Cellulolytic and fermentative guilds in eutrophic soils of the Florida Everglades

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Abstract

The northern Florida Everglades has been subject to eutrophication in recent years, resulting in well-documented changes in microbial ecology and a shift in the dominant plant species. This study investigated effects of plant quality and eutrophication on activities and composition of cellulolytic and fermentative guilds in soils. Most probable numbers of cellulolytic bacteria in eutrophic (F1) and transition (F4) soils were 10-fold higher than in oligotrophic soils (U3). Higher potential methanogenesis was observed from cellulose in microcosms with soils from F1 and F4, compared to U3 soils. Nutrient status of soil, rather than plant type, was the major factor controlling methanogenesis rates, although numbers of fermentative bacteria were higher in microcosms supplemented with ground cattail (dominant in F1 and F4) than with sawgrass (dominant component of soil in U3), regardless of soil origin. DNA sequence analysis indicated *Clostridium* assemblage composition correlates with soil nutrient status.

Introduction

The Florida Everglades is one of the largest freshwater marshes in North America and was historically a low-nutrient system. For over 35 years, the northern Everglades has been subject to nutrient loading and eutrophication associated with runoff from the nearby Everglades Agricultural Area. Agricultural runoff into the marsh formed a gradient in phosphorus concentrations ranging from 1500 mg total P kg^{-1} soil adjacent to the Everglades Agricultural Area to approximately 500 mg total P kg^{-1} soil in the relatively oligotrophic regions of the marsh. Prior to eutrophication, the Everglades was adapted to very low phosphorus concentrations (water column concentrations of less than 10 ppb), and phosphorus was the limiting nutrient for plant growth.

A number of significant changes in the ecology of the Everglades occurred as a result of eutrophication, and are evident along the nutrient gradient. Among the most significant of these changes is replacement of the dominant vegetation, sawgrass (*Cladium jamaicense*) in ridges and spike rush (*Eleocharis cellulosa*) in sloughs, by dense stands of cattail (*Typha domingensis*). Increased plant biomass and phosphorus in the eutrophic regions of the gradient resulted in increased rates of microbial activity, including microbial respiration, methanogenesis, and sulfate reduction (Castro *et al.*, 2002, 2004, 2005; Chauhan *et al.*, 2004).

Previous work in our laboratory demonstrated that the compositions and activities of assemblages of sulfate-reducing prokaryotes, methanogens, and syntrophs differ between eutrophic and oligotrophic regions of the marsh (Castro *et al.*, 2002, 2004, 2005; Chauhan *et al.*, 2004). Methanogenic and sulfate-reducing guilds depend in large part on decomposition of plant material by other microorganisms, particularly cellulolytic and fermentative species. Fermentation products formed from cellulose decomposition products are utilized by other anaerobes, including those mentioned above. The amount and type of fermentation products can control the activities and composition of guilds of these prokaryotes, such that changes in the activities of cellulolytic and fermentative bacteria would likely affect the activities and compositions of sulfate-reducing and methanogenic guilds. Previous work on these sites suggested a greater diversity of available fermentation products in F1 and F4 than U3 soils, which affected the composition of sulfate-reducing guilds (Castro *et al.*, 2002,

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2005). The compositions of methanogenic guilds also differ in these sites, possibly a result of differing H_2 concentrations produced from fermentation (Castro *et al.*, 2002, 2005).

Considerable information is available on the roles of cellulolytic and fermentative bacteria in the decomposition of plant material in human and animal intestines (Franks *et al.*, 1998; Hayashi *et al.*, 2002) and rice paddies (Hengstmann *et al.*, 1999; Weber *et al.*, 2001); however, little is known of the ecology of these guilds in natural wetlands or of the possible effects of plant quality or eutrophication on their activities and composition.

The primary objective of this study was to utilize a combination of culture- and nonculture-based approaches to characterize the activities, numbers, and composition of assemblages of cellulolytic and fermentative species in eutrophic and oligotrophic regions of the Everglades to gain a more complete picture of nutrient impacts on carbon cycling in this marsh.

Materials and methods

Site characteristics, sampling and biogeochemical characterization

Soil samples were collected from eutrophic, transition, and oligotrophic regions of the Florida Everglades Water Conservation Area 2A (WCA-2A) under flooded conditions in Spring 2002. Eutrophic regions (F1) are dominated by cattail and transition regions (F4) by mixtures of cattail and sawgrass. Oligotrophic regions (U3) are dominated by sawgrass on ridges and by spike rush in sloughs. Soil cores were collected in triplicate from each site and transferred on ice to the laboratory. After removal of the detrital layer, cores were sectioned and soils corresponding to 0–10 cm depth were separated, and replicate soil samples manually mixed to create a composite sample for each site. Subsamples to be used for microcosm experiments and enumeration were stored at 4 °C until analysis (within 2–7 days after sampling). Subsamples intended for DNA analysis were frozen at −70 °C. Total nitrogen, extractable ammonium-N, total phosphorus, total inorganic phosphorus, extractable organic carbon, and microbial biomass carbon were determined by the Wetland Biogeochemistry Lab at the University of Florida, as described previously (Wright & Reddy, 2001a; Castro *et al.*, 2002; Chauhan *et al.*, 2004).

Microbial enumeration

The most probable number technique with five replicates per dilution was used for enumeration studies. The most probable number medium for both fermentative and cellulolytic most probable numbers contained peptone ($10\,g\,L^{-1}$), NaCl ($5\,g\,L^{-1}$) and bromocresol purple ($0.0085\,g\,L^{-1}$), cysteine-sodium sulfide (2%, to provide final redox potential of −110 to −200 mV), plus the appropriate carbon substrate. Glucose (20 mM) and cellulose powder (0.3% weight in volume; Avicel PH-101 microcrystalline cellulose; FMC Biopolymer, Philadelphia, PA) were added to separate most probable number tubes for enumeration of fermentation bacteria and cellulolytic bacteria, respectively. For fermentative most probable number, a color change from purple to yellow due to acidity was counted as positive. For cellulose most probable number, tubes showing visual change in conformation of cellulose were counted as positive. The visual change included a shift from a dispersible powder to a more dense opaque form. This observed shift in form was confirmed as degradation by incubation of the powder as sole carbon source for cellulose degrading organisms (data not shown).

