gram of soil from the 0-to-10-cm soil layer was mixed with 2 ml of anoxic basal carbonate yeast extract trypticase medium (60) under a N_2 stream in anaerobic culture tubes that were later closed with butyl rubber stoppers and aluminum seals. The tubes were preincubated for 2 weeks before substrates were added. Acetate and formate (20 mM each) were added from anaerobic sterile concentrated stock solutions. The tubes were fitted with three-way Luer stopcocks (Cole-Parmer, Vernon Hills, Ill.) for gas sampling and incubated in the dark at 25°C with shaking at 100 rpm. Methane in the headspace was measured by gas chromatography with a Shimadzu 8A gas chromatograph equipped with a Carboxen 1000 column (Supelco, Bellefonte, Pa.) and a flame ionization detector operating at 110°C. The carrier gas was N_2, and the oven temperature was 160°C. All determinations were carried out in triplicate. Headspace pressure was measured using a digital pressure indicator (DPI 705; Druck, New Fairfield, Conn.). Methane determinations were conducted in quintuplicate. Statistical analyses were performed by using JMP statistical software (version 4.04; SAS Institute, Cary, N.C.). Two-way analysis of variance was performed with substrate addition and site type as factors. Subsequently, the Tukey-Kramer honestly significant difference means test ($P = 0.05$) was used for comparisons of the treatments.

Nucleic acid extraction and PCR amplification. Nucleic acids were extracted from 0.25 g of the 0-to-10-cm soil layer with UltraClean soil DNA kits (MoBio, Solana Beach, Calif.) according to the manufacturer's instructions. PCR was conducted using the primer set designed by Luton et al. (40), which amplifies a ca. 465- to 490-bp fragment of *mcrA*; this set consisted of primers mcrA-f (5′-GGTGGTGTMGGATTCACACARTAYGCWASCGC-3′) and mcrA-r (5′-TTCATTGCRTAGTTWGGRTAGTT-3′). The reaction mixture used for PCR amplification contained 7 μl of distilled H_2O, 1 μl of each primer (10 pmol/μl), 10 μl of HotStar*Taq* Master Mix (QIAGEN, Valencia, Calif.), and 1 μl of diluted DNA solution. PCR amplification was carried out in a GeneAmp PCR system 2400 (Perkin-Elmer Applied Biosystems, Norwalk, Conn.). The initial enzyme activation and DNA denaturation were performed for 15 min at 95°C, followed by five cycles of 30 s at 95°C, 30 s at 55°C, and 30 s of extension at 72°C, and the temperature ramp between the annealing and extension segment was decreased to 0.1°C per s from the default 1°C per s because of the degeneracy of the primers. After this, 30 cycles were performed with the following conditions: 30 s at 95°C, 30 s at 55°C, and 30-s extension at 72°C, and a final extension of 72°C for 7 min. The PCR products were electrophoresed on 2% agarose gels in Tris-acetate-EDTA buffer to confirm amplification of expected size product.

The primer set combination 23F and 1492R was used for 16S rRNA gene amplification (2, 32). The initial enzyme activation and DNA denaturation was performed for 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 60-s extension at 72°C, and a final extension of 72°C for 7 min. PCR products were electrophoresed on 0.7% agarose gels in Tris-acetate-EDTA buffer to confirm amplification of the expected size of product.

Cloning of *mcrA* and 16S rRNA genes and RFLP analysis. Fresh PCR amplicons were ligated into pCRII-TOPO cloning vector and transformed into chemically competent *Escherichia coli* TOP10F′ cells according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif.). Individual colonies of *E. coli* were screened by direct PCR amplification with the *mcr* or 16S rRNA primers using the previously described PCR programs. Random fragment length polymorphism (RFLP) analyses were conducted using the restriction enzyme TacI for *mcrA* and 16S rRNA gene amplification products. Digests were analyzed by agarose gel electrophoresis using 2% and 4% gels for 16S rRNA and *mcrA* digests, respectively. Clone libraries were analyzed by analytic rarefaction using the software aRarefactWin (version 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens [http://www.uga.edu/~strata/software/]).

Sequencing and phylogenetic analysis. Selected clones were sequenced at the DNA Sequencing Core Laboratory at the University of Florida with the mcrA-f and 23F primers. A total of 76 16S rRNA gene clones were partially sequenced (approximately 500 bp). For *mcrA*, 52 clones were partially sequenced (480 bp, which translated into a deduced amino acid sequence of approximately 160 amino acids). Deduced amino acid sequences of the MCR α-subunit were aligned and analyzed with ClustalX version 1.81 (59). Since Luton et al. (40) did not find differences between different *mcrA* phylogenetic analyses, data presented here are from a neighbor-joining analysis using ClustalX 1.81. *Methanopyrus kandleri* sequence was used as an outgroup in the phylogenetic analysis (40). Bootstrap analysis was performed with 100 resamplings of the amino acid sequences. 16S rRNA gene sequence alignments were also evaluated with PAUP* version 4.0b8 using parsimony-based algorithms (D. L. Swofford, Sinauer Associates, Sunderland, Mass.). Several phylogenetic approaches yielded similar results with only minor changes in the placement of some sequences within the major cluster. Trees were constructed using heuristic searches with 10 random stepwise additions of taxa and by tree-bisection reconnection branch swapping. The characters were weighted to give more weight to characters with lower levels of homoplasy. Bootstrap analysis was performed with 100 resamplings. *M. kandleri* sequence was used as the outgroup, because this species is the suggested outgroup for the *mcrA* analysis (40). The nomenclature of the *Archaea* domain is according to the taxonomic outline of the prokaryotic genera of *Bergey's Manual of Systematic Bacteriology* (19).

