水田土壌の主要なメタン生成古細菌群の解析

（課題番号　１４５６００５１）

平成１４年度～平成１５年度科学研究費補助金（基盤研究(C)(2)）研究成果報告書

平成１６年３月

研究代表者　　浅　川　　晋
（名古屋大学大学院生命農学研究科助教授）

はしがき

メタンは地球温暖化ガスの一つであり，水田はその重要な発生源である。そのため，水田からのメタンの発生制御は，地球規模での環境問題解決のための緊急かつ重要な課題である。一方，水田土壌を含む嫌気生態系では，メタン生成反応は有機物の最終的な分解過程に位置づけられ，極めて重要な反応である。すなわち，無酸素条件下では，多糖，タンパク，脂質等の複雑な有機物は，それぞれ種類の異なる微生物が関与する加水分解，有機酸・アルコール生成，水素・酢酸生成といった反応によって順次分解され，水素，酢酸等のメタン生成基質が生じ，最終的にメタン生成反応によりメタンへと変換される。水素や酢酸が過剰に蓄積するとそれ以前の段階の反応を阻害するため，メタン生成反応は，水稲根の生育阻害要因となりうる酢酸や低級脂肪酸等の蓄積を防止するだけにとどまらず，有機物の嫌気的分解を全体として円滑に進行させることにより，水田土壌中の物質代謝に極めて大きな役割を果たしている。従って，水田土壌中におけるメタン生成を理解することは，地球温暖化ガスであるメタンの発生制御を目指す環境保全面へ貢献するだけではなく，土壌中で生じている物質代謝を明らかにするという土壌学の基本的課題の解決のためにも，極めて重要である。そのためには，メタン生成反応を担うメタン生成古細菌の種類や動態などの水田土壌中における生態を明らかにすることが必須である。しかしながら，メタン生成古細菌は，培養に高度な嫌気度を要求し，取り扱いが煩雑な絶対嫌気性の微生物ということもあり，菌の分離・同定まで含めた水田土壌中の生態についての総合的な研究はほとんどない。本研究は，このような水田土壌中のメタン生成古細菌の生態の総合的解明の一環として，環境試料から直接抽出・増幅したDNAの解析を行う，いわゆる分子生態学的手法により，水田土壌中に生息する主要なメタン生成古細菌群を明らかにすることを目的とした。具体的には，既に培養法と細胞構成成分分析法により解析が行われた実際の水田圃場を対象とし，その圃場から分離・同定した菌株を活用して，メタン生成古細菌の16S　rDNAを対象とした変性剤濃度勾配ゲル電気泳動（DGGE）法を確立し，水田土壌中のメタン生成古細菌群集の解析を行った。

研究の遂行に当たり，試験圃場の利用を快諾下さいました愛知県農業総合試験場安城農場技術センター松家一夫室長，内田利治総括研究員，林　元樹技師，籾井隆志技師，および独立行政法人農業技術研究機構九州沖縄農業研究センター水田作研究部土屋一成室長，原　嘉隆研究員，草　佳那子研究員に深く感謝致します。また，菌株やDNAの提供などで多くの方々に御協力を頂きました。記して謝意を表します。

この報告書により，本研究の成果が，水田土壌のみならず，他の様々な環境中のメタン生成古細菌群集の研究に活用されることを期待する次第である。

研究組織

研究代表者：浅川　晋　（名古屋大学大学院生命農学研究科助教授）

研究協力者：木村　眞人　（名古屋大学大学院生命農学研究科教授）
村瀬　潤　（名古屋大学大学院生命農学研究科助手）
渡邉　健史　（名古屋大学大学院生命農学研究科修士課程）
中村　明日美（名古屋大学大学院生命農学研究科修士課程）
松家　一夫　（愛知県農総試安城農業技術センター室長）
内田　利治　（愛知県農総試作物研究部水田利用グループ総括研究員）
林　元樹　（愛知県農総試安城農業技術センター技師）
籾井　隆志　（愛知県農総試安城農業技術センター技師）
土屋　一成　（九州沖縄農業研究センター水田作研究部室長）
原　嘉隆　（九州沖縄農業研究センター水田作研究部主任研究官）
草　佳那子（九州沖縄農業研究センター水田作研究部研究員）

交付決定額（配分額）

（金額単位：千円）

	直接経費	間接経費	合　計
平成１４年度	１，８００	０	１，８００
平成１５年度	１，２００	０	１，２００
総　計	３，０００	０	３，０００

研究発表

（１）学会誌等

Watanabe T, Asakawa S, Nakamura A, Nagaoka K, Kimura M: DGGE method for analyzing 16S rDNA of methanogenic archaeal community in paddy field soil. *FEMS Microbiol Lett,* in press.

（２）口頭発表

浅川　晋，渡邉健史，中村明日美，木村眞人：水田土壌のメタン生成古細菌を対象としたPCR-DGGE法の検討，土と微生物，57(2)，157（2003）

渡邉健史，浅川　晋，中村明日美，木村眞人：PCR-DGGE法による水田土壌のメタン生成古細菌群の解析，日本土壌肥料学会講演要旨集，49，36（2003）

研究成果の概要

既に培養法と細胞構成成分分析法により解析が行われた実際の水田圃場を対象とし，その圃場から分離・同定した菌株を活用して，メタン生成古細菌の16S　rDNAを対象とした変性剤濃度勾配ゲル電気泳動（DGGE）法を確立し，水田土壌中のメタン生成古細菌群集を解析することを目的とした。これまで用いられてきた古細菌用のプライマーを基に，水田土壌からの分離菌株およびデータベースに登録されているメタン生成古細菌の16S　rDNAの塩基配列との比較を行い，メタン生成古細菌を対象としたPCR-DGGE用のプライマーを５種類（forward２種類，reverse３種類）設計した。これらのプライマーを用いて，１３種類の純粋培養菌株から抽出したゲノムDNAについて，PCR増幅およびDGGEの条件を検討した。その結果，２組のプライマー対（0357F-GC，0691R; 0348F-GC，0691R）でDGGE解析が可能であったが，プライマー対0348F-GC, 0691Rではいくつかのメタン生成古細菌以外の古細菌の16S　rDNAが増幅された。この２組のプライマー対を用い，メタン生成古細菌を分離した圃場（筑後）および比較対照の圃場（安城）の水田土壌試料より抽出したDNAを対象にDGGEを行ったところ明瞭なパターンが得られ，DGGE解析が可能であった。41のバンドを切り出し，塩基配列を決定したところ，全てメタン生成古細菌由来の16S rDNAであり，*Methanomicrobiales*, *Methanosarcinales*およびRice　clusterに近縁であった。以上の結果より，水田土壌中のメタン生成古細菌群集の解析には，今回確立したプライマー対0357F-GC，0691Rを用いたPCR-DGGE法が最適であると考えられた。現在，本方法により水田圃場におけるメタン生成古細菌群の季節変動の解析を行っている。

DGGE method for analyzing 16S rDNA of methanogenic archaeal community in paddy field soil

WATANABE Takeshi[1], ASAKAWA Susumu[1,2], NAKAMURA Asumi[1†], NAGAOKA Kazunari[2#] and KIMURA Makoto[1]

[1] Soil Biology and Chemistry, Graduate School of Bioagricultural Sciences, Nagoya University, Furocho, Chikusa, Nagoya 464-8601, Japan

[2] Laboratory of Soil Microbiology, Kyushu National Agricultural Experiment Station, Nishigoshi, Kumamoto 861-1192, Japan

Present address:

[†] Home, 1-11-60 Hamatake, Chigasaki, Kanagawa 253-0021, Japan

[#] Soil Organisms Laboratory, National Agricultural Research Center, Kannondai, Tsukuba, Ibaraki 305-8666, Japan

Abstract

The denaturing gradient gel electrophoresis (DGGE) method for analyzing 16S rDNA of methanogenic archaeal community in paddy field soil was presented. Five specific primers for 16S rDNA of methanogenic archaea, which were modified from the primers for archaea, were first evaluated by PCR and DGGE using genomic DNAs of 13 pure culture strains of methanogenic archaea. The DGGE analysis was possible with two primer pairs (0348aF-GC and 0691R; 0357F-GC and 0691R) of the five pairs tested although 16S rDNA of some nonmethanogenic archaea was amplified with 0348aF-GC and 0691R. These two primer pairs were further evaluated for use in analysis of methanogenic archaeal community in Japanese paddy field soil. Good separation and quality of patterns were obtained in DGGE analysis with the both primer pairs. Totally 41 DNA fragments were excised from the DGGE gels and their sequences were determined. All fragments belonged to methanogenic archaea. These results indicate that the procedure of DGGE analysis with the primer pair of 0357F-GC and 0691R is suitable for investigating methanogenic archaeal community in paddy field soil.