Laboratory microcosm studies

Composite soil samples (2 g, wet weight) were mixed with 50 mL basal carbonate yeast extract trypticase (BCYT) medium (Touzel & Albagnac, 1983) in 100 mL serum tubes. In each microcosm experiment, three or five replicates were used for each composite sample from F1, F4, and U3 soils. BCYT also included resazurin (1%), cysteine-sodium sulfide (2%), and a carbon source. Material used as a carbon source includes cellulose (0.162 g), or dried and crushed dead standing cattail or sawgrass plants (0.5 g). Phosphorus was not added to microcosms with plant material. All media, stock solutions, and microcosms were prepared under nitrogen gas stream to provide anaerobic conditions. Vials were closed with rubber stoppers and aluminum seals, and incubated at 28 °C. Liquid samples (1 mL) were collected weekly from microcosms. These samples were centrifuged, filtered through 0.2 μm filters, and stored at −20 °C until analysis. Fatty acids were measured with a high-pressure liquid chromatograph (HPLC) (Waters Corp., Milford, MA) equipped with a UV detector set at 210 nm. Aminex HP 87 H column (300 × 7.5 mm) was used with sulfuric acid (0.5 mM) as mobile phase at the flow rate of $0.6\,mL\,min^{-1}$. Methane formation in the headspace was determined by a Shimadzu 8A gas chromatograph equipped with a Carboxen 1000 column (Supelco, Bellefonte, PA) and a flame ionization detector set at 110 °C. Nitrogen was used as carrier gas and the oven temperature was 160 °C. The pressure in the headspace was measured with a digital pressure device (DPI 705; Druck, New Fairfield, CT).

Nucleic acid extraction and PCR amplification

DNA was extracted from soil samples using Ultra Clean Soil DNA kit (MoBio, Solana Beach, CA) according to manufacturer's instructions. DNA was extracted from the microcosms using this kit with the following modifications:

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approximately 1.5 mL supernatant from the microcosms were centrifuged at high speed in a microfuge, the supernatant discarded, and pellets resuspended in the kit's bead solution. The remainder of the purification followed the manufacturer's instructions. After extraction, DNA was analyzed by electrophoresis through between 0.7% and 1% agarose in Tris-Acetate-EDTA buffer.

Primer names, sequences, annealing temperatures, and target groups for amplification by PCR are presented in Table 1 (Lane, 1991; Franks *et al.*, 1998; Van Dyke & McCarthy, 2002). PCR reaction mixtures contained 10 μL of HotStarTaq master mix (Qiagen, Valencia, CA), 7 μL of distilled H_2O, 1 μL of each primer (10 pmol μL^{-1}) and 1 μL of diluted DNA solution. PCR cycling was performed at 94 °C for 1 min for denaturation and at 72 °C for 1 min for chain extension. Annealing was performed for 1 min for *Clostridium*-specific primers at temperatures shown in Table 1. Reaction mixtures were subjected to 40 cycles for *Clostridium*-specific primers in a Perkin-Elmer Model 2400 Thermal Cycler (Perkin-Elmer, Norwalk, CT). An initial activation step of 95 °C for 15 min was required for HotStarTaq master mix. An additional 7 min were added for chain extension at the end of reactions.

Cloning of 16S rRNA genes and restriction fragment length polymorphism analysis

Fresh PCR products were ligated into a pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA) and transformed into chemically competent *Escherichia coli* cells (TOP10F') according to the vendor's instructions. Individual colonies were screened by direct PCR amplification and restriction fragment length polymorphism (RFLP) analysis was performed using digestion enzymes *Hha*I+*Eco*RV for Cluster I, *Alu*I for Cluster III, and *Msp*I for Clusters IV and XIVab clones. Selection of digestion enzymes for RFLP was based on *in silico* analysis of previously identified 16S rRNA genes of *Clostridium* species in the National Center Biotechnology Information (NCBI) database using CLONEMAP software (version 2.11, CGC Scientific Inc, Ballwin, MO). Digestion reactions were analyzed in 2% agarose gels. Clone libraries were analyzed by analytical rarefaction with software ARAREFACTWIN [version 1.3, S Holland, Stratigraphy Lab, University of Georgia, Athens (http://www.uga.edu/~strata/software)] to confirm that sufficient numbers of RFLP groups were selected to represent the clone libraries.

Sequencing and phylogenetic analysis

Selected clones representing different RFLP patterns were sequenced by the University of Florida's Interdisciplinary Center for Biotechnology Research core sequencing facility. Sequences were compared with previously identified sequences in the NCBI database using BLAST (Altschul *et al.*, 1990). The sequences obtained in this study were initially aligned with closely matched sequences from the NCBI database using the Pileup function of GCG Package (Accelrys Inc., San Diego, CA) and adjusted manually with CLUSTALX version 1.8 (Thompson *et al.*, 1997). Phylogenetic trees were generated with TREECON (Van de Peer & De Wachter, 1994, 1997) using a neighbor-joining method. Bootstrap analysis was performed with 100 resamplings of the DNA sequences to estimate the confidence of tree topology.

Nucleic acid accession numbers

The GenBank accession numbers obtained in this study for *Clostridium* 16S rRNA gene sequences are AY650400–AY650408 and DQ168144–DQ168309.

Terminal RFLP analysis

Terminal RFLP (T-RFLP) analysis was conducted on 136 soil samples collected from F1, F4, and U3 regions of WCA-2A between April 2001 and August 2002. Three replicate cores from each site were collected on an approximately monthly basis for over 1 year (Castro *et al.*, 2005). DNA was extracted from soil samples by using Ultra Clean Soil DNA kit (MoBio) according to manufacturer's instruction. For PCR, primers Erec-0482-a-S-19 and Ccoc-1112-a-A-19 (Table 1) targeting 16S rRNA gene of *Clostridium* Cluster XIV species were used. The forward primer Erec-0482-a-S-19 was labeled with 6-FAM (6-carboxyfluorescein) by the vendor (Invitrogen). The same PCR cycling conditions, except annealing temperature, were used as described in previous sections. The annealing temperature was set to be 53 °C instead 55 °C. The PCR reaction mixture contained 25 μL of HotStarTaq master mix (Qiagen, Valencia, CA), 17.5 μL of distilled H_2O, 2.5 μL of each primer (10 pmol mL^{-1}) and 2.5 μL of diluted DNA solution. After confirming the expected PCR product size by electrophoresis through 0.7% agarose gels, products were cleaned and concentrated with QIAquick PCR purification kits (Qiagen) to 30 μL.