Diversity index. The cumulative number of phylotypes was calculated by fitting the rarefaction curves to a hyperbolic model with the formula $y = ax/(b + x)$ using Datafit software (version 8.0.32; Oakdale Engineering, Oakdale, Pa.), where y represents the number of phylotypes and x is the number of individuals.

TABLE 2. Potential methanogenic rates and CH_4 accumulated in eutrophic (F1) and oligotrophic (U3) Everglades soils

Date and addition	F1		U3	
	Rate[a]	μmol of CH_4[b]	Rate	μmol of CH_4
Dec. 2001				
None	0.015 (0.002)	2.0 (0.4)	0.003 (0.001)	0.2 (0.01)
Acetate	0.057 (0.003)	8.8 (0.3)	0.007 (0.001)	0.4 (0.04)
Jan. 2002				
None	0.026 (0.003)	1.4 (0.2)	0.004 (0.001)	0.4 (0.04)
Acetate	0.049 (0.005)	7.8 (0.3)	0.016 (0.003)	2.9 (0.9)
May 2002				
None	0.026 (0.002)	3.2 (0.3)	0.003 (0.001)	0.4 (0.1)
Acetate	0.040 (0.002)	7.1 (1.2)	0.003 (0.001)	0.2 (0.1)
Formate	0.084 (0.005)	7.0 (0.5)	0.005 (0.001)	1.2 (0.3)

[a] Potential methanogenic rates (in micromoles per gram per hour); for details see the section on methanogenesis in Materials and Methods. Standard errors of the means are shown in parentheses ($n = 5$).
[b] Micromoles of CH_4 accumulated at the end of 6 days of incubation.

TABLE 3. Expected and recovered phylotypes and diversity indices for archaeal 16S rRNA and *mcrA* clone libraries for eutrophic and oligotrophic Everglades soils

Clone library	No. of expected phylotypes[a]	No. of recovered phylotypes[b]	Shannon's index
Archaeal 16S rRNA			
F1, summer	36.0 ± 0.8	20 (38)	1.25
F1, spring	86.7 ± 2.5	23 (32)	1.29
U3, summer	28.0 ± 0.8	16 (38)	1.06
U3, spring	32.8 ± 0.8	17 (36)	1.10
mcr			
F1, summer	22.7 ± 0.6	14 (35)	1.03
F1, spring	36.1 ± 0.8	18 (37)	1.12
U3, summer	13.2 ± 0.1	9 (29)	0.83
U3, spring	12.9 ± 0.1	10 (38)	0.83

[a] Value of constant a from hyperbolic equation $y = ax/(b + x)$ with the standard error.
[b] Values in parentheses represent the total number of clones screened.

Phylotypes were defined on the basis of unique restriction digestion patterns. Diversity of clone libraries was calculated using the Shannon index (45) according to the following formula: $H = -\Sigma p_i(\log p_i)$ from $i = 1$ to n, where p_i represents the proportion of a particular phylotype in the clone library and n is the total number of phylotypes.

Nucleotide sequence accession numbers. GenBank accession numbers for partial *mcrA* sequences for F1 samples are AY458405 to AY458419 for spring samples and AY459307 to AY459319 for summer samples. Partial *mcrA* sequences for U3 samples are AY458420 to AY458427 for spring and AY460212 to AY460220 for summer samples. GenBank accession numbers for partial 16S rRNA sequences for F1 samples are AY456713 to AY456731 for spring and AY475640 to AY457657 for summer samples. Partial 16S rRNA sequences for U3 samples are AY456732 to AY456748 for spring and AY457658 to AY457673 for summer samples.

RESULTS

Methanogenic rates. Intrinsic initial methanogenesis rates were highly affected by the addition of acetate, site eutrophication status, and the interaction effect of acetate addition and eutrophication status (two-way analysis of variance; $P < 0.0001$). Intrinsic methanogenic rates were five to nine times higher in F1 soils than in U3 soils (Table 2), and total methane accumulated at the end of 6 days of incubation was 4 to 10 times higher for the F1 soils than for U3 soils ($P < 0.05$). Addition of acetate resulted in an increase in methanogenesis rates in soils from both sites, with the exception of U3 samples taken in May 2002. The rates with acetate addition were 3 to 12 times higher for F1 than for U3 soil samples.

Hydrogen has been shown to be an important electron donor in eutrophic zones of this marsh (61). Approximately 60% of cultured hydrogenotrophic methanogens are considered to be capable of using formate as an electron donor (18), and use of formate as a surrogate for H_2-CO_2 is a common practice in anaerobic digestion studies because diffusion problems from the gas headspace to the liquid media are avoided (13). We employed formate as a surrogate for H_2-CO_2 to test the response of these soils to H_2-CO_2, and we found that addition of formate resulted in a 17-fold increase in F1 samples compared with U3 soil samples.

Diversity indices for cloned *mcrA* and 16S rRNA sequences. Samples from eutrophic soils were more diverse in both libraries when evaluated by either the cumulative expected phylotypes index or Shannon's index. Archaeal 16S rRNA gene libraries were more diverse than were *mcrA* clone libraries (Table 3).

