

## ORIGINAL ARTICLE

# Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing

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Little is known about the archaeal diversity of fermented seafood; most of the earlier studies of fermented food have focused on lactic acid bacteria (LAB) in the fermentation process. In this study, the archaeal and bacterial diversity in seven kinds of fermented seafood were culture-independently examined using barcoded pyrosequencing and PCR–denaturing gradient gel electrophoresis (DGGE) methods. The multiplex barcoded pyrosequencing was performed in a single run, with multiple samples tagged uniquely by multiplex identifiers, using different primers for *Archaea* or *Bacteria*. Because PCR–DGGE analysis is a conventional molecular ecological approach, this analysis was also performed on the same samples and the results were compared with the results of the barcoded pyrosequencing analysis. A total of 13372 sequences were retrieved from 15898 pyrosequencing reads and were analyzed to evaluate the diversity of the archaeal and bacterial populations in seafood. The most predominant types of archaea and bacteria identified in the samples included extremely halophilic archaea related to the family Halobacteriaceae; various uncultured mesophilic Crenarchaeota, including Crenarchaeota Group I.1 (CG I.1a and CG I.1b), Marine Benthic Group B (MBG-B), and Miscellaneous Crenarchaeotic Group (MCG); and LAB affiliated with genus *Lactobacillus* and *Weissella*. Interestingly, numerous uncultured mesophilic Crenarchaeota groups were as ubiquitous in the fermented seafood as in terrestrial and aquatic niches; the existence of these Crenarchaeota groups has not been reported in any fermented food. These results indicate that the archaeal populations in the fermented seafood analyzed are diverse and include the halophilic and mesophilic groups, and that barcoded pyrosequencing is a promising and cost-effective method for analyzing microbial diversity compared with conventional approaches.

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## Introduction

Salted and fermented seafood products ('jeotgal' in Korean) are widely produced and consumed in Asia (Lee *et al.*, 1993). Jeotgal is made of fish or shellfish, such as shrimp, oysters, fish roes, and fish tripes with lots of salt, followed by a fermentation period so that the jeotgal develops its own distinct taste through the endogenous enzyme activities in the fish or microbes that degrade fish proteins (Cha and

Lee, 1985; Lee, 1994). Various kinds of jeotgal were made and consumed; specifically, the 145 different kinds of fermented seafood were classified on the basis of the raw materials available in Korea (Suh and Yoon, 1987). Fermented seafood is often used as a side dish as well as an ingredient to improve the taste of other foods, such as kimchi, a famous fermented vegetable product of Korea. To date, researches aimed at isolating novel strains in the Asian fermented seafood have identified more than 15 novel species in archaea and bacteria (<http://www.bacterio.net>) (Euzéby, 1997). However, little is known about the microbial diversity and their function in the fermented seafood products, compared with those in fermented dairy products, which have been widely investigated over the past century (Lee *et al.*, 1993; Lee, 1997) even though the use of fermented seafood products, including liquid

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fish sauce and salt-fermented seafood, is currently widespread in Asia. Most of the existing studies with fermented foods emphasized the lactic acid bacteria (LAB) in the fermented food ecosystem because of their unique physiological and metabolic characteristics (Caplice and Fitzgerald, 1999). Bacterial diversity was partially exploited in fermented seafoods, such as fermented fish sauces or fish products, through culture-based approaches (Sands and Crisan, 1974; Crisan and Sands, 1975; Lee, 1997; Paludan-Muller *et al.*, 1999).