Between 100 and 150 ng of amplification product were digested with *Hinc*II according to vendor's instruction (Promega, Madison, WI). Selection of digestion enzyme for T-RFLP was based on *in silico* analysis of *Clostridium* sequences cloned from soil samples by using CLONEMAP software (version 2.11, CGC Scientific Inc.). From digestion reactions, between 1 and 1.5 μL aliquots were processed by the University of Florida's Interdisciplinary Center for Biotechnology Research core sequencing facility. T-RFLP analysis was conducted manually by scoring presence and absence of the peak corresponding to expected fragment size. A general linear model was applied using PROC GLM (SAS Institute Inc., Cary, NC). Duncan's Multiple Range

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Table 1. Primers and annealing temperatures used in this study

Primer (sequence 5′-3′)*	Target gene†	Annealing temperature (°C)	References
27F (AGAGTTTGATCMTGGCTCAG)	Universal 16S rRNA gene	58	Lane, (1991)
1492R (TACGGYTACCTTGTTACGACTT)	Universal 16S rRNA gene		Lane, (1991)
Chis-0150-a-S-23 (AAAGGRAGATTAATACCGCATAA)	*Clostridium* Cluster I 16S rRNA gene	58‡	Franks *et al*. (1998)
Cbot-0983-a-A-21 (CARGRGATGTCAAGYCYAGGT)	*Clostridium* Cluster I 16S rRNA gene		Van Dyke & McCarthy, (2002)
Cther-0650-a-S-23 (TCTTGAGTGYYGGAGAGGAAAGC)	*Clostridium* Cluster III 16S rRNA gene	60	Van Dyke & McCarthy, (2002)
Cther-1352-a-A-19 (GRCAGTATDCTGACCTRCC)	*Clostridium* Cluster III 16S rRNA gene		Van Dyke & McCarthy, (2002)
Clos-0561-a-S-17 (TTACTGGGTGTAAAGGG)	*Clostridium* Cluster IV 16S rRNA gene	60	Van Dyke & McCarthy, (2002)
Clept-1129-a-A-17 (TAGAGTGCTCTTGCGTA)	*Clostridium* Cluster IV 16S rRNA gene		Van Dyke & McCarthy, (2002)
Erec-0482-a-S-19 (CGGTACYTGACTAAGAAGC)	*Clostridium* Cluster XIVab 16S rRNA gene	55	Franks *et al*. (1998)
Ccoc-1112-a-A-19 (TGGCTACTRDRVAYARGGG)	*Clostridium* Cluster XIVab 16S rRNA gene		Van Dyke & McCarthy, (2002)

*Y, T/C; V, G/C/A; R, A/G.
†Based on clustering system described by Collins *et al*. (1994).
‡Originally reported as 65 °C.

Table 2. Selected biogeochemical parameters for soils from eutrophic (F1), transition (F4), and oligotrophic (U3) regions of WCA-2A during Spring 2002*

Sampling site	Moisture content (%)	TP ($mg\,kg^{-1}$)	TP_i ($mg\,kg^{-1}$)	TC ($g\,kg^{-1}$)	MBC ($mg\,kg^{-1}$)	Extractable TOC ($mg\,kg^{-1}$)	TN ($g\,kg^{-1}$)	NH4-N ($mg\,kg^{-1}$)
F1	92 (1)	1110 (352)	366 (128)	446 (24)	7705 (1534)	2404 (204)	28.8 (2.1)	90 (13)
F4	93 (1)	767 (49)	310 (72)	357 (10)	8933 (1529)	2436 (284)	25.3 (2.6)	107 (16)
U3	93 (2)	449 (161)	221 (131)	230 (42)	2627 (128)	1973 (450)	32.8 (2.8)	103 (33)

*Standard deviations based on three replicates are presented in parentheses. Concentrations are expressed per kg (dry weight) soil.
TP, total phosphorus; TP_i, total inorganic phosphorus; TC, total carbon; MBC, microbial biomass carbon; TOC, total organic carbon; TN, total nitrogen; NH_4-N, extractable ammonium.

Test was used to determine significance of differences in T-RFLP data between soil samples.

Results and discussion

Biogeochemical characterization

Biogeochemical parameters for phosphorus, carbon, and nitrogen at the study sites are presented in Table 2. In general, soils from the eutrophic area contain higher levels of nutrients than soils from the oligotrophic area. Total phosphorus and total inorganic phosphorus were higher in soil from the eutrophic area (F1), followed by soils from the transition (F4) and oligotrophic (U3) zones. Similarly, total carbon was higher in F1 soils, followed by F4 and U3 soils. Extractable organic carbon was similar in F1 and F4 soils, which were higher than in U3 soil. Microbial biomass carbon was highest in F4 soils followed by F1, and U3 soil has the lowest microbial biomass carbon. These data are in agreement with previously published reports (Wright & Reddy, 2001a, b; Castro *et al*., 2002, 2004; Chauhan *et al*., 2004).

Table 3. Most probable numbers of cellulolytic and fermentative bacteria in Everglades soils

Soil	Cellulose	Fermentation
F1	2.39×10^5 (0.76–7.60)*	5.42×10^6 (1.79–14.19)
F4	3.47×10^5 (1.17–10.16)	9.17×10^6 (2.67–22.01)
U3	2.43×10^4 (0.78–7.40)	1.72×10^6 (0.43–4.97)

*Confidence levels (95%) are presented in parentheses.

Enumeration of cellulolytic and fermentative bacteria

Most probable numbers of cellulolytic bacteria were 10-fold higher in F1 and F4, than in U3 (Table 3). No significant differences in most probable numbers of fermentative bacteria were observed between F1, F4, and U3 soils. Higher numbers of cellulolytic bacteria are expected in F1 and F4 than in U3 due to the greater input of plant material to those soils. Numbers of fermenters are also likely higher in F1 and F4 than in U3 soils, although the large confidence intervals associated with the most probable number method do not allow this conclusion to be made with confidence. Analyses

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using this method are estimates of true numbers at best, and may significantly underestimate the true numbers of organisms that grow on substrates such as cellulose.

Microcosm studies

Cellulose microcosms

The production of fermentation products and methane from cellulose provides an indication of rates and potential pathways for carbon decomposition for the different regions. Methane production in the soil microcosms with added cellulose (Fig. 1) occurred in two stages. In the first stage, methane was produced in small amounts and gradually increased beginning the first week of the experiment. Sharp increases in methane production corresponding with sharp declines in acetate concentrations were observed for the F1 microcosm in the third week, and for the F4 microcosm in the fifth week. Methane was likely produced by hydrogenotrophic methanogens during the first stage, and by acetotrophs during the second stage. Similar trends have also been observed in microcosms containing rice roots (Lehmann-Richter *et al.*, 1999). Drake and co-workers (Drake *et al.*, 1996) reported similar results for Everglades soils, and attributed the lag to inhibition of acetotrophs by accumulation of H_2. Acetotrophic methanogens are inhibited by H_2; when CO_2 was added to microcosms in their studies, H_2 was consumed with CO_2 by homacetogens to form acetate, and acetotrophic methanogenesis increased significantly.

