A deeply branched novel phylotype found in
Japanese paddy soils

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Novel 16S rDNA clones which possibly constitute a sister clade from the two known archaeal lineages, Crenarchaeota and Euryarchaeota, were found in paddy soil environments. Overall signature sequences showed that the clone sequences shared a majority of signature sequence features with the Archaea and Eukarya. However, there were at least nine nucleotides which distinguished the novel clones from the domains Archaea and Eukarya. Phylogenetic trees, drawn by maximum-parsimony, neighbour-joining and maximum-likelihood methods, also supported the unique phylogenetic position of the clones. Both signature sequence and phylogenetic analyses strongly suggest that the novel organisms constitute a new group and their phylogenetic positions are distant from the Crenarchaeota and Euryarchaeota. A specific primer set was designed to detect the presence of the novel group of organisms in terrestrial environments. Specific DNA fragments were amplified from all paddy soil DNAs, suggesting that the novel organisms are widely distributed in rice paddy fields in Japan.

Keywords: novel soil clones, unculturable micro-organisms, paddy soil, novel phylotype

INTRODUCTION

The three-domain system (Woese et al., 1990; Wheelis et al., 1992) has been widely accepted, in contrast to the eucaryotic tree (Lake, 1991), for describing the relationships of the major lineages. Within this system, the three phylogenetic lineages are categorized as the domains Eukarya, Bacteria and Archaea (Woese et al., 1990). Each domain is further subdivided into kingdoms. The domain Archaea contains two kingdoms, Euryarchaeota and Crenarchaeota. The kingdom Euryarchaeota (Woese et al., 1990; Wheelis et al., 1992) includes methaneproducing archaea, extremely halophilic archaea and some other genera, such as Archaeoglobus, Thermococcus and Thermoplasmata. The kingdom Crenarchaeota (Woese et al., 1990; Wheelis et al., 1992) consists of extremely thermophilic and sulfur-dependent archaea, such as Sulfolobus, Thermoproteus, Pyrodictium and Desulfuroccoccus.

Organisms in many disparate environments still remain uncultured because of the difficulty of cultivation based on conventional techniques. In recent years, the phylogenetic positions of uncultured bacteria have been revealed by 16S rDNA sequence analyses (Ward et al., 1990, 1992; Weller & Ward, 1991; Fuhrman et al., 1992). Uncultured archaeal organisms have been detected in different biotopes, including marine environments (DeLong, 1992; Fuhrman et al., 1992), hot springs (Barns et al., 1994, 1996), abyssal environments (McInerney et al., 1995), freshwater (Schleper et al., 1997) and soils (Bintrim et al., 1997; Großkopf et al., 1998). Crenarcheotal lineages, which are branched very deeply from cultured Crenarchaeota, were revealed to be predominant in marine and abyssal environments (Fuhrman et al., 1992; McInerney et al., 1995). In addition to this bunch of deeply branched crenarchaeotal lineages, a number of phylotypes within the Crenarchaeota have also been detected in non-extreme environments, such as marine, freshwater and terrestrial soils (DeLong, 1998). These findings indicate that members of this kingdom of the Archaea are globally distributed and they are playing an ecologically important role in their habitats. Euryarchaeotal clones have also been detected in marine environments.

Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the novel soil clones and their aligned data set are D88480–D88489 and ds36901, respectively.