Although the composition and dynamics of the microbial populations in fermented foods were shown through culture-dependent methods, recent molecular ecological investigation showed that the microbial communities of most foods are more diverse and complex than originally thought (Fleet, 1999). Thus, the development of more sensitive, quantitative, and culture-independent tools are needed to explore the microbial ecology of fermented foods (Cheigh and Park, 1994; Giraffa and Neviani, 2001) with their theoretical accuracy detouring 'the great plate count anomaly' (Staley and Konopka, 1985). Although molecular methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis, fluorescence *in situ* hybridization, direct epifluorescence technique, terminal restriction fragment length polymorphism, length heterogeneity-PCR, and microarray (Giraffa, 2004) have been widely applied to study food fermentation and given us a lot of new ecological insights without cultivation bias (ben Omar and Ampe, 2000; Ampe *et al.*, 2001; Cocolin *et al.*, 2001; Randazzo *et al.*, 2002; van Beek and Priest, 2002; Lafarge *et al.*, 2004); these approaches do not allow one to comprehensively analyze all microbial populations within multiple samples (Ercolini, 2004). Thus, new additional in-depth analysis method for quantitatively analyzing the involvement of microbial populations in the process of food fermentation is warranted.

Although most of studies have focused on culture-dependent and culture-independent investigation of bacterial diversity, there is still a scarcity of information regarding the archaeal diversity in fermented seafoods. On the other hand, archaeal diversity was investigated in the various marine and terrestrial environments. Recent studies using cultivation-independent methods have uncovered abundant mesophilic crenarchaeal lineages in marine environments that have not yet been cultivated (Teske and Sorensen, 2008). The crenarchaeal lineages currently consist of several groups: the Marine Group I (MG-I), Marine Benthic Group A, B, D (MBG-A, B, D), Miscellaneous Crenarchaeotic Group (MCG), South African Goldmine Euryarchaeotal Group (SAGMEG), Ancient Archaeal Group (AAG), and Marine Hydrothermal Vent Group (MHVG). Some members of MG-I that were affiliated with the Crenarchaeota Group I.1 (CG I.1) (Ochsenreiter *et al.*, 2003) were known as aerobic,

non-extremophilic, ammonia-oxidizing, and autotrophic archaea (Francis *et al.*, 2005; Könneke *et al.*, 2005; Hallam *et al.*, 2006a,b). The ammonia-oxidizing archaea that have ammonia monooxygenase  $\alpha$ -subunit (*amoA*) genes are able to oxidize ammonia into nitrite and are considered to be important to global carbon and nitrogen cycles (Francis *et al.*, 2005; Könneke *et al.*, 2005). Many metagenomic studies have shown that ammonia-oxidizing archaea are abundant and ubiquitous not only in terrestrial ecosystems, such as various soils (Leininger *et al.*, 2006), alkaline sandy loam (Shen *et al.*, 2008), and rhizosphere (Herrmann *et al.*, 2008), but also in aquatic ecosystems, such as marine water columns and sediments (Francis *et al.*, 2005), mucus of corals (Siboni *et al.*, 2008), deep marine sediments (Inagaki *et al.*, 2006), and oceans (Wuchter *et al.*, 2006; Lam *et al.*, 2007; Mincer *et al.*, 2007).

The parallel high throughput pyrosequencing technique, a next-generation DNA sequencing platform based on the detection of pyrophosphate released during nucleotide incorporation, was developed by Margulies *et al.*, (2005) and provides a new way of dramatically reducing the costs associated with sequencing. The pyrosequencing method was applied to microbial ecology, and much more in-depth studies of microbial diversity have been carried out in various ecosystems such as deep mines (Edwards *et al.*, 2006), soil (Roesch *et al.*, 2007), deep marine biospheres (Huber *et al.*, 2007), chronic wounds (Dowd *et al.*, 2008a), tidal flats (Kim *et al.*, 2008), and human oral microflora (Keijsers *et al.*, 2008). Although pyrosequencing produces a very large number of reads from a single run, it is not appropriate to analyze multiple samples at a time. Recently, to overcome the limitation in the number of samples that can be sequenced in parallel, sample-specific key sequences called 'barcode' or 'tag' consisting of smaller sequences of two or four nucleotides (Binladen *et al.*, 2007; Hoffmann *et al.*, 2007; Kasschau *et al.*, 2007) were adapted for use with the pyrosequencing platform. The sample-specific barcode system using 8 or 10 nucleotides was subsequently developed for pyrosequencing analyses so that more samples could be encoded with greater accuracy (Parameswaran *et al.*, 2007; Dowd *et al.*, 2008b; Hamady *et al.*, 2008). The multiplex barcoded pyrosequencing analysis enables us to analyze an increased number of samples at a time, to reduce the cost per sample, and to perform in-depth analyses for studies of comparative microbial ecology (Binladen *et al.*, 2007; Hoffmann *et al.*, 2007; Kasschau *et al.*, 2007; Parameswaran *et al.*, 2007; Dowd *et al.*, 2008b; Hamady *et al.*, 2008). Along with the modified pyrosequencing approaches, efficient and automated bioinformatics pipeline were also used to achieve consistent, rapid, and accurate taxonomic assignments from the 16S rRNA sequence reads (Liu *et al.*, 2008).