Production of propionate and butyrate may be related to relatively high fermentation rates (Schink, 1997), as one might expect in soils with greater activities of fermentative bacteria, such as those observed in F1 and F4. Propionate was detected in all microcosms, and F1 and F4 cellulose microcosms accumulated more propionate than butyrate. A similar trend was reported for rice paddies (Conrad & Klose, 1999).

Consumption of propionate and butyrate leading to methane production requires the cooperation of secondary

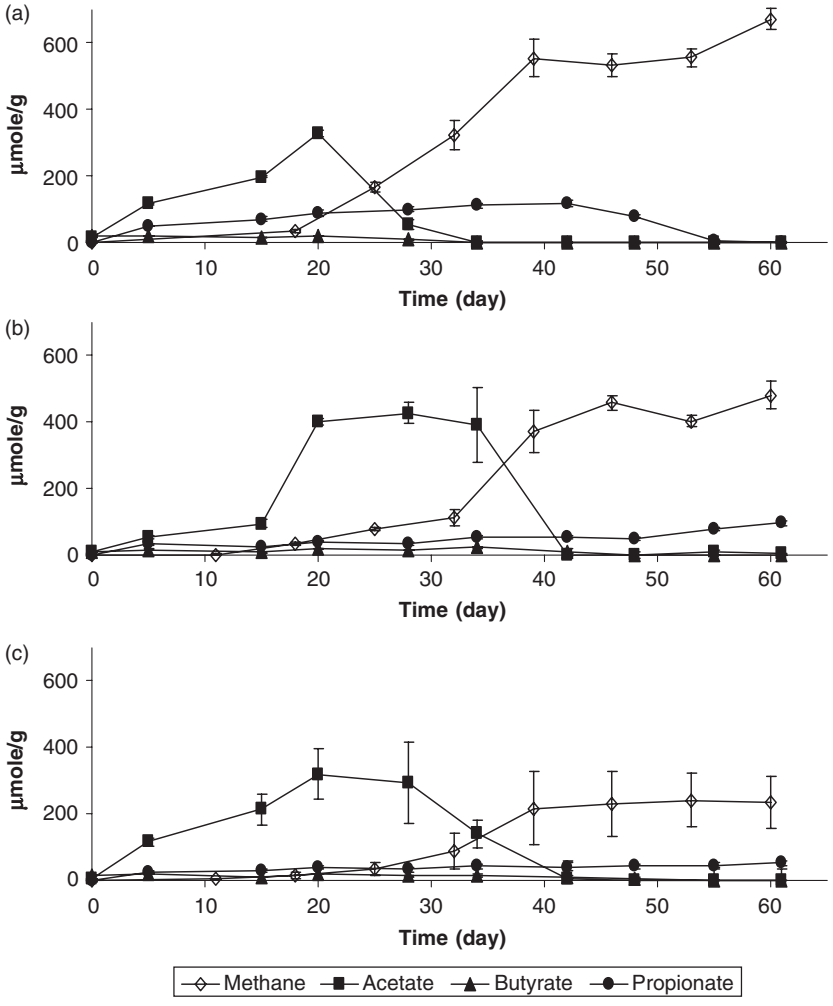


Fig. 1. Effect of cellulose on fermentation products and methane production in eutrophic and transition soils of the Everglades. Error bars represent standard errors based on three replicates. (a) F1-cellulose microcosms. (b) F4-cellulose microcosms. (c) U3-cellulose microcosms. Control values (no carbon added other than BCYT) were subtracted from values reported here.

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fermenters (syntrophs) and methanogens (Schink, 1997). Chauhan *et al.* (2004) reported higher rates of propionate- and butyrate-induced methanogenesis in F1 and F4 than in U3 soils. They also found that the composition of syntrophic consortia in F1 and F4 differed significantly from those found in U3 soils. In the present study, propionate consumption was only observed in F1 cellulose microcosms (Fig. 1), and only after long incubation times. This may be due to disruption of syntroph–methanogen consortia during mixing, which require close physical proximity to function (Schink, 1997). An alternate explanation was provided by Fukuzaki *et al.* (1990), who demonstrated that acetate has an inhibitory effect on propionate degradation.

Effect of plant type as carbon source on carbon cycling

Much of the readily decomposable material in plants is cellulose, but the relative degradation rates of cellulose and other decomposable compounds may depend on the type of plant. The dominant plants in F1 and F4 are cattail, and most of U3 is dominated by sawgrass. To test the relative impact of plant type on decomposition and methanogenesis, microcosms with ground cattail and sawgrass were established and products monitored. Table 4 presents chemical parameters for plant material used in these experiments (Rowland & Roberts, 1994; Inglett, 2005). The cellulose and lignin contents of plant material were similar, and the hemicellulose content of sawgrass was *c.* 6% higher than that of cattail. The primary difference between plant materials was their total nitrogen and total phosphorus contents. Cattails contain twice more total nitrogen and six times more total phosphorus than sawgrass used in this study.

Acetate production and consumption trends and methane formation in plant microcosm experiments are presented in Fig. 2, and most probable number enumeration for fermentative and cellulolytic bacteria at the end of the experiments are presented in Table 5. No significant differences were noted with regard to acetate formation and methanogenesis for the different plant sources. Soil was the major determinant in this experiment, and not plant type.

Most probable number enumeration indicated that fermentative bacteria were stimulated, not inhibited, by cattail (Table 5); 100-fold higher fermentative bacterial numbers were observed in cattail microcosms compared to sawgrass microcosms, regardless of the origin of soil samples.

It is not clear why similar differences were not observed in methane production (Fig. 2). It is possible that the additional numbers of fermenters were not large enough to produce a measurable difference by the methods used here, or that some products were processed through pathways that did not lead to acetate formation or methanogenesis. Sulfate reduction provides an alternative route for a limited amount of carbon in these soils (Castro *et al.*, 2005).

Phylogenetic analysis of cloned *Clostridium* 16S rRNA gene sequences

Previous studies (Franks *et al.*, 1998; Schwarz, 2001; Weber *et al.*, 2001; Van Dyke & McCarthy, 2002) conducted in various anaerobic environments emphasized the importance of *Clostridium* species for cellulose degradation and fermentation, particularly those species belonging to Clusters I, III, IV and XIV (Weber *et al.*, 2001; Van Dyke & McCarthy, 2002). The highest positive-scoring cellulose-degrader most probable number tubes in this study were dominated by sequences clustering with *Clostridium* species, such that this genus formed the basis for molecular studies on the distribution of cellulose degraders in WCA-2A soils. The genus *Clostridium* is a phylogenetically diverse group, such that no single PCR primer set has been designed to target all *Clostridium* species. PCR primers specific to the individual clusters proposed by Collins *et al.* (1994) for the genus *Clostridium* were used to investigate the diversity of *Clostridium* in F1, F4, and U3 soils (Table 1). Sequences from plant microcosms were also analyzed to investigate whether the type of plant material influences *Clostridium* assemblage composition.