Keywords: Methanogenic archaea; DGGE; Paddy field; 16S rDNA

Introduction

Methanogenesis is a final degradation process of organic matter in anoxic environments including paddy fields. In these environments, organic matters are degraded to methanogenic

substrates such as acetate, CO_2 and H_2 by diverse bacteria and CH_4 is formed and released to the atmosphere [1]. Methane is one of the green house gases whose effect is estimated to be 25 times higher than CO_2 [2]. Paddy fields are a major source of CH_4 emission, which accounted for 11 % of the total CH_4 emission and 16 % of the emission from anthropogenic sources [3]. Therefore, it is important to understand the mechanism of methanogenesis and in particular ecology of methanogenic archaea in paddy field soil. Methanogenic archaea belong to Euryarchaeota together with halophilic (Halobacteriales) and thermophilic (Archaeoglobales, Thermococcales and Thermoplasmatales) archaea [4]. Five orders (Methanobacteriales, Methanococcales, Methanomicrobiales, Methanopyrales and Methanosarcinales) of methanogenic archaea have been described so far and each of them forms a distinct lineage within Euryarchaeota [5-7].

In recent years, molecular ecological methods, for example restriction fragment length polymorphism (RFLP), fluorescent in situ hybridization (FISH), etc., have been widely used for analyzing microbial community structures in various environments. The denaturing gradient gel electrophoresis (DGGE) of SSU rDNA is one of these methods [8], which has the advantages that we can obtain phylogenetic information on the existed microorganisms and the community structural changes by analyzing bands migrated separately on DGGE gels. As for archaeal community, DGGE analysis was first applied to lake water sample [9] and then has been used in many studies on archaeal communities in various environments, most of which target 16S rDNA of both Euryarchaeota and Crenarchaeota and some do only Crenarchaeota. Methanogenic community has been intensively investigated in paddy soil microcosm by using clone library and terminal-RFLP (T-RFLP) techniques [10-18] and not a few studies have been conducted by cultivation method (e. g. [19]). However, there is no work that analyzes the community of methanogenic archaea in paddy field soil by the DGGE method.

Hence, the objective of this work is to establish DGGE method for analyzing the community of methanogenic archaea in paddy field soil. For this objective, the specific primers for 16S rDNA of methanogenic archaea were modified from the primers for archaea [9, 20] and first evaluated by using genomic DNAs of 13 strains of methanogenic archaea which belonged to Methanobacteriales, Methanosarcinales and Methanomicrobiales and included three isolates from Japanese paddy soil. The selected primers were further evaluated for use in analysis of methanogenic archaeal community in Japanese paddy field soil.

Materials and Methods

Archaeal and bacterial strains and DNA extraction

The strains used were 13 strains of methanogenic archaea, 4 strains of archaea other than methanogens and 3 strains of bacteria. *Methanobrevibacter arboriphilus* SA (=DSM 7056, JCM 9315, OCM 783), *Methanosarcina mazei* TMA (=DSM 9195, JCM 9314, OCM 784) and *Methanoculleus chikugoensis* MG62^T (=DSM 13459^T, JCM 10825^T, OCM 785^T) were isolated from paddy field soil in the Kyushu National Agricultural Experiment Station, Chikugo, Fukuoka, Japan [21-23] and maintained in our laboratory. *Methanothermobacter thermoautotrophicus* ΔH^T (=DSM 1053^T), *Methanosarcina mazei* S-6^T (=DSM 2053^T), *Methanosarcina mazei* LYC (=DSM 4556), *Methanoculleus thermophilus* CR-1^T (=DSM 2373^T), *Methanoculleus palmolei* INSULZ^T (=DSM 4273^T), *Methanoculleus bourgensis* MS2^T (=DSM 3045^T) *Methanoculleus bourgensis* RC/ER (=DSM 2772; formerly "*Methanoculleus olentangyi*") [24], *Methanoculleus bourgensis* CB1 (=DSM 6216; formerly "*Methanoculleus oldenburgensis*") [24] and *Methanoculleus marisnigri* JR1^T (=DSM 1498^T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Cells of *Methanothermobacter wolfeii* DSM 2970^T were obtained from Morii H. of the Department of Environmental Management, University of Occupational and Environmental Health Japan, Kitakyushu, Fukuoka, Japan. *Escherichia coli* K-12 (= IAM 1264) was obtained from the Institute of Molecular and Cellular Biosciences (formerly Institute of Applied Microbiology), the University of Tokyo, Tokyo, Japan. Genomic DNAs of these strains were extracted as described previously [21-23]. Genomic DNAs of *Metallosphaera sedula* JCM 9064, *Thermoproteus tenax* JCM 9277^T, *Pyrococcus horikoshii* JCM 9975 and "*Haloarcula aidinensis*" JCM 10025 were given by the courtesy of Ito T. of the Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Wako, Saitama, Japan. Genomic DNAs of *Bacillus subtilis* IFO 16412 and *Pseudomonas aeruginosa* IFO 12689 were gifts from Murase J. of our laboratory.

Soil

The sampling sites were two paddy field plots (D1 and E2) (Anthraquic Yellow Soil; Oxiaquic Dystrochrepts: total C content, 13 g kg^{-1}; total N content, 1.1 g kg^{-1}; pH [H_2O], 6.3) in Anjo Research and Extension Station, Aichi-ken Agricultural Research Center, Anjo, Aichi, Japan and four plots (Gray Lowland Soil; Endoquepts: total C content, 22-30 g kg^{-1}; total N

content, 2.1-2.9 g kg^{-1}; pH [H_2O], 5.9-6.2 [Yoo et al. (25)]), which were treated with chemical fertilizer (CF), rice straw plus chemical fertilizer (RS), rice straw compost plus chemical fertilizer (RSC) and wheat straw plus chemical fertilizer (WS), respectively, in the paddy field with long term application of organic matter [19] in the National Agricultural Research Center for Kyushu Okinawa Region (formerly Kyushu National Agricultural Experiment Station), Chikugo, Fukuoka, Japan. The sampling dates for D1, E2 and CF, RS, RSC and WS plots were 3 June 2002, 15 July 2002 and 12 September 2002, respectively. The D1 plot was with mono cropping of rice as summer crop (fallow in winter) and the transplanting of rice was conducted on 30 May 2002. The E2, CF, RS, RSC and WS plots were under double cropping conditions (rice in summer and wheat in winter) and rice was transplanted on 21 June 2002 for E2 and on 12 June 2002 for CF, RS, RSC and WS plots, respectively. The soil sample was taken from the plow layer of flooded soil using a trowel into a polyethylene bag and transported to the laboratory at 4 °C. Then soil samples were passed through a 2-mm mesh sieve and stored at 4 °C until use.