RFLP patterns of the 16S rRNA gene clones suggested a significant degree of sequence diversity in summer and spring samples. The number of phylotypes observed were lower than the predicted number of phylotypes according to a hyperbolic model fit to the data; however, covering all the diversity would require sequencing an extremely high number of clones, making the study economically unfeasible.

A significant degree of sequence diversity in summer and spring samples was observed with *mcrA* RFLP. However, diversity was lower than with the archaeal 16S rRNA gene libraries, since the *mcr* set of primers only targeted methanogenic prokaryotes and not the total archaeal population targeted with the archaeal primers. In the case of *mcr* sequences, the recovered phylotypes were more similar to the expected phylotypes than was the case for the 16S rRNA analysis (Table 3). In both cases, F1 samples exhibited a slightly higher degree of diversity as judged by the slope of the rarefaction curves (data not shown), which was in good agreement with the calculated Shannon's indices.

Phylogenetic analysis of cloned 16S rRNA sequences. Cloned archaeal sequences were distributed in a total of eight clades: one clade in the phylum *Crenarchaeota* and seven clades in the phylum *Euryarchaeota* (Fig. 1 and 2). The overall and major clades of the archaeal phylogeny were in very good agreement with previously published archaeal phylogeny (5, 18, 20). The seven *Euryarchaeota* clades included methanogens of the orders *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* and three putative clades composed of sequences from uncultured microorganisms.

The ARC-1 cluster is related to the order *Methanomicrobiales* and uncultivated *Methanomicrobiales* sequences recovered from a variety of ecosystems (16, 41, 46, 47). The ARC-2 cluster is related to sequences (26, 62) referred to in GenBank as unclassified/uncultured *Archaea* (environmental samples) or unclassified/uncultured *Methanomicrobiales* (43). The phylogenetic affiliation of these clones remains uncertain, but they may represent a deeply divergent branch of the *Methanomicrobiales* order. ARC-3 clusters with cultivated and uncultivated ar-