Cluster I

Cluster I is one of the largest of the *Clostridium* clusters, and is considered to be the core cluster for the genus (Collins *et al.*, 1994). Members of this group are metabolically diverse, and include cellulolytic, saccharolytic, and proteolytic members. In a recent study (Weber *et al.*, 2001), Cluster I species were shown to be the dominant *Clostridium* species active in rice straw decomposition, accounting for 24% of all bacterial cells in the study, strongly suggesting that Cluster I

Table 4. Biochemical parameters for plant material used in microcosm experiments

Plant material	Cellulose (%DW)	Hemicellulose (%DW)	Lignin (%)	Ash (%DW)	ND soluble* (%DW)	TN (%DW)	TP (%DW)
Cattail	40.3	13.8	15.3	1.5	29.3	0.75	0.035
Sawgrass	41.8	19.3	15.2	3.7	20.1	0.40	0.006

*Neutral detergent soluble fraction.
DW, dry weight.

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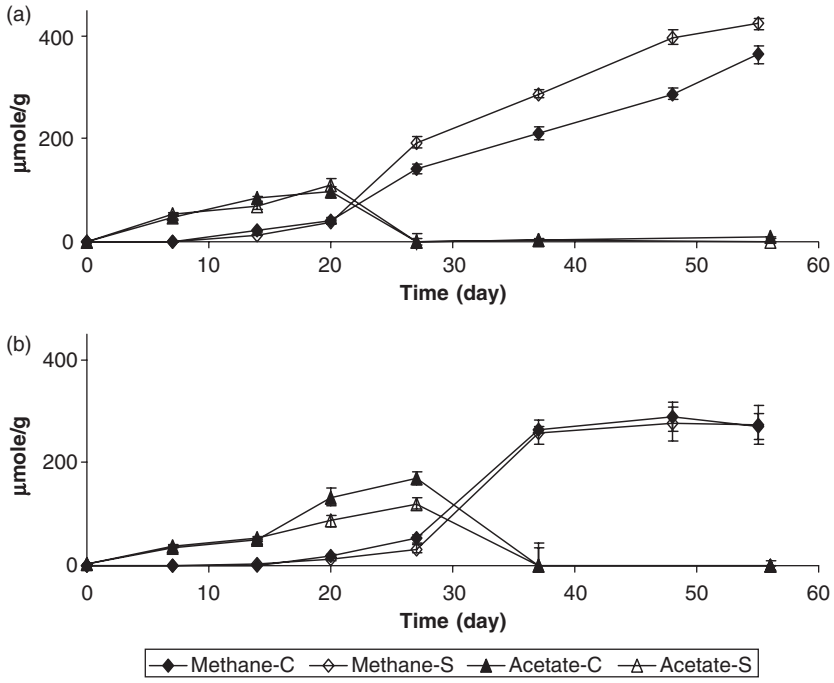


Fig. 2. Effect of plant materials on fermenta tion products and methane production in Everglades soils in the absence of phosphorus in the media. Error bars represent standard errors based on five replicates. (a) F1-plant microcosm. (b) U3-plant microcosm (C, cattail; S, sawgrass). Control values (no carbon added other than BCYT) were subtracted from values reported here.

Table 5. Most probable numbers of cellulolytic and fermentative bacteria in plant microcosms containing soils from F1 and U3 regions of the Everglades

Microcosm	Cellulose	Fermentation
F1-Cattail	2.40×10^8 (0.48–9.65)*	2.14×10^{11} (0.34–9.00)
F1-Sawgrass	4.27×10^7 (1.03–13.80)	3.05×10^9 (0.50–1.00)
U3-Cattail	9.33×10^7 (2.06–27.10)	4.62×10^{11} (1.16–5.00)
U3-Sawgrass	9.33×10^7 (2.06–27.10)	3.05×10^9 (0.50–11.00)

*Confidence levels (95%) are presented in parentheses.

species are responsible for fermentation of rice straw hydrolysis products in rice paddy environments.

Cluster I sequences obtained from WCA-2A soils included a novel branch (Fig. 3) composed exclusively of sequences from F1 and U3 soils; all sequences from F4 clustered separately with known Cluster I species. Clones from F1, F4, and U3 clustering in the branch with known Cluster I species were distributed relatively evenly. Sequence similarity of clones to known species in this branch ranges from 95% to 99%. Clones grouped with species such as *Clostridium quinii*, *Clostridium butyricum*, *Clostridium acetobutyricum*, and *Clostridium saccharobutyricum* are likely to play an important role in fermentation of various carbohydrates released from cellulose or other polymeric carbon sources in these soils. Clone T26 and T24 sequences shared 99% sequence similarity with *Clostridium magnum*, which can perform both homoacetogenesis and carbohydrate fermentation (Karnholz *et al*., 2002). Clones U111, U105, and U107 grouped with *Clostridium tunisiense* and *Clostridium argentinense*, which can ferment proteins but not sugars (Suen *et al*., 1988; Thabet *et al*., 2004).

Cluster III

Cluster III is the only cluster that consists solely of cellulolytic species. No specific grouping was observed for individual sites within Cluster III (Fig. 4).

Cluster IV

Significant clustering on the basis of site was observed for Cluster IV sequences (Fig. 5). Clones U2, U3, and U24 (from the oligotrophic soil, U3) grouped with *Clostridium cellulosi*, a thermophilic cellulolytic species, in a branch containing other cellulolytic species, such as *Ruminococcus flavefaciens* and *Ruminococcus albus* (Rainey & Janssen, 1995). The presence of U3 sequences in a branch dominated by cellulolytic species may not necessarily be indicative of their cellulolytic capability. *Clostridium cellulosi*, the closest relative to these clones, ferments a greater range of carbohydrates than do other Cluster IV *Clostridium* species (He *et al*., 1991). Clones U2, U3, and U24 may exhibit similar features which may provide great advantage to these species in nutrient-limited environments such as U3 soils. Clones F11, T1, T18, and U1 grouped with noncellulolytic species, including *Ruminococcus bromii*, *Clostridium leptum*,