DNA extraction from soil and purification

DNA extraction was based on the method of Zhou et al. [26] by bead-beating with some modifications. The extraction was replicated three times. A 0.5 g of soil sample was mixed with 720 μL of extraction buffer (100 mM Tris·HCl [pH 8.0], 100 mM EDTA·2 Na·2 H_2O, 1.5 M NaCl, 1 % hexadecylmethylammonium bromide [CTAB]), 0.4 g of each glassbead with a diameter of 0.1-0.15 mm, 0.5 mm or 1 mm and 180 μL of 10 % sodium dodecyl sulfate (SDS) in a 2-mL tube with a screw cap and the tube was shaken horizontally at 2,500 rpm for 750 s with a Mini-Beadbeater (Biospec Product, Bartlesville, OK, USA). Then the suspension was incubated at 65 °C for 30 min with shaking by hand every 10 min and was centrifuged at 17,360 *g* for 5 min at room temperature. A 300 μL of the supernatant was transferred into a 10-mL centrifuge tube. A 600 μL of the PCI solution (phenol : chloroform : isoamyl alcohol = 25 : 24 : 1; v/v) was added into the 2-mL tube and the suspension was mixed horizontally at 2,500 rpm for 10 s with a Mini-Beadbeater. After centrifugation at 17,360 *g* for 5 min at room temperature, 400 μL of the supernatant was taken and added to the 300 μL of the supernatant in 10-mL centrifuge tube. A 400 μL of the extraction buffer was added to the mixture in the 2-mL tube and the step of mixing at 2,500 rpm for 10 s, centrifugation at 17,360 *g* for 5 min and the transfer of the 400-μL supernatant to the 10-mL centrifuge tube was repeated three times. A 1.9 mL of the CIA (chloroform : isoamyl alcohol = 24 : 1; v/v) solution was added to the 10-mL

tube and the suspension was shaken by hand. After keeping statically for 5 min, the mixture was centrifuged at 17,360 *g* for 5 min. The supernatant was transferred into a new 10-mL centrifuge tube and 1.5 mL of isopropanol was added. The solution was mixed well and left for 5 min. The mixture was centrifuged at 17,360 *g* for 20 min and the supernatant was removed. The pellet was washed with 1 mL of 70 % ethanol and then dried up. The crude DNA was dissolved in 100 μL of TE (10 mM Tris·HCl; 1 mM EDTA; pH 8.0) and purified by using Sephadex G-200 as described previously by Cahyani et al. [27] based on the method of Jackson et al. [28].

PCR amplification

Table 1 shows the primer used. Primers 0357F, 0505R and 0691R were designed by modifying the known ones, PARCH 340f, PARCH 519r and 0690aR, respectively [9, 20]. The modification was done by aligning them with the 77 sequences from the database and also by submitting them to the BLAST search program at the National Center for Biotechnology Information (NCBI) web site (www.ncbi.nlm.nih.gov). The sequences used for alignment were those of the order Methanobacteriales (AF028688, AF233586, X99046, AF095260, AF028689, AF095261, AF028690, AF093061, Y12592, AF095264, X99047, X99048, AF095265, AB065294, U62533, U41095, U82322, U55235, M59139, M59145, AF095273), Methanomicrobiales (AF095266, AF095267, M59147, AF095268, Z13957, AF033672, AF172443, AF095269, AB038795, AF028693, AF095270, Y16382, M59129, AF262035, AF095271, AF095272, M59130, M59131, M59142, M59143, U76631, M60880), Methanococcales (U39016, AF056938, M59125, AF025822, M59126, AF005049, M59128, M36507, M59290, AF051404, AB057722) and Methanosarcinales (AJ238002, X65537, M59127, U20149, X98192, M59133, U20152, M59132, U20148, U20154, M59135, U20155, AF120163, AJ276437, M59146, AB071701, U89773, M59140, U20150, M59137, AJ002476, AF411469, AJ012742). Based on these results, the degeneracy was eliminated when modifying the primers 0357F and 0505R from PARCH 340f and PARCH 519r, respectively. On the other hand, the primer 0691R included "R" instead of "G" and the length and position were modified to improve the specificity. The primers 0348aF and 0915aR did not need any modification according to the aligning and BLAST results. These primers have been used for sequencing of archaeal 16S rDNA [20] and the primers with similar or same sequences were used in PCR-single strand conformation polymorphism (SSCP) and clone library analyses in archaeal communities [29, 30].

PCR mixture (50 μL) contained 0.5 μL of forward and reverse primers (50 pmol μL^{-1} each),

2.5 or 5.0 U of Ex-Taq polymerase (TaKaRa, Tokyo, Japan), 5 μL of Ex-Taq buffer (with 20 mM Mg^{2+}, TaKaRa), 5 μL of dNTP mixture (2.5 mM each, TaKaRa), 1 or 3 μL of DNA template (85-750 pg), and filled up with MilliQ water. In some cases, the DNAs extracted from soil were diluted and twice amount of Taq polymerase was used. PCR amplification was performed by using a TaKaRa PCR Thermal Cycler (TaKaRa) under the condition with 94 °C for 3 min (initial denaturation), followed by prescribed cycles of denaturation at 94 °C for 1 min, annealing for 1 min and extension at 72 °C for 2 min, and the final extension at 72 °C for 8 min. The cycle number and annealing temperature were modified depending on a primer pair used (see Results and Discussion).

PCR product was observed on agarose gel (2.0 %) with 1×TAE buffer (40 mM Tris·HCl, 40 mM acetate, 1.0 mM EDTA) and ethidium bromide (0.5 μg mL^{-1}) under UV light.

DGGE analysis

DGGE was performed with a Dcode Universal Mutation Detection System (Bio Rad Laboratories, Hercules, LA, USA). PCR product (about 150 ng) was applied onto 8 % (w/v) polyacrylamide gel in 1×TAE. The denaturant gradient range of the gel, in which 100 % denaturant contained 7 M urea and 40 % (v/v) formamide, was modified depending on PCR products applied (see Results and Discussion). Electrophoresis was run for 14 h at 60 °C at 100 V. The gels was stained for 20 min with SYBR Green I nucleic acid gel stain (1 : 10,000 dilution) (Biowhittaker Molecular Applications, Rockland, ME, USA). The stained gel was immediately photographed under UV light.

Sequence and phylogenetic analysis

Nucleotide sequences of DNA fragments recovered from bands on DGGE gels were determined as the following method. The gel strip of a band was excised from a DGGE gel with a 1-mL pipet tip into a 1.5-mL tube and the DNA was eluted in 30-μL TE at 4 °C for over night. The DNA fragment was amplified from the eluted solution by PCR and subjected to check the mobility on DGGE gels as described above. The primer pair without GC clamp (0348aF or 0357F and 0691R) was used in the template amplification by PCR for the subsequent cycle sequencing. Primer 0365F (5'-GCA GCA GGC GCG AAA-3') was also used in the cycle sequencing of DNA fragments. The PCR products were sequenced with a 373S DNA Automated Sequencer (Applied Biosystems, Chiba, Japan) using Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Tokyo, Japan).

Close relatives and phylogenetic affiliation of the obtained sequences were detemined by using the BLAST search program at the National Center for Biotechnology Information (NCBI) web site (www.ncbi.nlm.nih.gov). Phylogenetic tree was constructed by 1000-fold bootstrap analysis using parsimony and neighbor joining methods with the nj plot and the Tree ViewPPC (version 1.6.6 Developmental) softwares. All sequences determined in this study were deposited in the DDBJ databases under the accession numbers from AB113832 to AB113872.

The 16S rDNA sequences of *Methanobrevibacter arboriphilus* SA (=DSM 7056, JCM 9315, OCM 783), *Methanosarcina mazei* TMA (=DSM 9195, JCM 9314, OCM 784), *Methanosarcina mazei* LYC (=DSM 4556), *Methanoculleus thermophilus* CR-1^T (=DSM 2373^T) and *Methanothermobacter wolfeii* DSM 2970^T were determined as described previously [23] using the primers in the table by Achenbach et al. [20], M23'f (5'-TCC GTT TGA TCC TGS CGG A-3')[31], 0246R (5'-ACC CYA CCA ACT ASC T-3'), 0533F (5'-GTG CCA GCM GCC GCG GTA A-3'), 0707F (5'-GTG AAA TCC TGT AAT CC-3') and 0806F(5'-CCGGAT TAG ATA CCC GGG TAG TCC-3') (*E.coli* positions 8-26, 261-246, 515-533, 691-707 and 783-806), and have been deposited in DDBJ under the accession number of AB065294, AB065295, AB065296, AB065297 and AB104858, respectively.