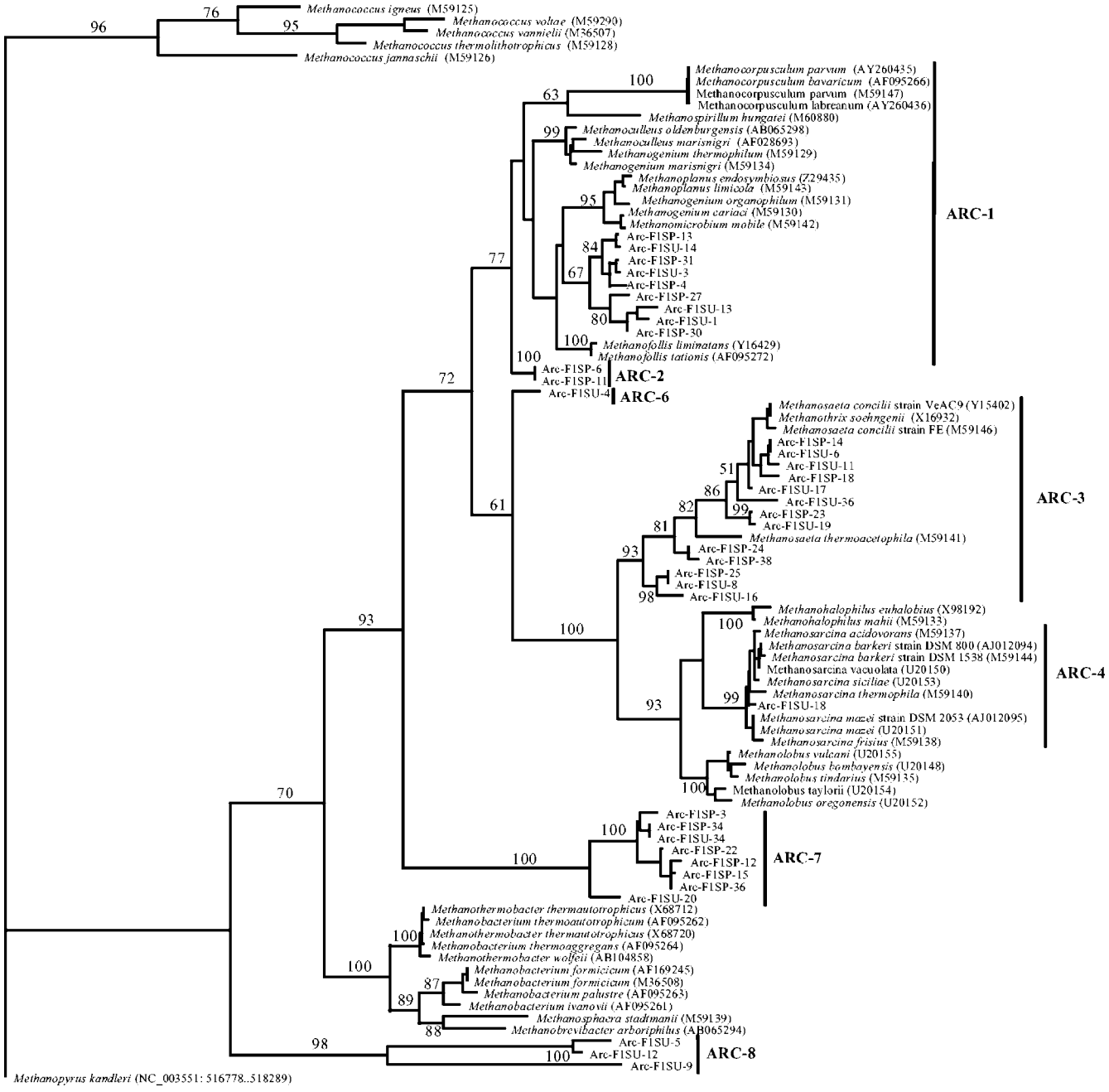

FIG. 1. Maximum parsimony tree for archaeal 16S rRNA gene sequences from eutrophic F1 sites of Everglades WCA-2A. The clones are named according to the site and time of sampling. The scale bar represents 1 nucleotide change per 100 sequence positions. Numbers at nodes represent percentages of bootstrap resamplings based on 100 replicates; only values above 50 are presented.

chaeal sequences related to *Methanosaeta* spp. recovered from a great variety of ecosystems (16, 20, 33, 39, 46–48, 55). The ARC-4 cluster was related to the genus *Methanosarcina*. The ARC-5 cluster was related to the *Methanosarcinales* order, although this cluster was deeply divergent from other *Methanosarcinales.* The ARC-6 cluster was composed of two clones that were deeply divergent in the *Methanosarcinales* order by parsimony analysis. However, these clones were placed as a very divergent deep branch grouping with the *Methanomicrobiales* in the neighbor-joining analysis. The GenBank BLAST report for these sequences returned representatives of the *Methanomicrobiales* and *Methanosarcinales* orders. More information is required to place these clones with a particular order. The ARC-7 cluster is deeply divergent from the *Methanosarcinales* and *Methanomicrobiales* orders, and clones related to this group have been reported in other environments (23, 29, 43, 56). The ARC-8 cluster is comprised of sequences related to the *Crenarchaeota* phylum. The *Crenarchaeota* phylum is