As far as we know, no studies have been carried out with the in-depth sequencing approach to evaluate bacterial, and especially archaeal, populations of food ecosystems associated with fermented products. Therefore, we focused our study on the microbial communities of the *Archaea* and *Bacteria* domains in fermented seafood products, which is a food ecosystem that is understudied by microbial ecologists. The archaeal and bacterial diversities in seven representative (best selling) kinds of fermented seafood were evaluated with a culture-independent approach. Results of the small subunit ribosomal rRNA (16S rRNA) gene sequencing analysis using multiplex barcoded pyrosequencing (Liu *et al.*, 2008) with uniquely tagged multiple amplicons in a single run were compared with the results obtained with the widely used PCR-DGGE fingerprinting method. The multiplex barcoded pyrosequencing technique enabled us to perform a comparative microbial ecological study by thoroughly analyzing several samples with reduced costs per sample.

## Materials and methods

### Sampling and DNA extraction

The seven kinds of seafood samples were purchased from the distributors of commercially available brands in Korea. These foods were made of the following raw materials: tiny shrimp (designated J1), shellfish (J2), cuttlefish (J3), oysters (J4), roe of pollack (J5), tripe of pollack (J6), or crab (J7). The bulk community DNAs were extracted separately from 1 ml volume of single samples of each seafood type, obtained just after they were opened, using the bead-beating method (Yeates *et al.*, 1998). All DNA samples were purified and quantified as described earlier (Bae *et al.*, 2005).

### PCR amplifications for pyrosequencing

The bulk community DNA sequences were amplified individually with universal primer pairs for the hypervariable V3 region of the 16S rRNA gene (Ashelford *et al.*, 2005). For *Archaea*, nested PCR amplification was performed using the arch20f-958r primer set (DeLong, 1992; DeLong *et al.*, 1999) for the first round of amplification, followed by the arch344f (Casamayor *et al.*, 2002)-519r (Ovreas *et al.*, 1997) primer set for the second round of amplification because some samples were not successfully amplified with the direct archaeal-specific primer set. For *Bacteria*, direct PCR amplification was performed using the 341f (Watanabe *et al.*, 2001)-518r (Muyzer *et al.*, 1993) primer set. All PCR amplifications were performed using the PCR mix (Maxime PCR PreMix Kit, iNtRON Biotechnology, Korea), supplemented with 1  $\mu$ l (50 ng  $\mu$ l<sup>-1</sup>) template DNA and 20 pmol of each primer, using a PTC-220 DNA Engine Dyad MJ

**Table 1** Sample information and multiplex identifier sequences used for the seven kinds of fermented seafood during the barcoded pyrosequencing analysis

Sample	NaCl (% w/v)	pH	Multiplex identifier
J1 (tiny shrimp)	32.8	7.0	ATATCGCGAG
J2 (shellfish)	36.0	5.0	CGTGTCTCTA
J3 (cuttlefish)	7.0	3.8	CTCGCGTGTC
J4 (oyster)	14.2	4.5	TAGTATCAGC
J5 (roe of pollack)	4.6	6.7	TCTCTATGCC
J6 (tripe of pollack)	5.0	4.0	TGATACGTCT
J7 (crab)	16.4	3.6	TACTGAGCTA

Research thermalcycler (PharmaTech, Seoul, Korea). The PCR conditions were as follows: 94 °C for 1 min; 30 cycles of denaturation (94 °C; 1 min), annealing (60 °C; 0.5 min), and extension (72 °C; 0.5 min); followed by the final elongation (72 °C; 10 min). The only exceptions were that the initial rounds of annealing (60 °C; 1 min) and extension (72 °C; 1.5 min) during the amplification process were modified for the archaeal sequences. After the DNA was quantified using a spectrophotometer (Nanodrop Technologies, Rockland, DE, USA), the archaeal and bacterial DNAs from the same sample were mixed in equivalent proportions.