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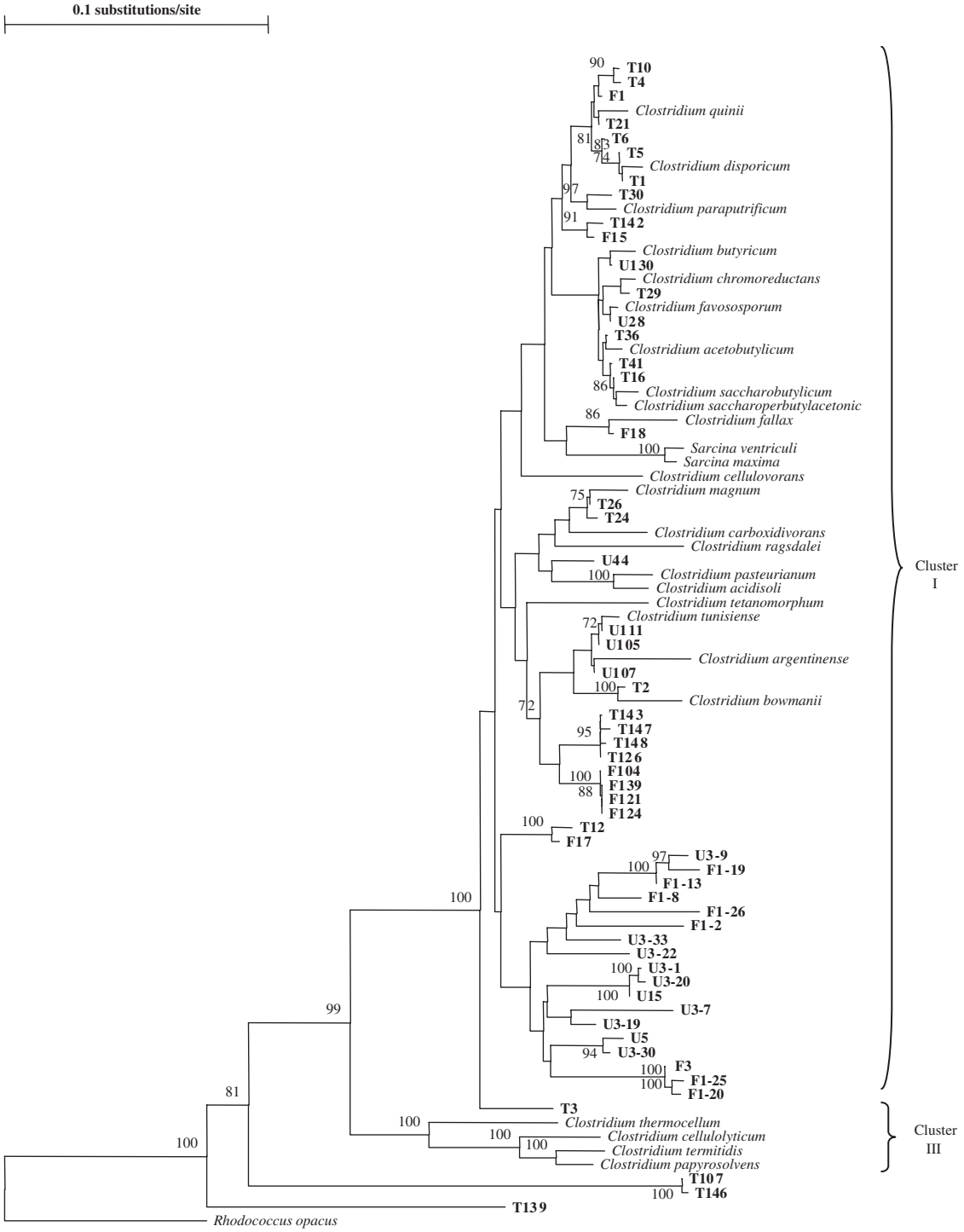


Fig. 3. Phylogenetic tree of *Clostridium* Cluster I 16S rRNA gene clone sequences obtained from Everglades soils (F1, F; F4, T; U3, U). Numbers at branch points refer to bootstrap analysis based on 100 resamplings.

Clostridium sporosphaeroides, and *Clostridium methylpentosum*. Other clones from F1 and F4 soils clustered with *Clostridium orbiscindens*, an asaccharolytic species (Winter *et al.*, 1991; Schoefer *et al.*, 2003).

Cluster XIV

Cluster XIV is the second largest *Clostridium* cluster and contains species belonging to genera such as *Ruminococcus*,

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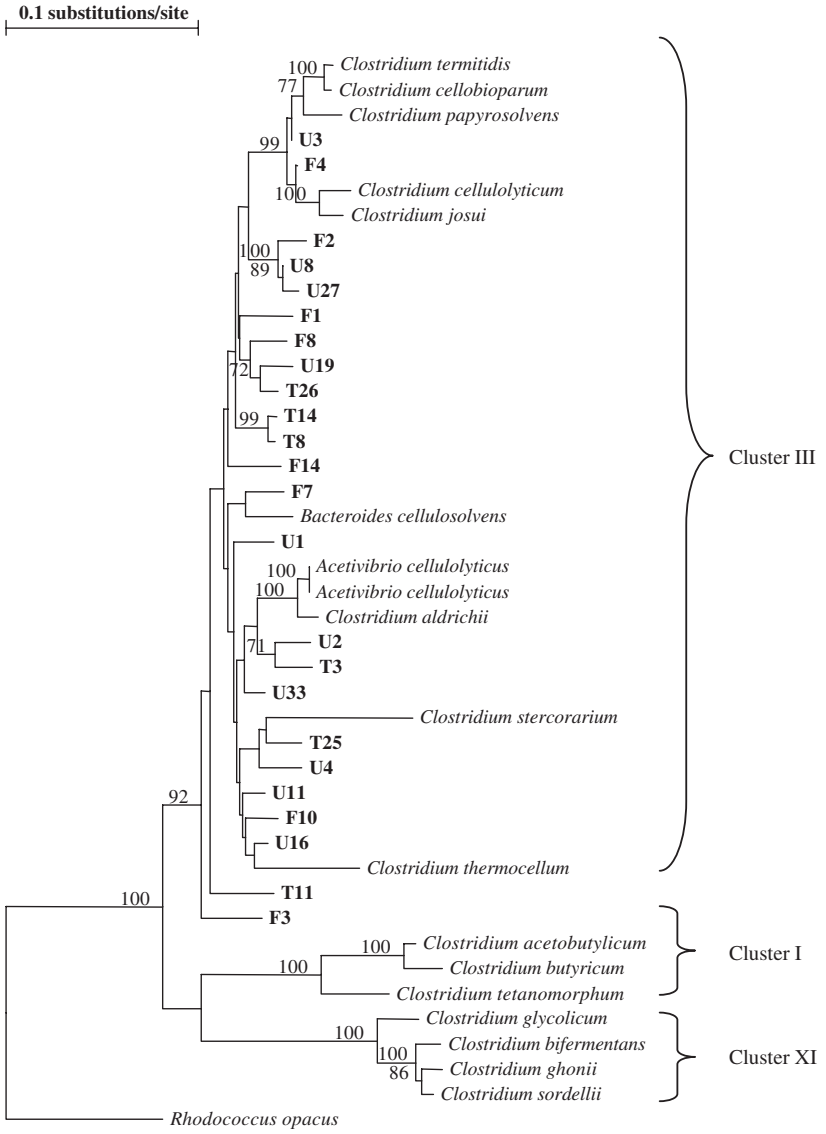


Fig. 4. Phylogenetic tree of *Clostridium* Cluster III 16S rRNA gene clone sequences obtained from Everglades soils (F1, F; F4, T; U3, U). Numbers at branch points refer to bootstrap analysis based on 100 resamplings.