Results and Discussion

Analysis of genomic DNA of methanogenic archaea by DGGE method

The three primer pairs (0348aF and 0691R; 0357F and 0691R; 0357F and 0915aR) of the five pairs evaluated showed good amplification by PCR with genomic DNAs from 13 methanogenic strains but not *E.coli* K-12 (Fig.1). 16S rDNA of *E. coli* K-12 and/or nonspecific DNA fragments were amplified with the other primer pairs (data not shown). Ratios of template to product varied among DNAs used. For example, the largest yield of 16S rDNA fragments from *Methanosarcina mazei* LYC and *Methanoculleus bourgensis* CB1 was expected to be amplified because the amounts of genomic DNAs of the two strains were largest (Table 2). The fragment from *Methanoculleus thermophilus* CR-1 was, however, most amplified with 0357F-GC and 0691R (Fig. 1b). In addition, less amount of the fragment from *Methanobrevibacter arboriphilus* SA was obtained than the expected one from the concentration of genomic DNA with 0348aF-GC and 0691R (Fig. 1a). There was no mismatch in the sequences between the primers and the genomic DNAs of 13 strains used. The size of genomic DNA and the copy number of 16S rRNA gene [32] and/or the different primer binding energies [33] may have

biased the PCR amplification although the number of rRNA operons has been reported only in several strains of methanogenic archaea including the ones whose whole genome sequences were determined; *Methanothermobacter thermoautotrophicus* ΔH and *Methanosarcina mazei* Gö1 have two and three copies, respectively, for example [34, 35]. The amount of PCR product from a genomic DNA varied among the primer pairs used (Fig. 1). Therefore, the effect of the different annealing temperature [36] and/or the interference from the DNA segments flanking target regions [37] may have also resulted in the observed biases.

Band patterns on DGGE gels with primer pairs of 0348aF-GC and 0691R and 0357F-GC and 0691R showed good resolution and separation (Fig. 2). On the other hand, clear bands were not obtained from PCR products with the primer pair of 0357F-GC and 0915aR even though some ranges of acrylamide concentration and denaturant gradient were tested (data not shown). The amplified fragments had approximately 600 bp length and might be too long for good separation. Further examination of other conditions will be needed to improve the separation with this primer pair.

The sequences of the fragments migrated to the same positions on DGGE gel were not necessarily identical. The fragments from *Methanosarcina mazei* TMA, *Methanosarcina mazei* S-6 and *Methanosarcina mazei* LYC, and the ones from *Methanothermobacter thermoautotrophicus* ΔH and *Methanothermobacter wolfeii* had 100 % similarity of sequences. However, the sequence similarity among the fragments from *Methanoculleus bourgensis* RC/ER, CB1 and MS2 was not 100 %, but 98.6 – 99.0 % and these fragments migrated to the same position (Fig. 2). These differences may be due to not only the G+C content of DNA but also the distribution of G and C (or A and T) in the fragment.

When a mixture of genomic DNAs from 8 strains were used as template, the respective bands were obtained as well though some nonspecific bands appeared, especially with the primer pair 0348aF-GC and 0691R (Fig. 2). This was probably due to the formation of chimeric DNA [38, 39] and the low annealing temperature (49 °C) with the large cycle number (40) in the PCR condition for 0348aF-GC and 0691R may have affected the specificity of amplification. When the mixing ratio of the genomic DNAs was varied, the intensity of band increased relative to the genomic DNA concentration (data not shown). These results indicate that the intensity of a certain band corresponds to the amount of respective template DNA, but that intensities of bands at the different locations cannot be compared to each other for estimating the amount of the template DNAs.

Specificity of the primer pairs used was examined with genomic DNAs from

nonmethnogenic archaeal and bacterial strains. Nonmethanogenic archaeal 16S rDNA fragments were amplified only with 0348aF-GC and 0691R and none of bacterial 16S rDNA was amplified with both primer pairs (Fig. 3). There was at least one base mismatch in the sequences between the primers and 16S rDNA of the tested strains with an exception that there was no mismatch between 0357F-GC and 0691R and 16S rDNA of "*Haloarcula aidinensis*" JCM 10025. The lower annealing temperature and larger cycle number in the PCR condition for 0348aF-GC and 0691R than those for 0357F-GC and 0691R may have affected again the specificity of amplification. Consequently, the primer pair 0357F-GC and 0691R was more specific for methanogenic archaea than 0348aF-GC and 0691R.

Analysis of methanogenic archaeal community in paddy field soil by DGGE method

DGGE band patterns with 0348aF-GC and 0691R and 0357F-GC and 0691R from paddy field soils at the two sites (Anjo and Chikugo) are shown in Fig. 4. Numbers of DNA fragments obtained from both fields with 0348aF-GC and 0691R and 0357F-GC and 0691R were 25 to 28 and 15 to 17, respectively. Although *Methanobrevibacter arboriphilus* SA, *Methanosarcina mazei* TMA and *Methanoculleus chikugoensis* MG62 were isolated from WS, WS and RSC plots, respectively, in the Chikugo paddy field, only the bands whose mobility was close to that of *Methanosarcina mazei* TMA were found in all samples from Chikugo field.

Representatives of bands, which were clear and had high intensity, were excised from both DGGE gels as many as possible and subjected to sequencing. The sequences of totally 41 DNA fragments were successfully determined. The formation of chimeric DNA fragment was not observed in those sequences. The phylogenetic relationships of sequenced 41 bands are shown in Fig. 5. All fragments belonged to methanogenic archaea. Most sequences were affiliated to Methanomicrobiales or Methanosarcinales, especially related to *Methanosaeta*. The sequences of E2-1a, E2-1b and RS-1b were close relatives to *Methanospirillum* sp. TM 20-1 which was isolated from a Japanese paddy field soil [40]. Several sequences belonging to "Rice clusters", which were reported to exist in only paddy fields [10, 15], were also obtained. Although the primer pair of 0348aF-GC and 0691R potentially amplifies 16S rDNA of nonmethanogenic archaea (Fig. 3), no fragment belonging to these taxa was obtained in the bands analyzed so far.

Kudo et al. [41] showed that *Methanosarcina*-, *Methanosaeta*- and *Methanogenium*-like clones dominated in paddy field soils in Japan by analyzing clone libraries of archaeal 16S rDNA. The analysis of ether-linked lipids and microscopic observation of MPN cultures suggested that the order Methanosarcinales was predominant in CF, RS and RSC plots in the

Chikugo field [19]. These results seem to support our sequence data although all of the fragments on DGGE gels were not analyzed. We will conduct ecological study on methanogenic archaeal communities in paddy field soil with the DGGE method presented here to assess the predominant species of methanogenic archaea in paddy field soil and monitor seasonal dynamics of the populations, etc. in the future paper.

In conclusion, the method for DGGE analysis of 16S rDNA described above is suitable for investigating methanogenic archaeal community in paddy field soil. Of the two primer pairs selected, 0357F-GC and 0691R seems to be better in view of its specificity for methanogenic archaea since the primer pair of 0348aF-GC and 0691R potentially amplifies 16S rDNA of nonmethanogenic archaea.

Acknowledgements

We thank Ito T. of the Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Japan, Morii H. of the Department of Environmental Management, University of Occupational and Environmental Health, Japan and Murase J. of our laboratory for providing their samples of DNAs and strains. We also thank the members of the Anjo Research and Extension Station, Aichi-ken Agricultural Research Center, Japan and the National Agricultural Research Center for Kyushu Okinawa Region, Japan for their help in taking soil samples. This work was supported in part by a Grant-in Aid for Scientific Research (C; No. 14560051) from Japan Society for the Promotion of Science and by a Grant for Environmental Research (No. 023146) from the Sumitomo Foundation.

Reference

[1] Bryant, M. P. (1979) Microbial methane production – theoretical aspects. J. Anim. Sci. 48, 193-201.

[2] Rodhe, H. (1990) A comparison of the contribution of various gases to the greenhouse effect. Science 248, 1217-1219.

[3] Prather, M., Derwent, R., Ehhalt, D., Fraser, P., Sanhueza, E. and Zhou, X. (1994) Other trace gases and atmospheric chemistry. In: Climate change 1994, Radiative forcing of climate change and an evaluation of the IPCC IS92 emission scenarios (Houghton, J. T., Filho, L. G. M., Bruce, J., Lee, H., Callander, B. A., Haites, E., Harris, N. and Maskell, K., Eds.), pp. 77-126. Cambridge University Press, New York.

[4] Garrity, G. M. and Holt, J. G. (2001) The road map to the manual. In: Bergey's manual of systematic bacteriology, 2nd edn, Vol. 1, The *Archaea* and the deeply branching and phototrophic *Bacteria* (Boone, D. R., Castenholz, R. W. and Garrity, G. M., Eds.), pp. 119-166. Springer, New York.

[5] Olsen, G. J., Woese, C. R. and Overbeek, R. (1994) The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. 176, 1-6.