### Pyrosequencing

To create a single-stranded DNA library for the Genome Sequencer FLX system (Roche, Mannheim, Germany), Multiplex Identifiers (MIDs; Roche) were used to label the end fragments of the purified PCR product DNA from the seven samples, according to the manufacturer's instructions. Specifically, the end fragments were blunted and tagged on both ends with one of seven ligation adaptors that contained a unique 10-bp sequence (Table 1) and a short 4-nucleotide sequence (TCAG) called a sequencing key, which were recognized by the system software and the priming sequences. The quality of the single-stranded DNA libraries generated using GS FLX Standard DNA Library Preparation Kit (Roche) was evaluated using DNA 1000 LabChip software with a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). Subsequently, all seven single-stranded DNA libraries with different sample-specific adaptors were pooled in equimolar amounts. The uniquely tagged, pooled DNA samples were immobilized onto DNA capture beads, amplified through emulsion-based clonal amplification (emPCR), and sequenced together in a single region (1 of 16 lanes) of a PicoTiterPlate device on a Genome Sequencer FLX system that has the read length of ~250 bases, according to the manufacturer's instructions (Roche).

### Pyrosequencing data analysis

All sequences were compared against a non-redundant GenBank database constructed in the local BLAST database with BLASTN search (Altschul *et al.*, 1990). Sequences shorter than 100 bp were excluded. The sequences with a greater than 75% identity with the best hit from the BLASTN search and a match length greater than 90% of the query length were selected for further analysis to remove the unspecific or non-rRNA gene sequences. The selected sequences were assigned as *Archaea* or *Bacteria* based on the taxonomic annotation of the sequences for the nearest neighbor from the BLASTN search. Directions of the strands were adjusted properly according to the BLASTN results. The sequences were aligned by the NAST program (DeSantis *et al.*, 2006) in the Greengenes (<http://greengenes.lbl.gov>). The primer sequences were excluded after alignment. The DNA distance matrices were calculated using the DNADIST program in PHYLIP (version 3.68). The matrices were used to define operational taxonomic units with 1% dissimilarity for determination of the coverage percentage by Good's method (Good, 1953), abundance-based coverage estimator (ACE), bias-corrected Chao1 richness estimator, Jackknife estimator of species richness, and the Shannon–Weaver and Simpson diversity indices by the DOTUR program (Schloss and Handelsman, 2005). Rarefaction analysis was also performed by DOTUR program (Schloss and Handelsman, 2005). Good's coverage percentage was calculated as  $[1 - (n/N)] \times 100$ , where  $n$  represents the number of single-member phylotypes and  $N$  represents the number of sequences. To determine the value of dissimilarity, we performed *in silico* modeling using the pre-aligned near full-length 16S rDNA sequence set from the Greengenes database. The 1000 sequences in front of the pre-aligned set were selected as model sequences and the model sequences were trimmed to make two sets of trimmed model sequences simulating the pyrosequencing reads with primers and without primers. The three sets of sequences were analyzed through same procedure as described above. The value of dissimilarity for the trimmed model sequences showing similar results with the 3% dissimilarity for full-length sequences was determined as a criterion to calculate the richness estimators and diversity indices of the jeotgal samples. Clustering analyses of seafood samples and the principal coordinates analysis (PCA) were performed using the UniFrac service (Lozupone *et al.*, 2006). Unique sequences (showing 100% similarity with each other) were selected from the pyrosequencing reads aligned with the NAST program. The neighbor-joining tree was constructed using the MEGA 3.01 (Kumar *et al.*, 2004) program based on the Jukes–Cantor model and used for the UniFrac analysis. The *Haloterrigena* sp. (AM285297) and *Lactobacillus curvatus* (EU855223) strains were used as outgroups for

*Archaea* and *Bacteria*, respectively. The online ribosomal database project classifier (Cole *et al.*, 2007) was used for the taxonomic assignments.