Eubacteria, *Syntrophococcus, Roseburia*, and *Epulopiscium*. This large cluster is divided into two deep branches, Clusters XIVa and XIVb (Fig. 6). Cluster XIVa species are versatile in their ability to utilize various carbohydrates, including polymeric carbon sources such as cellulose and xylan. Xylans, like cellulose, constitute a major part of plant material and, unlike cellulose, form the complex polymers classified as hemicelluloses (Uffen, 1997). Cluster XIVa contains strains exhibiting diverse abilities with regard to degradation of plant polymers. *Clostridium populeti* can degrade xylan, cellulose, and glucose (Sleat & Mah, 1985). Its close relative *Clostridium xylanovorans* can utilize xylan but not cellulose (Mechichi *et al*., 1999; Warnick *et al*., 2002). *Eubacterium xylanophilum* can ferment xylan and cellobiose, but cannot grow on glucose or cellulose (Van Gylswyk & Van der Toorn, 1985), in contrast to *Eubacterium cellulosolvens*, which can utilize cellulose but not xylan (Van Gylswyk & Van der Toorn, 1986). In our libraries, Cluster XIVa contained a mixture of sequences from all three sites, and included some deeply branching clades.

Cluster XIVb was dominated by sequences from F1 and F4, with no U3 sequences clustering in this group (Fig. 6). Cluster XIVb contains species from various environments that exhibit an array of characteristics regarding their ability to attack and utilize plant polymers. *Clostridium lentocellum*, isolated from a river sediment containing paper-mill waste, has the ability to utilize cellulose, xylan and glucose (Murray *et al*., 1986). Others, such as *Clostridium colinum* and *Clostridium piliformi*, are pathogens (Berkhoff, 1985).

Sequences from plant microcosms clustered with sequences from the appropriate soil samples, indicating that the type of plant material is not the main factor controlling

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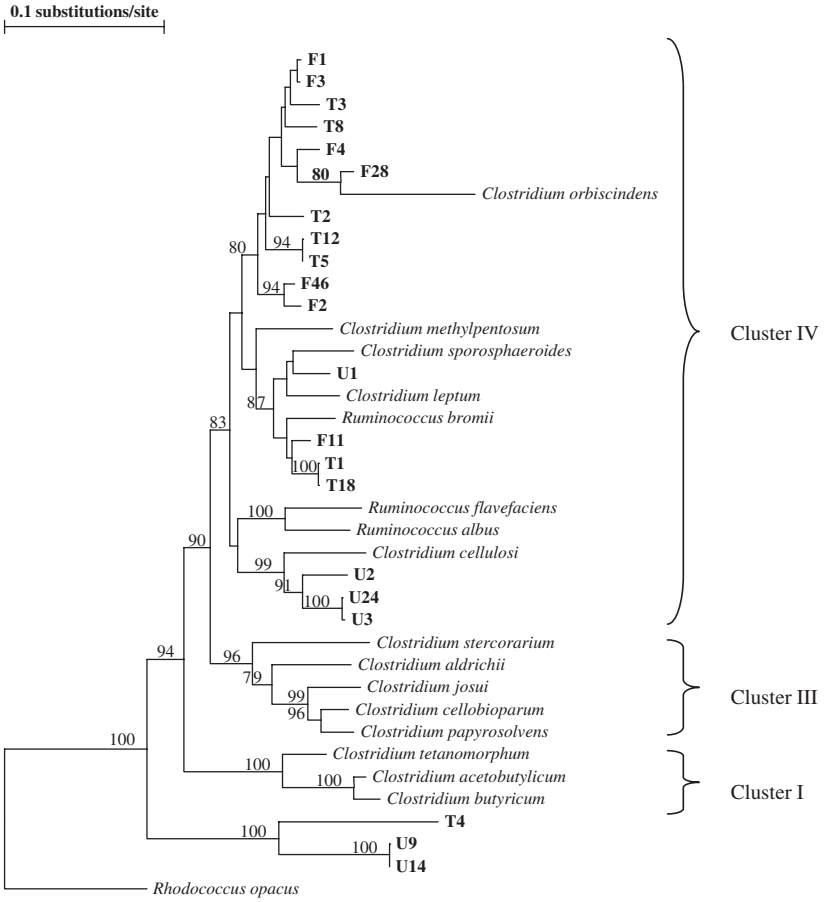


Fig. 5. Phylogenetic tree of *Clostridium* Cluster IV 16S rRNA gene clone sequences obtained from Everglades soil (F1, F; F4, T; U3, U). Numbers at branch points refer to bootstrap analysis based on 100 resamplings.

distribution of Cluster XIV phylotypes in these soils. All sequences, with the exception of clone FS2, were 96% or less similar to known Cluster XIV sequences, indicating that the Everglades soils harbor novel *Clostridium* species.

T-RFLP analysis of *Clostridium* Cluster XIV species

The greatest separation between F1, F4, and U3 sequences was observed for Clusters I and XIV, suggesting that strains belonging to these clusters may be selected by the nutrient status of the soil. To confirm this, a T-RFLP method was developed and evaluated for screening samples taken monthly from F1, F4, and U3 over the course of over 1 year. None of the 40 enzymes tested *in silico* distinguished Cluster I sequences on the basis of soil nutrient status; however, digestion with *Hinc*II provided clear separation between Clusters XIVa and XIVb. When digested with *Hinc*II, Cluster XIVb sequences produced a fragment of approximately 379 bp, whereas no digestion sites for this enzyme were identified for Cluster XIVa sequences. No U3 sequences were found in Cluster XIVb, such that this T-RFLP system might distinguish U3 samples from F1 and F4.