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sequences in the available databases using the program CLUSTAL W version 1.7 (Thompson et al., 1994). The compared sequences were positions 632–820, 880–996, 1041–1132 and 1142–1229. A maximum-parsimony (MP) tree reconstruction was performed using the PAUP software version 3.1.1 (Swofford, 1991). The applied ratio of transition and transversion was 2:1. A heuristic search was used with a random stepwise addition sequence of 100 replicates, tree-bisection-reconnection branch swapping and the MULPAR option. A further analysis was run with 100 bootstrap replicates, each consisting of 10 additional random replicates. A maximum-likelihood (ML) analysis was carried out using a program package, MOLPHY version 2.3b (Adachi & Hasegawa, 1996). A ML distance matrix was calculated using NucML, and a neighbour-joining (NJ) topology as the starting tree for the ML method was reconstructed by NJDist in MOLPHY. A ML tree was obtained using NucML with R (local rearrangement search) option based on the HKY model (Hasegawa et al., 1985). Local bootstrap probabilities were estimated by the RELL (resampling of estimated log-likelihood) method (Kishino et al., 1990; Hasegawa & Kishino, 1994). NJ analysis was performed using the PHYLIP software version 3.572 (Felsenstein, 1995). DNADIST from this program package was used to create a distance matrix based on the two-parameter method of Kimura (1980). This distance matrix was used to construct a NJ tree using the NJ algorithm from NEIGHBOR (PHYLIP version 3.572), and the bootstrap analyses utilized 1000 replicate data sets.

To assess the phylogenetic placement of the novel soil clones, nucleotide signature analysis was done by using the ARB program package (O. Strunk & W. Ludwig; http://www.mikro.biologie.tu-muenchen.de/pub/ARB/; Technische Universität München, Germany) with data from the Ribosomal Database Project (RDP) (Maidak et al., 1999).

Phylogenetic analysis. Soil clone sequences were compared to sequences in the available databases using the BLASTN algorithm (Altschul et al., 1997) to search for close evolutionary relatives (the last survey was performed in March 21, 2000). As no sequences in the DNA database showed similarities higher than 80%, representative organisms were selected from the Archaea, Eukarya and Bacteria. Partial 16S rDNA sequences of these organisms were then compiled for phylogenetic analyses with those of soil clones. The data set was aligned manually using 16S rRNA secondary structure to remove some unalignable regions on the domain level and sequences of identified homologous regions were realigned using the program CLUSTAL W version 1.7 (Thompson et al., 1994). The compared sequences were positions 632–820, 880–996, 1041–1132 and 1142–1229. A maximum-parsimony (MP) tree

METHODS

Sample collection and DNA manipulations. Soil DNA was extracted from several paddy soil samples collected at Kagoshima-city (31° 34’ N 130° 33’ E), Yamaguchi-city (33° 57’ N 130° 56’ E), Kumagaya-city (35° 9’ N 139° 23’ E), Tsukuba-city (36° 05’ N 140° 08’ E), Kumamoto-city (32° 49’ N 130° 43’ E), Tokyo-city (35° 42’ N 139° 46’ E) and Yamagata-city (38° 15’ N 140° 21’ E). The DNA was purified by agarose gel electrophoresis and submitted to PCR. A volume of 10–50 ng DNA was used for each amplification. Sampling procedures and PCR conditions are described in detail by Kudo et al. (1996). Primer sets were synthesized specifically to amplify the unusual 16S rDNA. The forward primers synthesized were AS11F (5’-TTC GTC GAC GGT TGA TCC YGS CRG AGG C-3’), which corresponds to positions in Escherichia coli) 11–23 of archaeal 16S rRNA; AS343F (5’-TTC GTC GAC TAC GGG GCG CAS CAG GC-3’), which corresponds to positions 343–359; and AS564F (5’-AAC CGT CGA CTG GCC CTG AAG CGY CCG TAG C-3’), which corresponds to positions 564–584. Reverse primers (complementary to 16S rRNA) were synthesized to be specific to the unusual 16S rDNA base on the determined partial 16S rDNA sequences (Kudo et al., 1996). The reverse primers, which had sequences complementary to positions 1247–1229, were SC13 (5’-CGG CGA ATT CTC ACC CAT TGT TGC GCG-3’) and SW42 (5’-CGG CGA ATT CCC TAT CAT TGT TGC GCG-3’). These primers had EcoRI or SalI restriction sites on the 5’ end. The DNA amplified by primer sets of AS564F-SC13 and AS564F-SW42 was cloned into pBluescript vector, and 10 clones were sequenced with a DNA sequencer (Shimadzu DQQ-1000).