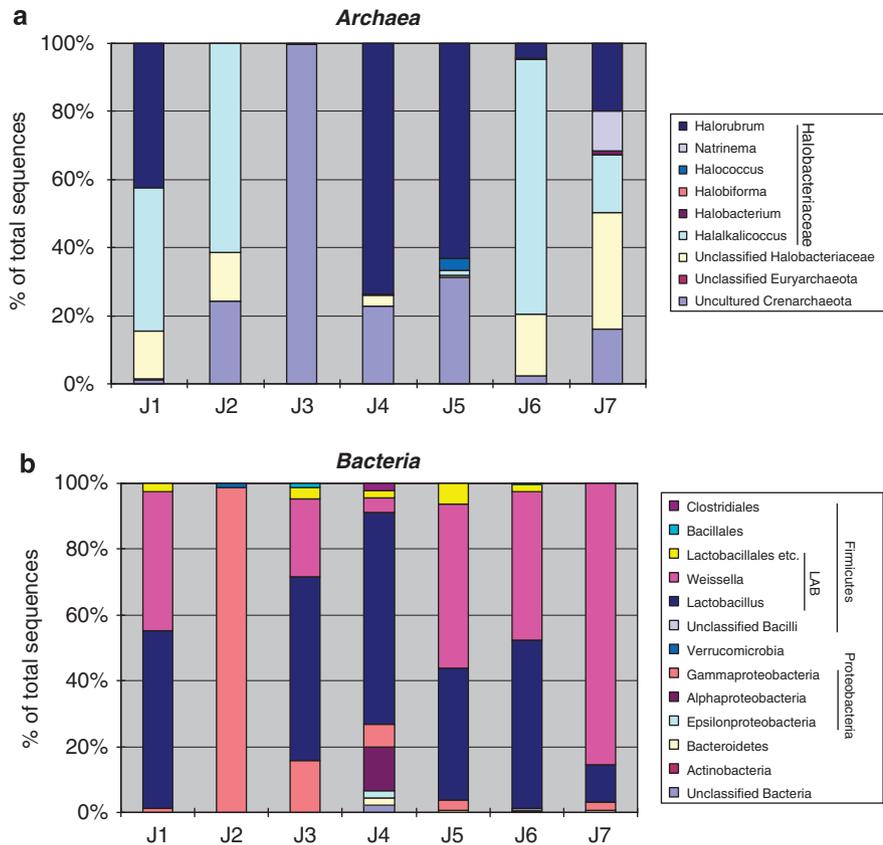
### PCR–DGGE and phylogenetic analysis

To amplify the archaeal 16S rRNA genes for the DGGE analysis, PCR amplifications were performed as described above, but with the following exceptions: archaeal sequences were amplified with the archGC340f-519r primer set (Ovreas *et al.*, 1997) for the second round of amplification as described earlier (Ferris *et al.*, 1996) and bacterial sequences were amplified with the GC338f-518r primer set (Muyzer *et al.*, 1993) as described earlier (Henckel *et al.*, 1999). The DGGE analysis and the sequence analysis of the excised DGGE bands were carried out as described earlier (Chang *et al.*, 2008; Nam *et al.*, 2008). The phylogenetic analysis of the DGGE band sequences was performed as described earlier (Roh *et al.*, 2008).