The results of T-RFLP analysis are presented in Table 6. A significant difference ($P < 0.05$) was observed between sites based on the presence or absence of the peak corresponding to the 379 bp fragment. In all, 32, 23, and 18 soil samples from eutrophic, transition, and oligotrophic regions, respectively, that yielded positive PCR amplification were included in the statistical analysis. All soil samples from eutrophic and transition regions showed the presence of the expected peak, whereas 39% of the soil samples from the oligotrophic region were counted as positive.

The inability of this T-RFLP system to completely resolve *Clostridium* phylotypes in oligotrophic soils from those in eutrophic soils is not surprising. The degree of resolution for most of the sequences in all *Clostridium* clusters, including Cluster XIV, was very poor, suggesting little selection for particular *Clostridium* phylotypes on the basis of the nutrient status of the soil. This is in contrast with a previous study (Castro *et al.*, 2005) on the distribution of methanogens and sulfate-reducing prokaryotes in these samples, which showed a robust separation of genotypes on the basis

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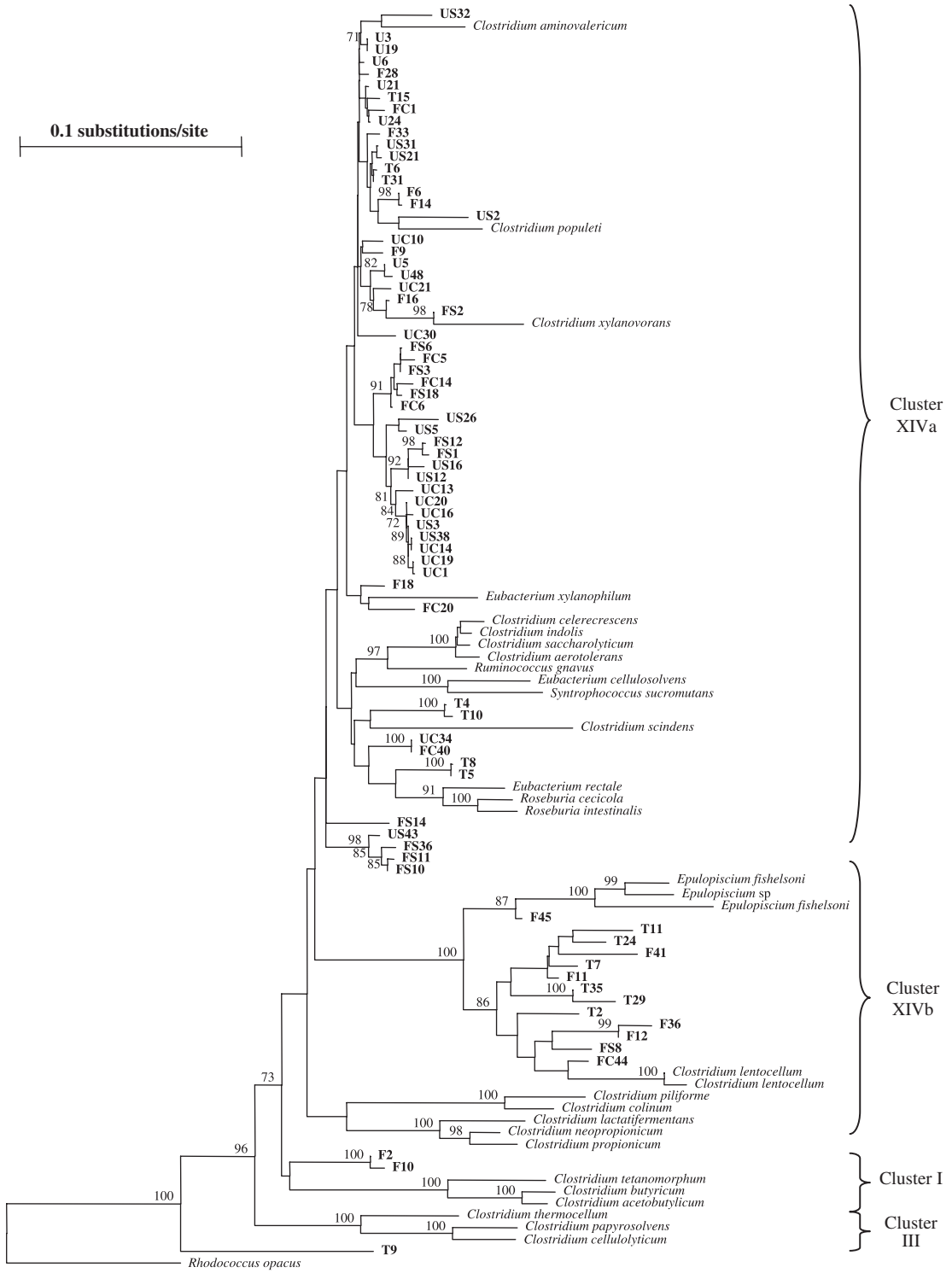


Fig. 6. Phylogenetic tree of *Clostridium* Cluster XIV 16S rRNA gene clone sequences obtained from Everglades soils and plant microcosms (F1, F; F4, T; U3, U; cattail, C; sawgrass, S). Numbers at branch points refer to bootstrap analysis based on 100 resamplings.

of nutrient status. That study employed functional genes characteristic of methanogens (*mcrA*) and sulfate-reducing prokaryotes (*dsrAB*), which are more variable than 16S rRNA genes and provide greater resolution between phylotypes than would be likely for a system based on the highly conserved 16S rRNA gene. In addition, sulfate-reducing

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Table 6. Result of terminal restriction fragment length polymorphism application for soil samples from the Everglades

	F1	F4	U3
No. of samples	32	23	18
Positive terminal restriction fragments (%)	100	100	39
Negative terminal restriction fragments (%)	–	–	61
Duncan classification*	A	A	B

*Soils in the same Duncan classification group are not significantly different ($P < 0.05$).

prokaryotes and methanogens may be more sensitive to a variety of factors that differ with nutrient status, including the amount and types of fermentation products, than would *Clostridium* species.

Conclusions

Rates of cellulose degradation and fermentation, and the composition of guilds involved in these processes, differed significantly between eutrophic and oligotrophic soils. Our findings and previously published reports on carbon cycling in the Everglades strongly suggest that sulfate-reducing prokaryotes and fatty-acid-consuming bacteria associated with hydrogen-scavenging methanogens play an important role in carbon cycling, and this association is likely to be affected by the activities and types of fermentative and cellulolytic organisms that supply electron donors to these groups. The type of plant material (cattail vs. sawgrass) did not appear to select specific phylotypes of *Clostridium* in these soils, although other plant associated carbon, such as root exudates, may be responsible for the selection observed. This study contributed to a greater understanding of carbon cycling in wetland environments and provided a molecular database that will contribute to monitoring ecosystem restoration.

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