[6] Garcia, J.-L., Patel, B. K. C. and Ollivier, B. (2000) Taxonomic, phylogenetic, and ecological diversity of methanogenic *Archaea*. Anaerobe 6, 205-226.

[7] Whitman, W. B., Boone, D. R., Koga, Y. and Keswani, J. (2001) Taxonomy of methanogenic *Archaea*. In: Bergey's manual of systematic bacteriology, 2nd edn, Vol. 1, The *Archaea* and the deeply branching and phototrophic *Bacteria* (Boone, D. R., Castenholz, R. W. and Garrity, G. M., Eds.), pp. 211-213. Springer, New York.

[8] Muyzer, G., de Waal, E. C. and Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695-700.

[9] Øvreås, L., Forney, L., Daae, F. L. and Torsvik, V. (1997) Distribution of bacterioplankton in meromictic lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. 63, 3367-3373.

[10] Großkopf, R., Stubner, S. and Liesack, W. (1998) Novel euryarchaeotal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms. Appl. Environ. Microbiol. 64, 4983-4989.

[11] Großkopf, R., Janssen, P. H. and Liesack, W. (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl. Environ. Microbiol. 64, 960-969.

[12] Chin, K.-J., Lukow, T., Stubner, S. and Conrad, R. (1999) Structure and function of the methanogenic archaeal community in stable cellulose-degrading enrichment cultures at two different temperatures (15 and 30 °C). FEMS Microbiol. Ecol. 30, 313-326.

[13] Chin, K.-J., Lukow, T. and Conrad, R. (1999) Effect of temperature on structure and function of the methanogenic archaeal community in an anoxic rice field soil. Appl. Environ. Microbiol. 65, 2341-2349.

[14] Lehmann-Richter, S., Großkopf, R., Liesack, W., Frenzel, P. and Conrad, R. (1999) Methanogenic archaea and CO_2-dependent methanogenesis on washed rice roots. Environ. Microbiol. 1, 159-166.

[15] Lueders, T. and Friedrich, M. (2000) Archaeal population dynamics during sequential reduction processes in rice field soil. Appl. Environ. Microbiol. 66, 2732-2742.

[16] Fey, A., Chin, K. J. and Conrad, R. (2001) Thermophilic methanogens in rice field soil. Environ. Microbiol. 3, 295-303.

[17] Ramakrishnan, B., Lueders, T., Dunfield, P. F., Conrad, R. and Friedrich, M. W. (2001) Archaeal

community structures in rice soils from different geographical regions before and after initiation of methane production. FEMS Microbiol. Ecol. 37, 175-186.

[18] Weber, S., Lueders, T., Friedrich, M. W. and Conrad, R. (2001) Methanogenic populations involved in the degradation of rice straw in anoxic paddy soil. FEMS Microbiol. Ecol. 38, 11-20.

[19] Asakawa, S., Akagawa-Matsushita, M., Koga, Y. and Hayano, K. (1998) Communities of methanogenic bacteria in paddy field soils with long-term application of organic matter. Soil Biol. Biochem. 30, 299-303.

[20] Achenbach, L. and Woese, C. (1995) 16S and 23S rRNA-like primers. In: Archaea, a laboratory manual, methanogens (Sowers, K. R. and Schreier, H. J., Eds.), pp. 521-523. Cold Spring Harbor Laboratory Press, New York.

[21] Asakawa, S., Morii, H., Akagawa-Matsushita, M., Koga, Y. and Hayano, K. (1993) Characterization of *Methanobrevibacter arboriphilicus* SA isolated from a paddy field soil and DNA-DNA hybridization among *M. arboriphilicus* strains. Int. J. Syst. Bacteriol. 43, 683-686. (Erratum 44, 185 [1994])

[22] Asakawa, S., Akagawa-Matsushita, M., Morii, H., Koga, Y. and Hayano, K. (1995) Characterization of *Methanosarcina mazeii* TMA isolated from a paddy field soil. Curr. Microbiol. 31, 34-38.

[23] Dianou, D., Miyaki, T., Asakawa, S., Morii, H., Nagaoka, K., Oyaizu, H. and Matsumoto, S. (2001) *Methanoculleus chikugoensis* sp. nov., a novel methanogenic archaeon isolated from paddy field soil in Japan, and DNA-DNA hybridization among *Methanoculleus* species. Int. J. Syst. Evol. Microbiol. 51, 1663-1669.

[24] Asakawa, S. and Nagaoka, K. (2003) *Methanoculleus bourgensis*, *Methanoculleus olentangyi* and *Methanoculleus oldenburgensis* are subjective synonyms. Int. J. Syst. Evol. Microbiol. 53, 1551-1552.

[25] Yoo, C.-H., So, J.-D., Ida, A., Tanaka, F. and Nishida, M. (1992) Effect of long-term organic matter application on the fine textured paddy soils of double cropping system in temperate area. J. Korean Soc. Soil Sci. Fert. 25, 325-333.

[26] Zhou, J., Bruns, M. A. and Tiedje, J. M. (1996) DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62, 316-322.

[27] Cahyani, V. R., Matsuya, K., Asakawa, S. and Kimura, M. (2003) Succession and phylogenetic composition of bacterial communities responsible for the composting process of rice straw estimated by PCR-DGGE analysis. Soil Sci. Plant Nutr. 49, 619-630.

[28] Jackson, C. R., Harper, J. P., Willoughby, D., Roden, E. E. and Churchill, P. F. (1997) A simple, efficient method for the separation of humic substances and DNA from environmental samples. Appl.

Environ. Microbiol. 63, 4993-4995.

[29] Leclerc, M., Delbes, C., Moletta, R. and Godon, J.-J. (2001) Single strand conformation polymorphism monitoring of 16S rDNA Archaea during start-up of an anaerobic digester. FEMS Microbiol. Ecol. 34, 213-220.

[30] Delbès, C., Moletta, R. and Godon, J.-J. (2001) Bacterial and archaeal 16S rDNA and 16S rRNA dynamics during an acetate crisis in an anaerobic digestor ecosystem. FEMS Microbiol. Ecol. 35, 19-26.

[31] Hiraishi, A. (2001) Sequences of ribosomal RNA gene. In: Manual for classification and identification of microorganisms (Suzuki, K., Hiraishi, A. and Yokota, A. Eds.), pp. 48-64. Springer-Verlag Tokyo, Tokyo.

[32] Farrelly, V., Rainey, F. A. and Stackebrandt, E. (1995) Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl. Environ. Microbiol. 61, 2798-2801.

[33] Polz, M. F. and Cavanaugh, C. M. (1998) Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. 64, 3724-3730.

[34] Smith, D. R., Doucette-Stamm, L. A., Deloughery, C., Lee, H., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K., Harrison, D., Hoang, L., Keagle, P., Lumm, W., Pothier, B., Qiu, D., Spadafora, R., Vicaire, R., Wang, Y., Wierzbowski, J., Gibson, R., Jiwani, N., Caruso, A., Bush, D., Safer, H., Patwell, D., Prabhakar, S., McDougall, S., Shimer, G., Goyal, A., Pietrokovski, S., Church, G. M., Daniels, C. J., Mao, J.-I, Rice, P., Nölling, J. and Reeve, J. N. (1997) Complate genome sequence of *Methanobacterium thermoautotrophicum* ΔH: functional analysis and comparative genomics. J. Bacteriol. 179, 7135-7155.

[35] Deppenmeier, U., Johann, A., Hartsch, T., Merkl, R., Schmitz, R. A., Martinez-Arias, R., Henne, A., Wiezer, A., Bäumer, S., Jacobi, C., Brüggemann, H., Lienard, T., Christmann, A., Bömeke, M., Steckel, S., Bhattacharyya, A., Lykidis, A., Overbeek, R., Klenk, H.-P., Gunsalus, R. P., Fritz, H.-J. and Gottschalk, G. (2002) The genome of *Methanosarcina mazei*: evidence for lateral gene transfer between bacteria and archaea. J. Mol. Microbiol. Biotechnol. 4, 453-461.

[36] Ishii, K. and Fukui, M. (2001) Optimization of annealing temperature to reduce bias caused by a primer mismatch in multitemplate PCR. Appl. Environ. Microbiol. 67, 3753-3755.

[37] Hansen, M. C., Tolker-Nielsen, T., Givskov, M. and Molin, S. (1998) Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. FEMS Microbiol. Ecol. 26, 141-149.