## Results

### Sequence analysis by pyrosequencing

A total of 15 898 reads were obtained from a single lane of a 16-lane PicoTiterPlate on a Genome Sequencer FLX system. Of these, 13 372 sequences (84%) were identified as archaeal or bacterial sequences. Specifically, 12 035 or 1337 sequences from the 13 372 reads were classified as *Archaea* or *Bacteria*, respectively. Average read lengths were 147 or 182 bases for *Archaea* or *Bacteria*, respectively. The smaller percentage of bacterial sequences (11%) compared with archaeal sequences was not expected because the same amount of archaeal and bacterial DNA was mixed from each sample before preparing the single-stranded DNA library for pyrosequencing. It can be assumed that the biased ratio of the two domains was not only because of an inefficiency in the emPCR technique using different amplicon sizes for *Archaea* and *Bacteria* (147 and 182 bp, respectively), but also because of the impurities or unknown compounds disturbing the accurate measurement of DNA amount. For some of the samples (J3–J7), 9.2–14.6% of the sequences were identified as eukaryotic sequences composed mainly of yeast (*Pichia fabianii* and *Candida tropicalis*) and some chloroplasts. Detection of chloroplasts was expected because plant materials, such as hot pepper or garlic, are used as ingredients in the fermented seafood products and the chloroplasts contain 16S rRNA genes. Coamplification of non-target eukaryotic 18S rDNA by commonly used universal 16S rDNA-targeting PCR primers has been reported and is probably because of the low specificity of the primer sets used in earlier studies (Ampe *et al.*, 1999; Ampe and Miambi, 2000; Huys *et al.*, 2008; Rivas *et al.*, 2004).



**Figure 1** Phylogenetic classification for the pyrosequencing analysis obtained from a ribosomal database project classifier analyses. The fermented seafood sample in each lane is as follows: J1, shrimp; J2, shellfish; J3, cuttlefish; J4, oyster; J5, roe of pollack; J6, tripe of pollack; and J7, crab. (a) *Archaea*, (b) *Bacteria*.

### Microflora in food samples observed with pyrosequencing

The phylogenetic classification of sequences from the seafood samples is summarized in Figure 1. Except for sample J3 (cuttlefish jeotgal; 0.4%), the archaeal sequences primarily belonged to the family Halobacteriaceae of the phylum Euryarchaeota (68.6–98.4%). Fermented seafood samples with high NaCl concentration (J1, J2, J4, J7; more than 10% of NaCl) had a larger number of members from the family Halobacteriaceae, and various members in the class Halobacteria were detected in each fermented seafood sample. Genera *Halorubrum* and *Halalkalicoccus* occurred frequently in most samples. The abundance of genus *Halorubrum* in the archaeal reads was 42.5%, 73.7%, 63.3%, and 19.9% in samples J1, J4, J5, and J7, respectively, and genus *Halalkalicoccus* comprised 42.0%, 61.2%, 74.8%, and 17.0% of samples J1, J2, J6, and J7, respectively. Genus *Halococcus* was a minor component of samples J5 (3.3%) and J6 (0.2%). Genera *Natrinema* (11.8%) and *Halobacterium* (0.9%) were found only in sample J7, whereas genus *Halobiforma* (0.1%) was detected in sample J4 only. A significant number of pyrosequencing reads belonged to the unclassified Halobacteriaceae (14.0%, 14.4%, 18.2%, and 34.4% in samples J1,

J2, J6, and J7, respectively). On the other hand, archaeal sequences classified as uncultured Crenarchaeota were found in all samples: 1.3%, 24.3%, 99.6%, 22.8%, 31.4%, 2.3%, and 16.0% for samples J1, J2, J3, J4, J5, J6, and J7, respectively. Interestingly, sample J3 (cuttlefish) had the greatest percentage of uncultured Crenarchaeota (99.6%) and the smallest percentage of Halobacteriaceae (0.4%) compared to the other samples.

Except for sample J2, most of the pyrosequencing reads (71.1–98.7%) of the bacterial amplicons belonged to the order Lactobacillales, which is related to the LAB. Fermented seafood samples with a low pH (J3, J4, J6, and J7; pH 4.5 or less) contained a larger number of members from the order Lactobacillales. However, only genera *Weissella* and *Lactobacillus*, which are LAB, were major components of the seafood samples. The ratio of the two genera varied among the samples. Specifically, samples J1, J3, J5, and J6 contained a similar proportion of each genus, whereas one genus was more predominant than the other genus in samples J4 and J7. Sample J2 had a significantly different bacterial population than the other samples; this sample contained 98.8% Gammaproteobacteria, which consisted of the order Vibrionales (90.1%), which contained the genus *Salinivibrio* (89.5%), and