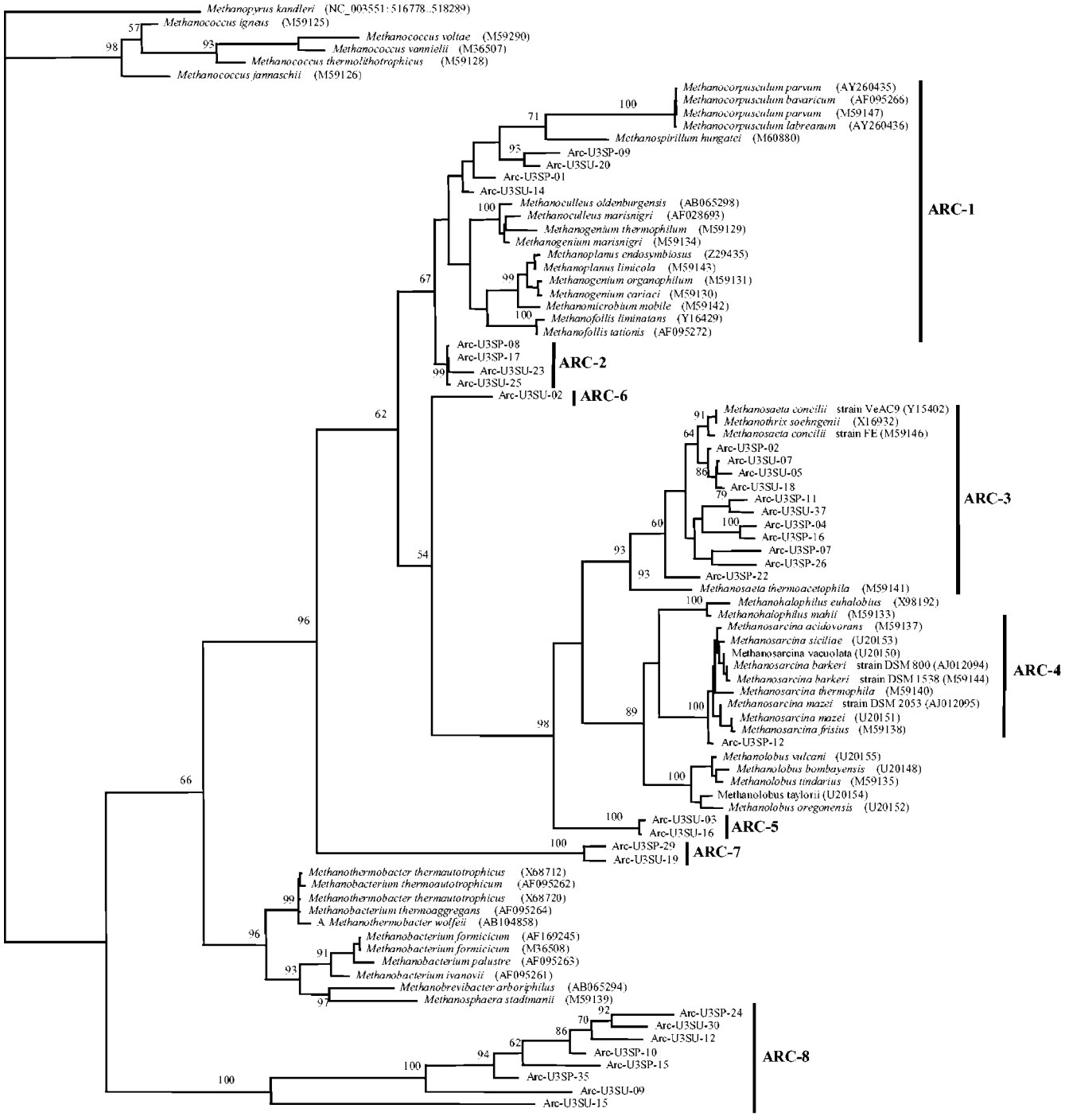

FIG. 2. Maximum parsimony tree for archaeal 16S rRNA gene sequences from oligotrophic U3 sites of Everglades WCA-2A. The clones are named according to the site and time of sampling. The scale bar represents 1 nucleotide change per 100 sequence positions. Numbers at nodes represent the percentages of bootstrap resamplings based on 100 replicates; only values above 50 are presented.

composed of extreme hyperthermophilic prokaryotes (51, 57); however, related sequences have been found in cold and moderate-temperature environments (39, 55). No clone related to the *Methanococcales* or *Methanobacteriales* order was found in clone libraries from any season or site in this study.

Effect of eutrophication and season on the dynamics of archaeal sequences. Clone libraries for F1 sites were dominated by clusters ARC-3 (*Methanosaeta* cluster), ARC-1 (*Methanomicrobiales* cluster), and ARC-7 (uncultured cluster), representing almost 80% of the clone library sequences for summer

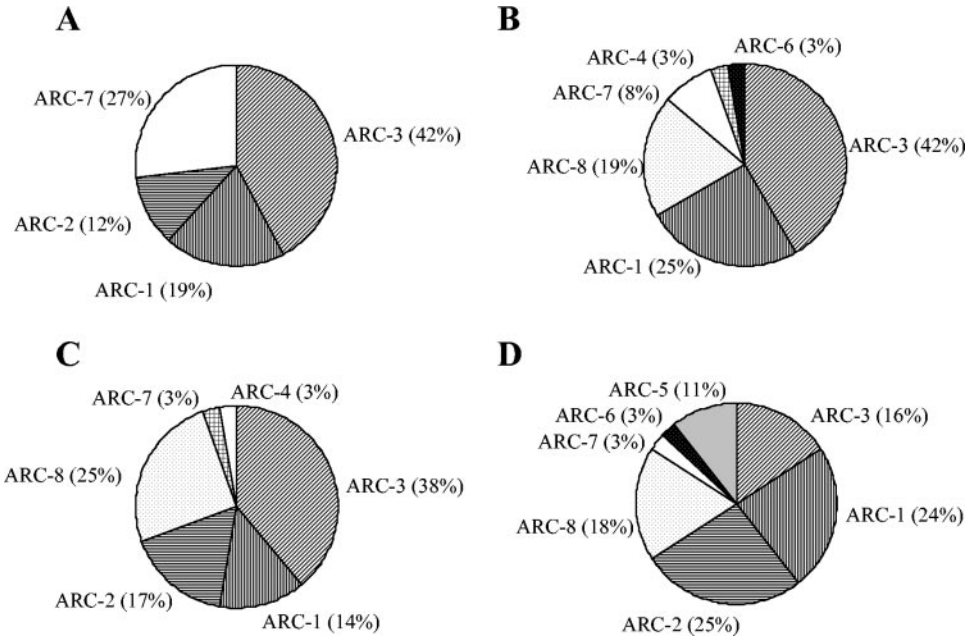

FIG. 3. Spatial and seasonal distributions of archaeal 16S rRNA clones in eutrophic soils for spring (A) and summer (B) and oligotrophic soils for spring (C) and summer (D).

samples and 90% for spring samples (Fig. 3). The summer F1 clone library also contained ARC-8 (*Crenarchaeota* cluster) and minor amounts of ARC-4 sequences (*Methanosarcina* cluster) and ARC-6 sequences (uncultured cluster) (Fig. 3). Seasonal changes between summer and spring for eutrophic F1 sites resulted in an emergence of clones of cluster ARC-2 (uncultured cluster) and disappearance of sequences of clusters ARC-4, ARC-6, and ARC-8 from summer to spring, but clusters ARC-1, ARC-3, and ARC-7 dominated clone libraries from both sampling sites.

U3 clone libraries were dominated by clusters ARC-3, ARC-1, ARC-2, and ARC-8, with minor amounts of cluster ARC-7 (Fig. 3). Seasonal changes from summer to spring resulted in some changes of the relative amounts of the major cluster and in the emergence of cluster ARC-4 and disappearance of clusters ARC-5 (uncultured cluster) and ARC-6.

Phylogenetic analysis of cloned *mcrA* sequences. Cloned MCR sequences were distributed among a total of seven clades encompassing the orders *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* and three putative clades composed of sequences from uncultured microorganisms (Fig. 4). The overall MCR phylogeny (Fig. 4) was in very good agreement with previously published MCR phylogenies.