[38] Liesack, W., Weyland, H. and Stackebrandt, E. (1991) Potential risks of gene amplification by PCR

as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microb. Ecol. 21, 191-198.

[39] Kopczynski, E. D., Bateson, M. M. and Ward, D. M. (1994) Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. Appl. Environ. Microbiol. 60, 746-748.

[40] Tonouchi, A. (2002) Isolation and characterization of a motile hydrogenotrophic methanogen from rice paddy field soil in Japan. FEMS Microbiol. Lett. 208, 239-243.

[41] Kudo, Y., Nakajima, T., Miyaki, T. and Oyaizu, H. (1997) Methanogen flora of paddy soils in Japan. FEMS Microbiol. Ecol. 22, 39-48.

Tabe 1. PCR of primers. The upper sequence was used in the present study and the lower was the original one. The modified base is shown in bold letter.

Primer	Sequence	*E. coli* position	*T* m (°C)	Reference
0357F	CCC TAC GGG GCG CAG CAG	340 - 357	64.9	
PARCH340f	CCC TAC GGG GYG CAS CAG	340 - 357		[9]
0348aF	TCC AGG CCC TAC GGG	333 - 348	52.0	[20]
0505R	TTA CCG CGG CGG CTG	533 - 519	52.0	
PARCH519r	TTA CCG CGG CKG CTG	533 - 519		[9]
0691R	GGA TTA CAR GAT TTC AC	707 - 691	49.0	
0690aR	TTA CAG GAT TTC ACT	704 - 690		[20]
0915aR	GTG CTC CCC CGC CAA TTC CT	934 - 915	62.5	[20]
GC clamp	CGC CCG CCG CGC GCG GCG GGC			
	GGG GCG GGG GCA CGG GGG G			[8]

Table 2. Methanogenic archaeal strains and DNA concentration used for PCR template.

Strain	Accession number of 16S rDNA	Concentration (ng /μL)
Methanobrevibacter arboriphilus SA (DSM 7056)	AB065294	0.17
Methanosarcina mazei TMA (DSM 9195)	AB065295	0.10
Methanoculleus chikugoensis MG62 (DSM 13459)	AB038795	0.18
Methanothermobacter thermoautotrophicus ΔH (DSM 1053)	AE000930,AE000940	0.19
Methanothermobacter wolfeii (DSM 2970)	AB104858	0.13
Methanosarcina mazei S-6 (DSM2053)	U20151	0.10
Methanosarcina mazei LYC (DSM 4556)	AB065296	0.23
Methanoculleus thermophilus CR-1 (DSM 2373)	AB065297	0.11
Methanoculleus palmolei INSLUZ (DSM 4273)	Y16382	0.09
Methanoculleus bourgensis RC/ER (DSM 2772)	AF095270	0.12
Methanoculleus bourgensis CB1 (DSM 6216)	AB065298	0.23
Methanoculleus bourgensis MS2 (DSM 3045)	AF095269	0.19
Methanoculleus marisnigri JR1 (DSM 1498)	M59134	0.12

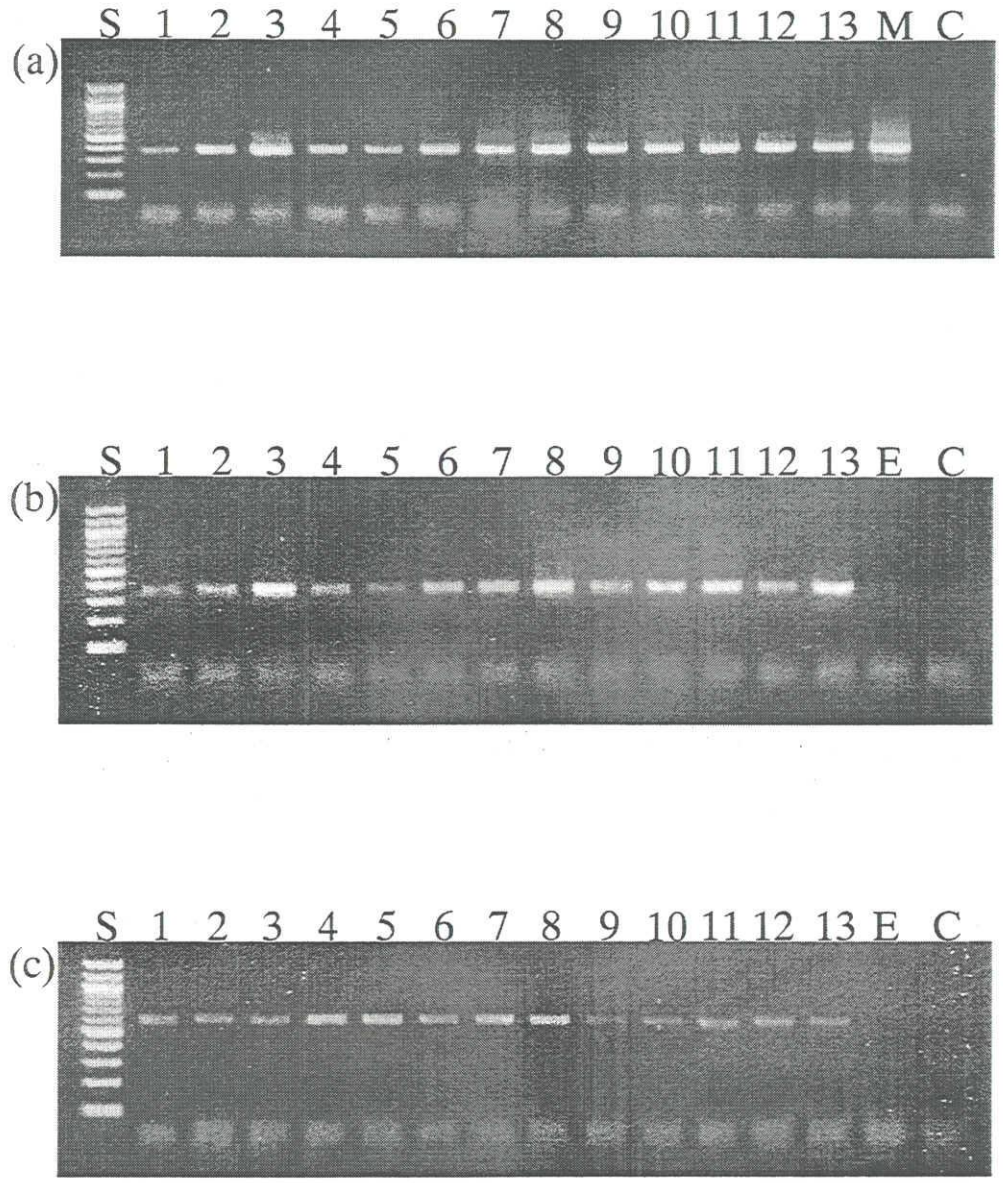

Fig. 1. PCR products amplified with selected primer pairs. (a) 0348aF-GC and 0691R. (b) 0357F-GC and 0691R. (c) 0357F-GC and 0915aR. S, 100 bp ladder; 1, *Methanobrevibacter arboriphilus* SA; 2, *Methanosaricina mazei* TMA; 3, *Methanoculleus chikugoensis* MG62; 4, *Methanothermobacter thermoautotrophicus* ΔH^T; 5, *Methanothermobacter wolfeii*; 6, *Methanosarcina mazei* S-6^T; 7, *Methanosarcina mazei* LYC; 8, *Methanoculleus thermophilus* CR-1^T; 9, *Methanoculleus palmolei* INSULZ^T; 10, *Methanoculleus bourgensis* RC/ER; 11, *Methanoculleus bourgensis* CB1; 12, *Methanoculleus bourgensis* MS2^T; 13, *Methanoculleus marisnigri* JR1^T; M, products amplified from a mixture of genomic DNAs of 8 methanogenic archaeal strains (1-4, 8-10, 13); E, *E. coli* K-12; C, negative control (PCR products without DNA template). The annealing temperature and cycle number for 0348aF-GC and 0691R, 0357F-GC and 0691R and 0357F-GC and 0915aR were 49 °C and 40, 53 °C and 35 and 65 °C and 25, respectively.