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Chimera check and prediction of rRNA secondary structure. Each sequence was submitted to the CHECK-CHEMERA program of the RDP (Maidak et al., 1999) to detect the presence of possible chimeric artefacts, and the putative partial secondary structure of 16S rRNA was predicted from the primary structure using the program RNA structure version 2.52 (Mathews & Burkard, 1997). The Zuker algorithm (1989) was used for RNA structure prediction based on the free energy minimization, and the putative secondary structure was constructed through comparison with that of published small-subunit rRNA (Neefs et al., 1993).

Distribution of novel soil organisms in the environment. To examine the distribution of the novel organisms in the environment, we designed an oligonucleotide primer, 970F (5’-AAT YYA ACT CAA CGC RGA G-3’), with a sequence identical to positions 959–976 of 16S rDNA of the novel clones. The presence of the novel soil organisms was tested with a primer set of 970F and SC13 for paddy soils, upland soils and forest soils collected in Japan. For each PCR reaction, approximately 10 ng purified soil DNA was used, and amplification was performed using AmpliTaq Gold (Perkin Elmer) in a thermal cycler (Hybaid) programmed for 9 min at 94 °C, followed by 55 cycles of 30 s at 94 °C, 20 s at 57 °C, and 25 s at 70 °C, and an additional incubation at 70 °C for 10 min. After amplification, 7 μl product was electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

Sequences of cloned 16S rDNA

Großkopf et al. (1998) analysed a number of archaeal 16S rDNA clones, covering novel euryarchaeotal groups, which were detected on rice roots and in anoxic rice paddy soil. Crenarchaeotal clones, which are divergent
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Fig. 1. Putative secondary structure of the partial 16S rRNA of SC4. Bold letters were used to indicate the novel soil clone unique nucleotide sequences. The corresponding nucleotides to these positions are highly conservative on the domain level and they are indicated in boxes. Bac, Euk and Arc in boxes indicate Bacteria, Eukarya and Archaea, respectively, and italic numerals are the numbers of the E. coli numbering system. The nucleotide 1 corresponds to the 585th nucleotide of the 16S rDNA in the E. coli numbering system.

from this phylogenetic line of descent, were also detected in soil environments (Bintrim et al., 1997). The discovery of new lineages in the soil ecosystem is not surprising considering the great genetic diversity of archaeal rDNA clones retrieved from various natural environments. We previously found novel 16S rDNA clones based on sequence identity (Kudo et al., 1996, 1997). Although the length of compared 16S rDNAs was approximately 270 bp, the sequences were extremely novel. Therefore, extended phylogenetic analysis was necessary to clarify the phylogenetic position of the novel soil clones.

To amplify nearly complete 16S rDNA fragments of the novel soil organisms, PCR amplification was performed with two primer sets, AS11F-SC13 and AS11F-SW42. However, these primer sets did not give any amplification products. AS343F as a forward primer was subjected to the PCR amplification instead of AS11F, but these primer sets also failed to amplify the target molecules. Partial 16S rDNA was obtained with primer sets AS564F-SC13 and AS564F-SW42, and these products were cloned. Sequences of 10 cloned DNA samples were determined for further analyses, and the sequences of each clone were 82–98% similar to each other. The length of sequenced DNA ranged from 644 to 647 nucleotides. Each sequence was folded into a putative secondary structure of 16S rRNA from positions 585 to 1228 (E. coli numbering system; Fig. 1), and they were found to be well-conserved in agreement with previously published models (Neefs et al., 1993). The correct secondary structure seemed to show some evidence that the individual sequences are a natural occurrence, although this evidence does not give complete proof that the sequences are not chimeric. CHECK-CHIMERA also did not detect chimeras among the novel soil sequences.