The MCR-1 cluster was related to the order *Methanobacteriales*, with similar clones found in rice paddies (38) and landfill material (40). The MCR-2 cluster was composed exclusively of our clones and sequences from uncultured microorganisms (15, 25), and their phylogenetic affiliation remains uncertain. The MCR-3 cluster was related to *Methanosarcina* sequences, with similar sequences also recovered from landfill and rice paddy soils (38, 40). The MCR-4 cluster was related to the genus *Methanosaeta* of the order *Methanosarcinales*. Sequences Mcr-U3SP-18, U3SU-16, and U3SU-33 exhibited ca. 85% similarity to sequences of rice cluster I, outside the *Methanosaeta* cluster described by Lueders et al. (38); however, bootstrap analysis did not support these as a separate cluster. More sequence information would clarify the assignment of these sequences as an individual cluster. Cluster MCR-5 was composed of sequences branching deeper in the *Methanomicrobiales* order. Sequences clustering with this group have been also found in landfill soils (40); however, they are deeply divergent from cultured *Methanomicrobiales* species. Similar sequences were also obtained from a variety of environments (15, 17, 25). These sequences could be divided into two clusters but were treated as a single group here because of the lack of information on physiological characteristics that might support division of the cluster into two groups. Cluster MCR-6 was related to *Methanomicrobiales*, and similar sequences were obtained from landfill soils (40) and rice paddies (38). The MCR-7 cluster was related to *Methanomicrobiales* but in a separate cluster from cultured *Methanomicrobiales*.

Effect of eutrophication and season on the dynamics of MCR sequences. Clone libraries for F1 sites were dominated by sequences in clusters MRC-7, MCR-6, and MCR-5 (related to *Methanomicrobiales*). The summer F1 clone library also contained MCR-1 (*Methanobacteriales* cluster), MCR-2 (uncultivated cluster), and minor amounts of MCR-3 sequences (*Methanosarcina* cluster) (Fig. 5). Seasonal changes between summer and spring for eutrophic sites resulted in an increase of MCR-6 and decrease of MCR-7, an emergence of MCR-4 clones (*Methanosaeta* cluster), and disappearance of MCR-1 sequences. *Methanobacteriales* was not recovered from archaeal libraries; however, these clones represented approximately 10% of the *mcrA* libraries. Screening of more clones in the archaeal libraries may yield clones within this group.

U3 clone libraries were more dynamic; summer samples were dominated by clones of the clusters MCR-5 and MCR-7, followed by MCR-4 and minor amounts of MCR-1 (Fig. 5).