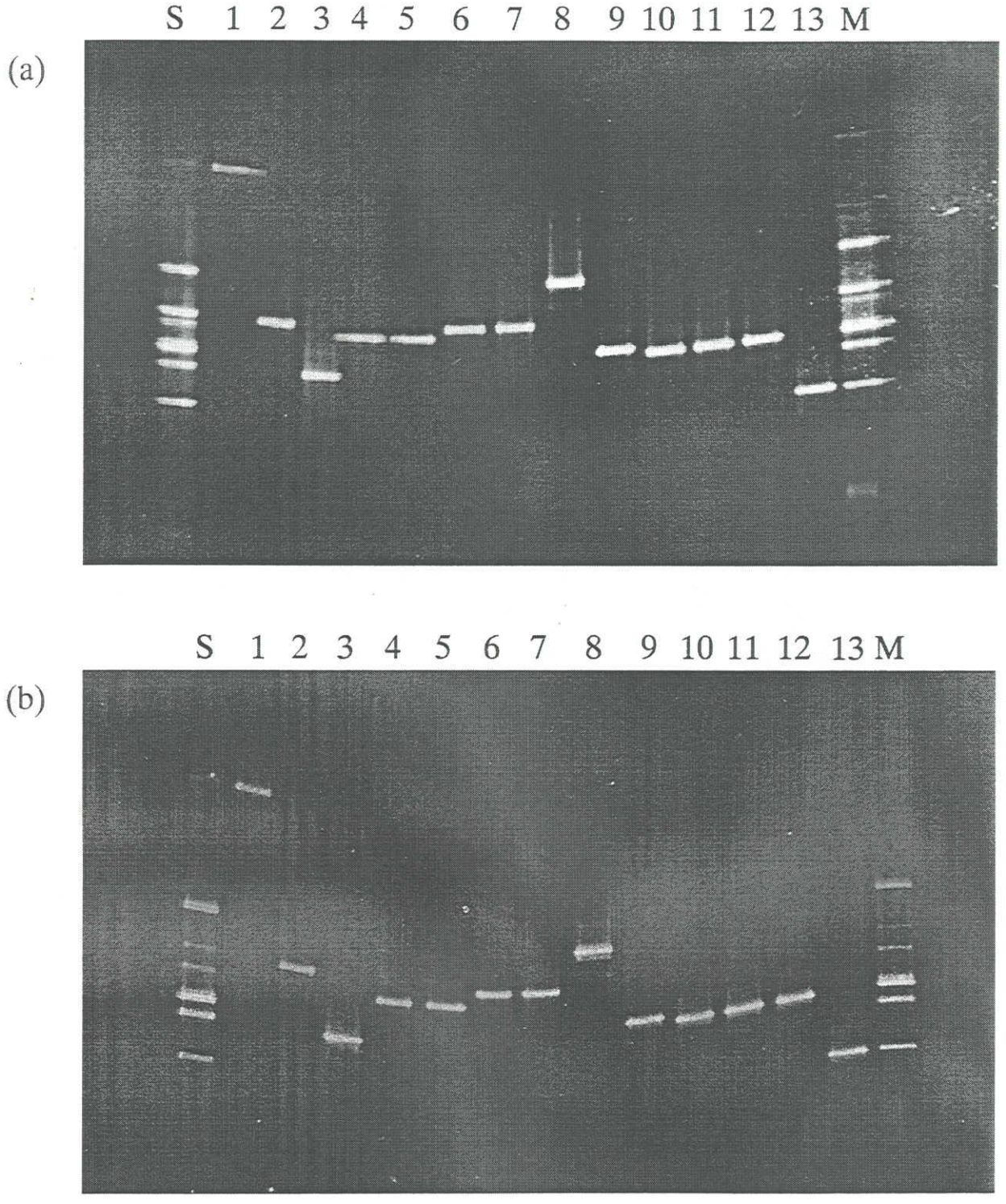

Fig. 2. DGGE band patterns of PCR products. (a) 0348aF-GC and 0691R. (b) 0357F-GC and 0691R. Lanes 1-13 and M are the same as in Fig. 1. S, mixture of equal amount of PCR products from the genomic DNAs of 13 methanogenic archaeal strains (1-13). The denaturant gradient ranges were from 25 to 65 % and from 25 to 60 % for (a) and (b), respectively.

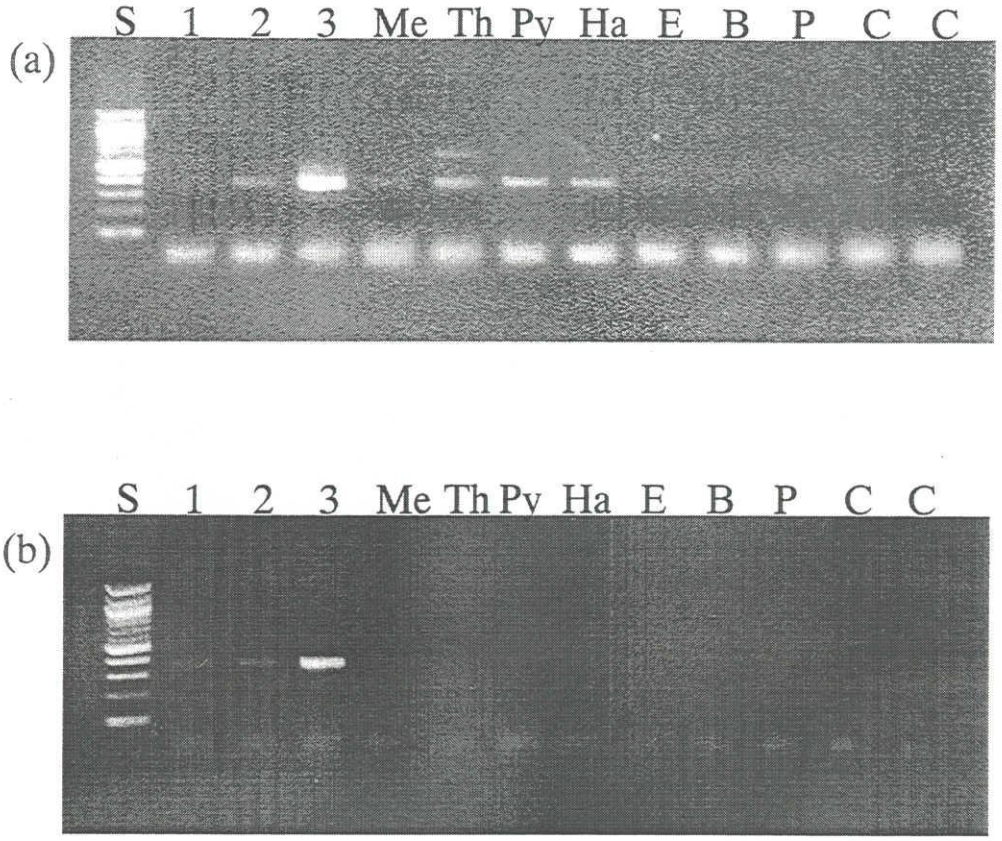

Fig. 3. Specificity of primer pairs for methanogenic archaea. (a) 0348aF-GC and 0691R. (b) 0357F-GC and 0691R. 1-3, S, C and E are the same as in Fig. 1. Me, *Metallospaera sedula* JCM 9064; Th, *Thermoproteus tenax* JCM 9277^{T}; Py, *Pyrococcus horikoshi* JCM 9975; Ha, "*Haloarcula aidinensis*" JCM 10025; B, *B. subtilis* IFO 16412; P, *P. aeruginosa* IFO 12689.