Phylogenetic analysis and signature analysis of the novel soil clones

Phylogenetic analyses by MP, NJ and ML all showed similar topologies (Fig. 2). Novel soil clones formed a monophyletic clade which was supported by >99% bootstrap proportions, and these phylotypes formed a sister group with the Euryarchaeota and Crenarchaeota in all the trees. However, the domain Archaea was ambiguously divergent by MP analysis. This unusual
Fig. 2. Phylogenetic relationships of the novel soil clones inferred from the three analytical methods. (a) Strict consensus tree of the six most parsimonious trees; (b) NJ tree; (c) ML tree (α/β^2 – 942). Numbers in the MP and NJ trees indicate bootstrap percentage and numbers in the ML tree indicate local bootstrap probabilities. Scale bars below the NJ and ML trees mean substitutions per site. The GenBank/EMBL/DDBJ accession numbers of the determined sequences are D88480–D88489. The accession numbers of compared sequences are as follows: Archaeoglobus fulgidus, X05567; Aquifex pyrophilus, M8358; Chloroflexus aurantiacus, D38365; Dictyostelium discoideum, X00134; Deinococcus radiodurans, M21413; E. coli, J01695; Giardia muris, X65036; Haloferax volcanii, K00421; Homo sapiens, X03205; Methanobacterium thermoautotrophicum, Z37156; Methanobacterium bryantii, M59124; Methanococcus jannaschii, M59126; Methanococcus thermolithotrophicus, M59128; Methanococcus vannielii, M36507; Methanococcus voltae, M59290; Methanogenium cariaci, M59130; Methanospirillum hungatii, M60880; Petrotoga metherma, L10657; Saccharomyces cerevisiae, M27607; Sulfolobus solfataricus, X03235; Thermococcus celer, M21529; Thermofilum pendens, X14835; Thermoproteus tenax, M35966; Thermotoga maritima, M21774; Tritrichomonas foetus, U17509; Vairimorpha necatrix, M24612; pip27, L25852; pip33, L25300; pip41, L25301; pip78, L25303; pip89, L25305; JM2, L24196; and JM8, L24201. Clones pip27, pip33, pip41, pip78 and pip89 were isolated from Yellowstone National Park hot springs (Barns et al., 1994); JM2 and JM8 were isolated from an abyssal deposit feeder (McInerney et al., 1995).
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Table 1. 16S rRNA sequence signature distinguishing Bacteria, Eukarya, Archaea and novel soil clones

<table>
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<tr>
<th>Position</th>
<th>Novel soil clones</th>
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<th>Eukarya</th>
<th>Bacteria</th>
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<td>Y</td>
<td>C</td>
<td>C</td>
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<td>Y</td>
<td>A</td>
</tr>
<tr>
<td>756</td>
<td>G</td>
<td>G</td>
<td>Mostly A</td>
<td>Mostly C</td>
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<tr>
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<td>U</td>
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<td>U</td>
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<td>G</td>
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<td>1235</td>
<td>A</td>
<td>U</td>
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* Including planktonic and abyssal clones and ‘Korarchaeota’.

branching was probably due to the limited number of informative sites. The monophyletic relationship of the Euryarchaeota and Crenarchaeota is supported by comparatively high local bootstrap probabilities (90%) in the ML tree; however, the bootstrap value for the NJ topology was lower than that of the ML method. The ML method is suitable for phylogenetic analysis among organisms which have multiple substitutions. The NJ method underestimates multiple substitutions, so it is possible that its internal branch lengths are shorter than those obtained by the ML method (Adachi & Hasegawa, 1995). In this case, the ML tree possibly estimates the right internal branch length and true evolutionary tree.