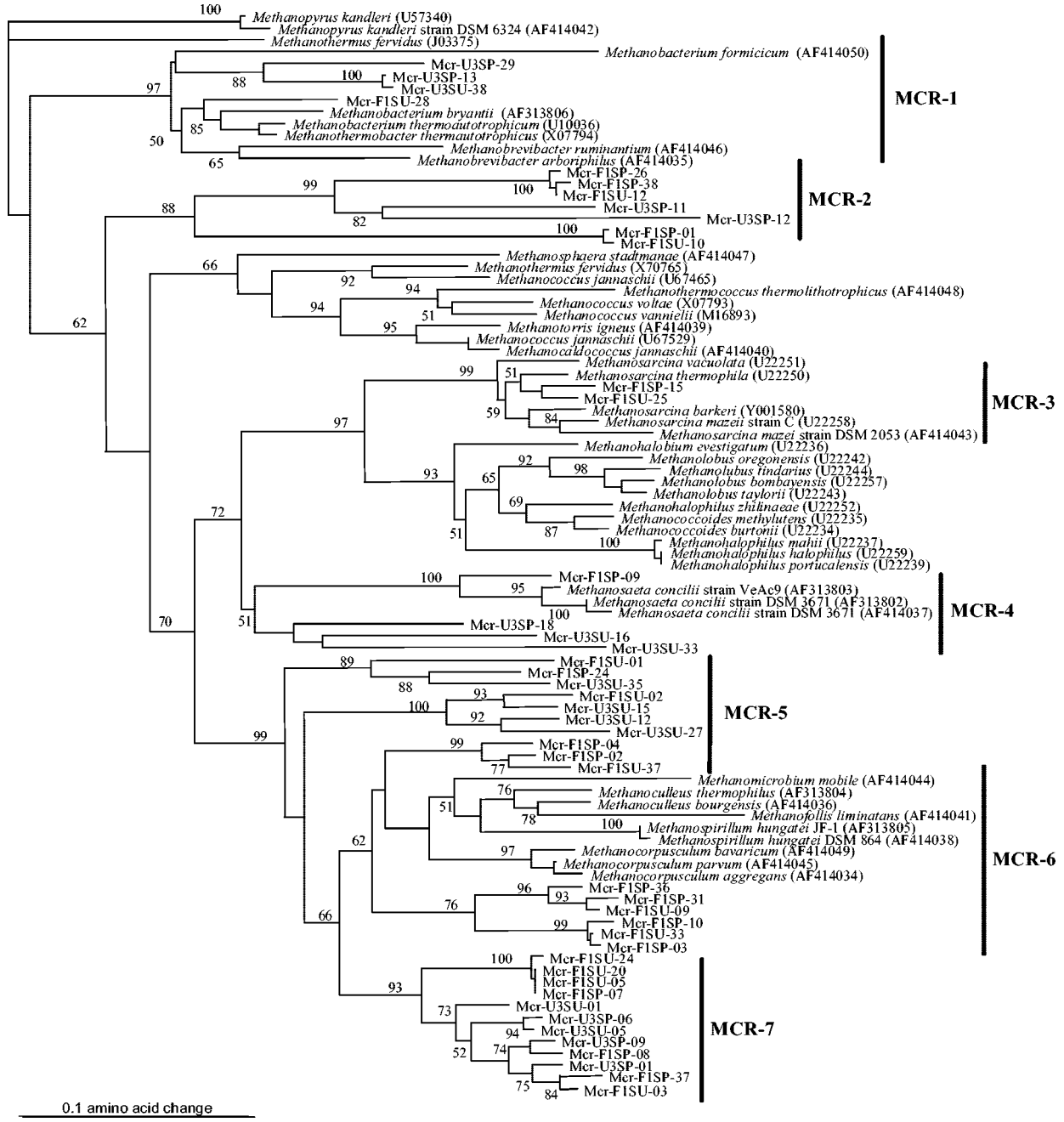

FIG. 4. Neighbor-joining MCR α-subunit tree. The clones are named according to the origin and time of sampling. The scale bar represents 10% sequence divergence. Numbers at nodes represent the percentages of bootstrap resamplings based on 100 replicates; only values higher than 50 are presented.

Seasonal changes from summer to spring resulted in an increase in the relative amount of MCR-7 sequences, the emergence of MCR-2 sequences, the disappearance of MCR-5 sequences (uncultured cluster), and minor changes in the frequencies of MCR-1 and MCR-4 sequences. Clearly, the dominance of clones related to *Methanomicrobiales* and the lower representation of *Methanosaeta* spp., a cluster present in significant numbers in the archaeal 16S rRNA gene libraries, may indicate a PCR bias for the *mcr* set of primers. Luton et al. (40) tested the set of primers with *Methanosaeta* and *Methanosarcina* spp. and obtained amplification of sequences related to these genera. However, most clones present in their libraries were related to species belonging to *Methanomicrobiales* and *Methanobacteriales.* Few clones were related to *Methanosarcina* spp., and none were related to *Methanosaeta* spp. The authors attributed these observations to PCR biases. Therefore, it seems that this set of *mcr* primers would be appropriate primers to study diversity of *Methanobacteriales* and *Methanomicro-*

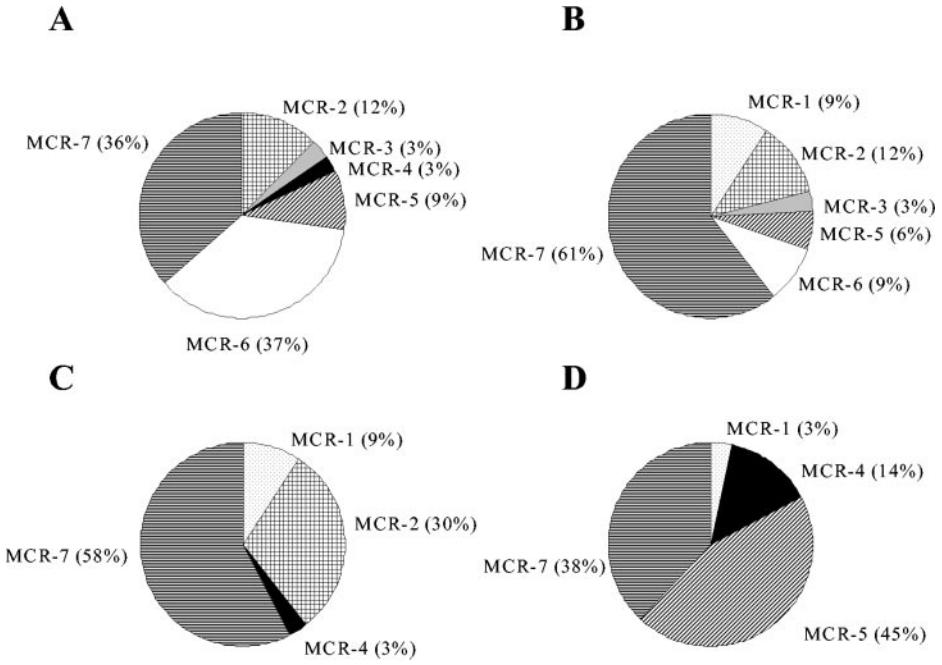

FIG. 5. Spatial and seasonal distribution of *mcrA* clones in eutrophic soils for spring (A) and summer (B) and oligotrophic soils for spring (C) and summer (D).

biales. The dynamics of these two methanogenic orders may be useful as indicators to assess the effects of eutrophication on microbial communities.