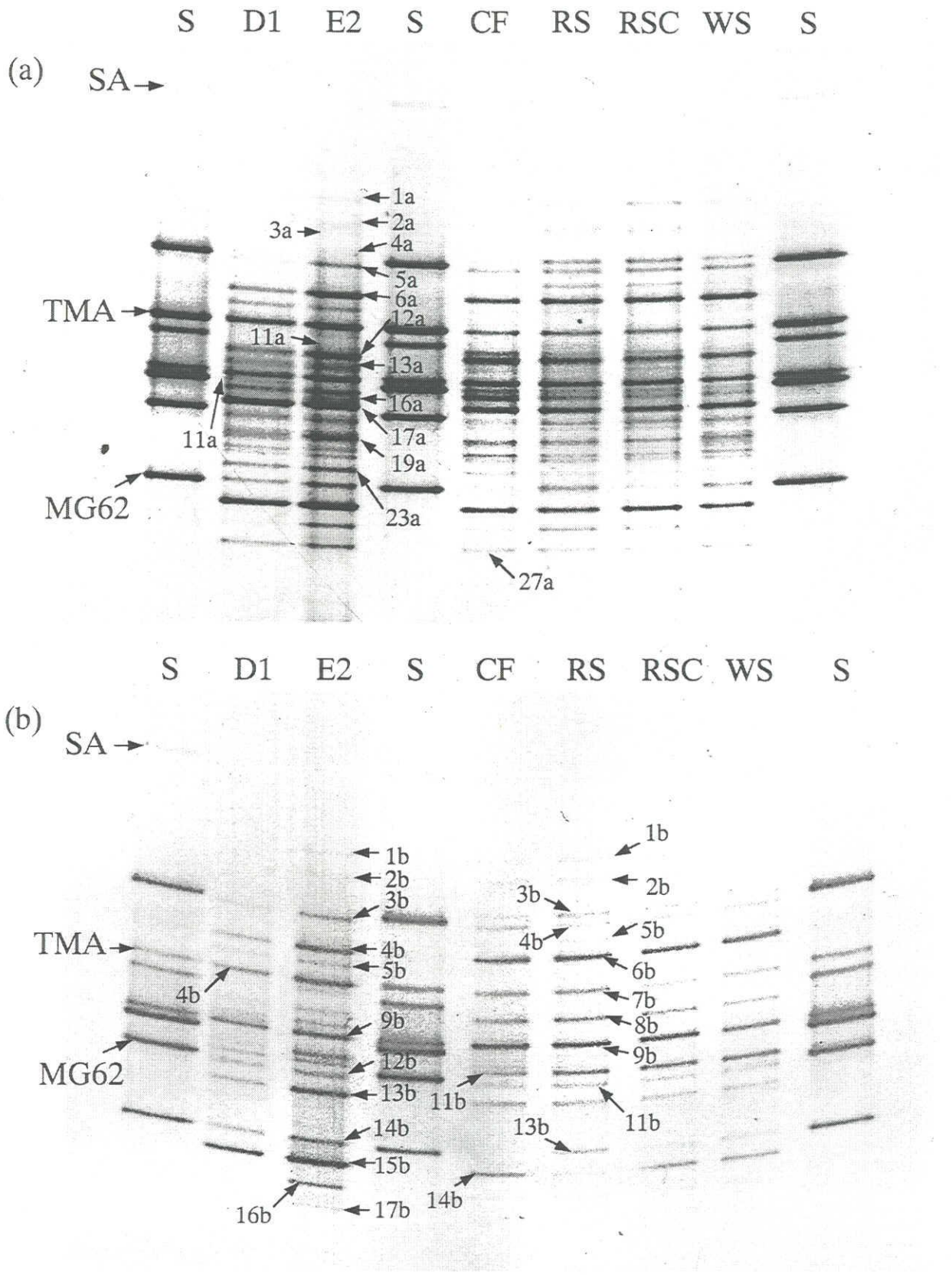

Fig. 4. DGGE band patterns (negatively converted) obtained from soil samples of paddy fields in Anjo and Chikugo. (a) 0348aF-GC and 0691R. (b) 0357F-GC and 0691R. S is the same as in Fig. 2. D1 and E2 are the plots at paddy fields in Anjo. CF, chemical fertilizer plot; RS, rice straw plus chemical fertilizer plot; RSC, rice straw compost plus chemical fertilizer plot; WS, wheat straw plus chemical fertilizer plot at paddy field in Chikugo. The numbers indicated by arrows show the bands whose sequences were determined and correspond with the ones on the phylogenetic tree.

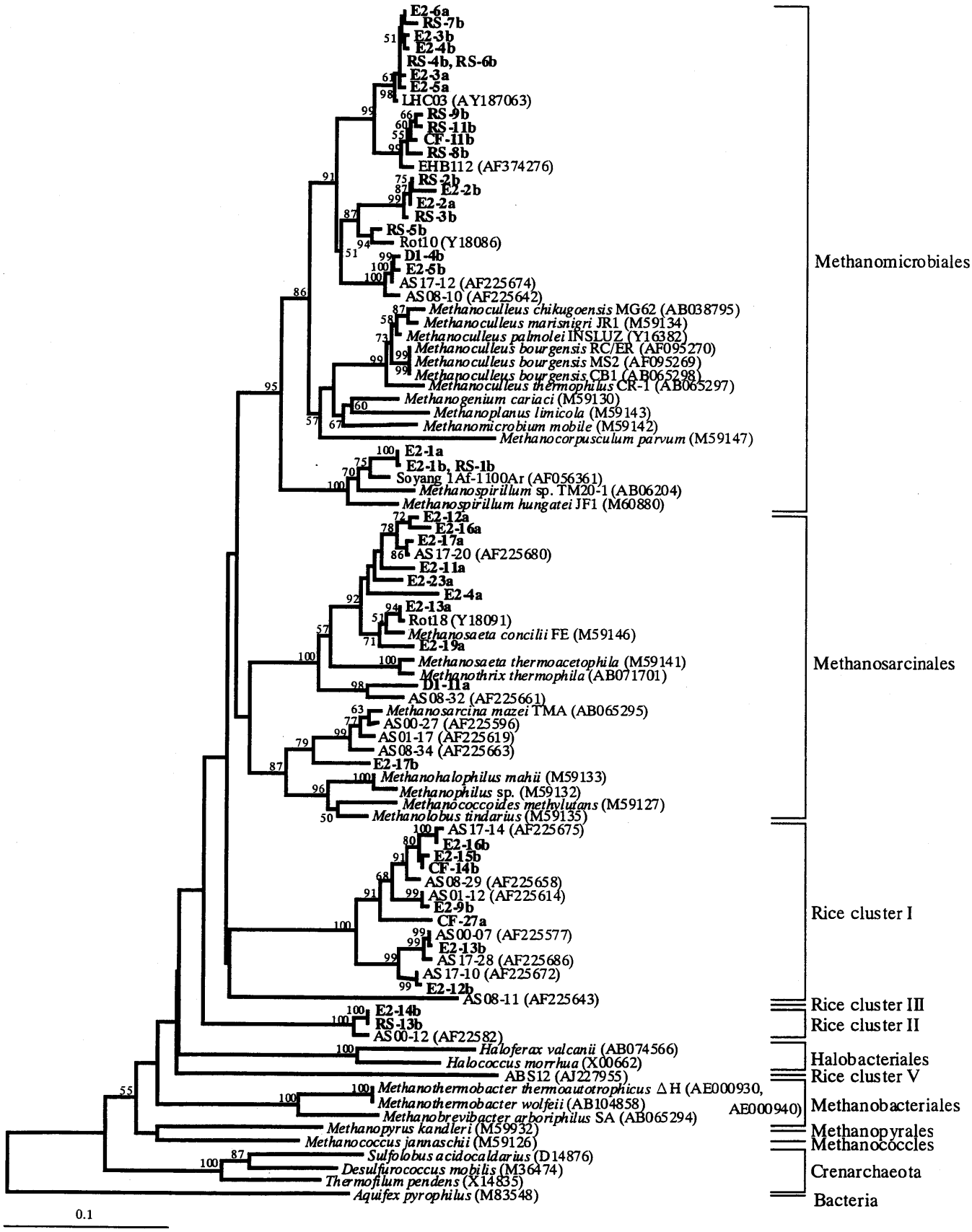

Fig. 5. Phylogenetic relationships of 16S rDNA sequences retrieved by DGGE method from paddy field soil in Anjo and Chikugo. The sequences obtained in the present study are shown in bold letters. a and b show the sequences with 0348aF-GC and 0691R and 0357F-GC and 0691R (*E. coli* positions 349-690 and 358-690), respectively. Phylogenetic relationships were compared among the sequences from the *E.coli* position 358 to 690. Bootstrap values (%) are shown at branch points (when more than 50 %). *Aquifex pyrophilus* was used as an out group. The scale bar represents 0.1 substitutions per nucleotide. Accession numbers of sequences are indicated in parentheses.