The topology of phylogenetic trees is not reliable when the bootstrap shows a moderate to low level. Deep branchings, such as those between bacterial phyla, usually show relatively low bootstrap values, and the different branching topology has been presented in various published papers, even though the same molecules were used for these analyses (Woese, 1987; Neefs et al., 1993; Olsen et al., 1994). However, they are clearly distinguished by signature sequences (Woese, 1987). We tried to find signature sequences (Woese, 1987) to distinguish the novel soil clones from archaea, including deeply branched archaeal phylotypes. The signature sequences are shown in Table 1. Among the deeply branched archaeal phylotypes, marine planktonic clones have been discovered from Pacific Ocean bacterioplankton samples (Fuhrman et al., 1992). Later, McInerney et al. (1995) also found clones belonging to the marine planktonic archaeal lineage from the midgut contents of a deep-sea marine holothurian. Fuhrman et al. (1992) reported that the planktonic clones belonged to the crenarchaeotal lineage, but McInerney et al. (1995) reported that the planktonic and abyssal clones formed a discrete phylogenetic cluster that was separate from the Crenarchaeota and Euryarchaeota. Signature sequence analysis (Table 1) revealed that the signature nucleotides of planktonic and abyssal clones and the ‘Korarchaeota’ were identical to those of the Archaea. In contrast, the novel soil clones showed signature sequences that appeared to be different from those of the Archaea (including planktonic and abyssal clones and ‘Korarchaeota’), Bacteria and Eukarya. The differences that distinguish these novel soil clones from the Archaea, Bacteria and Eukarya were found in positions 688, 693, 699, 946, 1050, 1114, 1186, 1208 and 1235 (Table 1). Overall signature sequences showed that the novel clone sequences shared more distinct signature nucleotides with the Archaea and Eukarya than with the Bacteria. Signature sequences that shared a majority of signature...
sequence features with Archaea and Eukarya were positions 716, 912, 931, 952, 966, 1086, 1109 and 1110. The mean sequence similarity of the novel soil clones to the Crenarchaeota and Euryarchaeota was 78.0%, a value much lower than the mean value (83.7%) between Crenarchaeota and Euryarchaeota.

The signature sequences shown in Table 1 are very conservative, and some of the nucleotides, at positions 688, 699, 946, 1050, 1208 and 1235, have been recognized as universally conserved among the domains Eukarya, Archaea and Bacteria (Woese, 1987). Our finding that the 10 novel soil clones exhibited non-universal sequences supports their connection to an ancient lineage of life; thus the acquisition of their full 16S rDNA sequences may help to resolve phylogenetic relationships of major lineages in the tree of life.

### Inference of novel soil micro-organisms

We know nothing about these organisms other than their 16S rDNA sequences, so it might be difficult to predict their phenotypic characteristics. However, it is expected that sequences derived from the paddy fields are from mesophilic organisms. Table 2 compares the G+C contents of the sequenced regions of the 16S rDNA of Archaea and the novel soil clones. The high G+C content of the Crenarchaeota is correlated with their thermophilic characteristic; thermophiles usually have rDNA G+C contents of >60 mol% whereas mesophiles generally have rDNA G+C contents of 55 mol% or less (Dalgaard & Garrett, 1993; Woese et al., 1991). Judging from the sampling environments and the G+C contents of the novel soil clones, the sequences probably come from mesophilic organisms.

The presence of the novel soil organisms was determined by using a primer set of 970F and SC13 for paddy soils, upland soils and forest soils collected in Japan. PCR amplification (Fig. 3) showed that the novel organisms were present in all paddy soil samples, but not in upland and forest soil samples. The archaeal community structure changes according to soil conditions (Chin et al., 1999). Likewise, the population of the novel microorganisms might change depending on the season and weather conditions. In the PCR detection, novel soil microorganisms were detected in anoxic soils but not in oxic soils. From these facts, we speculate that the sequences would come from anaerobes or facultative anaerobes.

### Conclusions

In the present study, we discovered a novel phylogenetic cluster of life in soil DNAs. This phylogenetic cluster showed a unique signature sequence in the 16S rDNA. The cluster was also diverse phylogenetically. We have not yet recognized the organisms from which the novel clones were derived. However, the organisms are presumed to inhabit Japanese paddy soils and this would indicate an important role in the soil anaerobic ecosystem. If these organisms are cultured, they will provide useful information, not only on microbial ecology, but also on the phylogenetic relationships of major lineages in the tree of life.

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