DISCUSSION

Although methanogenesis is one of the main processes responsible for terminal anaerobic organic matter mineralization in the Everglades, very little is known about the microbial groups involved in this process (66). Extensive biogeochemical research has shown that phosphorus loading has resulted in changes at the micro and macro levels in nutrient-impacted regions of WCA-2A (3, 11, 14, 66). However, this is the first complete report of which we are aware that has characterized assemblages of methanogens in oligotrophic and eutrophic zones of the northern Everglades. The only previous attempt to characterize methanogenic communities in the Everglades was conducted by Drake et al. (14) in similar sites. Those authors reported an enrichment of almost 6 orders of magnitude in acetoclastic methanogens in eutrophic regions. They also reported an enrichment of acetate-producing microorganisms and H_2-consuming microorganisms in the eutrophic zones of the marsh. The number of H_2-consuming acetogens was similar in eutrophic and oligotrophic sites, which may indicate that the increase of total H_2-consuming microorganisms is due to hydrogenotrophic methanogens. These differences in microbial enumerations correspond with lower methanogenic rates observed for oligotrophic sites than for eutrophic sites. In a more recent study, our investigators found that hydrogenotrophic methanogens were 1,000 and 100 times higher than acetoclastic methanogens in eutrophic and oligotrophic soils, respectively, and relative numbers of acetoclastic methanogens were similar between eutrophic and oligotrophic sites (4). Clearly, phosphorus loading of the Everglades WCA-2A is affecting, either directly or indirectly, the population size and activity of methanogens, resulting in greater numbers and methanogenic activities in phosphorus-enriched sites. Methanogenesis rates for samples of eutrophic sites measured using formate were ca. 2.5 times higher than methanogenesis rates measured using acetate as substrate. These data suggest that hydrogenotrophic methanogens are important in the decomposition of organic matter in eutrophic Everglades soils.

Acetate utilization is restricted to two genera in the order *Methanosarcinales*, *Methanosaeta* and *Methanosarcina*; all other species of methanogens use H_2. However, it is estimated that 70 to 80% of the methane produced in nature comes from conversion of acetate to methane by acetoclastic methanogens (27). Conrad reported that the contribution to H_2 or acetate to methane production is highly variable and that the relative proportions are reversed in cases where the hydrogen contribution is higher (7). In some cases, methanogenesis is exclusively driven by hydrogen. Examples include eutrophic lakes, hot spring mats, coastal marine sediments, temperate bogs, Antarctic water bodies, and lake sediments. Recently, Horn et al. (24) reported that hydrogen is the main methanogenic precursor in acidic peat, which is in agreement with other studies conducted in acidic peats (34, 64).

There are several possible explanations for the higher contribution of hydrogen as electron donor for methanogenesis, including additional sinks or loss of acetate by nonmethanogenic microorganisms and additional pools of hydrogen, such as production of high amounts of H_2 by fermentation of organic matter or geological inputs. Acetate can be consumed by nonmethanogenic microorganisms using electron acceptors such as oxygen, nitrate, ferric ion, and sulfate if these electron acceptors are available. We previously reported a slight com-

petition for acetate between methanogens and sulfate-reducing prokaryotes in eutrophic F1 soils (3). With the exception of sulfate reduction, these alternative terminal electron-accepting processes are not considered to be significant in WCA-2A soils compared with methanogenesis.

Clones in the archaeal 16S rRNA gene libraries related to previously cultured methanogens in groups ARC-1 (*Methanobacteriales*), ARC-3 (*Methanosaeta* spp.), and ARC-4 (*Methanosarcina* spp.) may provide insight into the dynamics of acetate in these soils. Two methanogens related to *Methanosarcina* spp. and *Methanosaeta* spp. were detected. *Methanosaeta* spp. are specialists that grow only on acetate and dominate at low acetate concentrations (7 to 70 μM); *Methanosarcina* spp. are generalists that grow on hydrogen and methyl compounds but require acetate at much higher concentrations (0.2 to 1.2 mM) (27) than do *Methanosaeta* spp. The dominance of *Methanosaeta*-like sequences strongly suggests that acetate concentrations are low in these soils. *Methanosarcina* sequences were observed in small proportions, which may indicate soil niches where acetate concentrations are locally high. Cluster ARC-1 sequences (related to the *Methanomicrobiales*) were observed in significant numbers in all clone libraries, indicating that this order may be responsible for hydrogenotrophic methanogenesis. *mcrA* clone libraries were dominated by sequences related to *Methanomicrobiales* (clusters ARC-5, ARC-6, and ARC-7) and were highly diverse for this particular microbial group. A similar enrichment of *Methanomicrobiales* and *Methanobacteriales*, hydrogenotrophic methanogens, was reported for peat soils where hydrogen was an important methanogenic precursor (24).

The partial pressure of hydrogen can be a primary factor controlling the products of fermentation. If the hydrogen partial pressure is kept below 10^{-3} atm, fermentation to acetate, H_2, and CO_2 occurs in some systems; however, if H_2 accumulates, the formation of more-reduced products such as fatty acids or alcohol is promoted (52, 67). This shift results in an increase in the pool of fatty acids (butyrate or propionate), which are syntrophically degraded to acetate and H_2-CO_2, increasing the pool of hydrogen for hydrogenotrophic methanogens. Hydrogen is converted to methane by species in the order *Methanomicrobiales*. Acetate in low concentrations may be used by sulfate-reducing prokaryotes and syntrophic acetate oxidizers (8, 35, 53, 68), which would increase the hydrogen pool. This would favor proliferation of the specialist *Methanosaeta* spp., rather than the generalist *Methanosarcina* spp.

Phosphorus enrichment plays a major role in the carbon cycle of the Everglades by increasing the input of organic matter, which consequently increases the activity and number of methanogens in these soils. The diversity of hydrogenotrophic methanogenic assemblages was also affected by eutrophication, which may indicate a selection of different hydrogenotrophic methanogens driven by different levels of hydrogen between eutrophic and oligotrophic sites of the Everglades. We are currently studying the response of other microbial groups, including syntrophic bacteria (4) and fermentative bacteria (61), to eutrophication, which will provide greater insight into the role of hydrogen as a major substrate of methanogenic communities in this type of ecosystem.

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