**Table 2** Comparison of phylotype coverage and diversity estimation of the 16S rRNA gene libraries from the pyrosequencing analysis

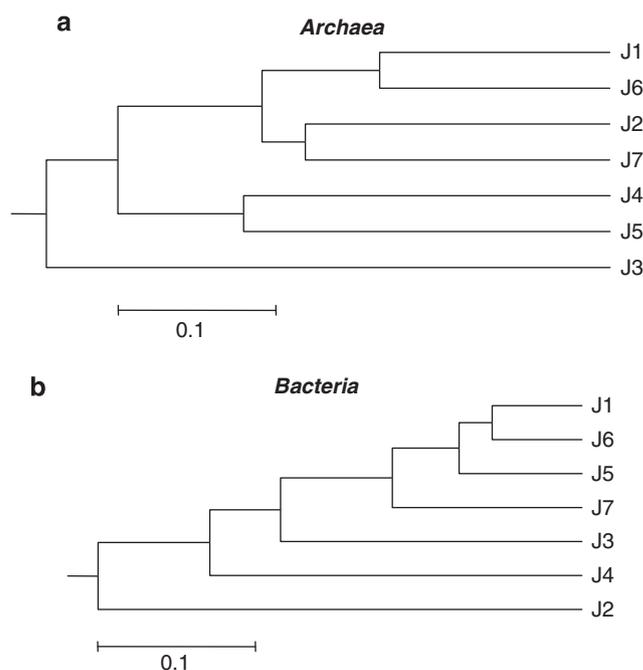
Sample	Reads	OTU <sup>a</sup>	Good <sup>b</sup>	Chao1	ACE	Jackknife	Shannon	Simpson
<i>Archaea</i>								
J1	388	83	79.5	160	193	172	2.59	0.207
J2	2132	282	84.4	561	570	663	2.91	0.277
J3	3258	340	83.5	547	541	672	2.75	0.336
J4	1116	200	78.5	324	367	365	3.17	0.219
J5	788	155	83.2	310	320	388	2.98	0.199
J6	3279	332	83.7	532	522	667	2.75	0.35
J7	1074	269	80.7	540	545	4334	3.95	0.061
<i>Bacteria</i>								
J1	78	8	87.5	11	17	12	1.22	0.367
J2	171	21	100	87	43	67	1.56	0.424
J3	146	18	88.9	30	31	30	1.74	0.3
J4	54	13	92.3	27	40	27	1.92	0.191
J5	129	21	90.5	61	77	67	1.55	0.335
J6	593	56	80.4	88	98	87	1.97	0.275
J7	166	21	85.7	38	47	37	1.32	0.527

<sup>a</sup>The operational taxonomic units (OTU) were defined with 1% dissimilarity.

<sup>b</sup>The coverage percentages (Good), richness estimators (ACE, Chao1, and Jackknife) and diversity indices (Shannon and Simpson) were calculated using Good's method (Good, 1953) and the DOTUR program (Schloss and Handelsman, 2005).

the order Oceanospirillales (7.6%), which contained the family Halomonadaceae (5.8%). Pyrosequencing reads belonging to Gammaproteobacteria were also found in samples J3 and J4, with the percentage of bacterial reads as follows: 15.9% (family Vibrionaceae was a major component) and 6.7% (the family Pseudomonadaceae), respectively. Only sample J4 contained the orders Rhizobiales (11.1%) and Rhodobacterales (2.2%) in Alphaproteobacteria.

To more clearly confirm that the results of our sequencing study was sufficient for ecosystem analysis, the number of operational taxonomic units and the statistical estimates of species richness were determined (see Table 2). From the *in silico* modeling, 1% dissimilarity of trimmed model sequences without primers showed most similar results corresponding to 3% dissimilarity of full-length sequences (Supplementary Table 1SM, 2SM, and 3SM). The number and estimated number of operational taxonomic units were less than the values obtained from other ecosystems such as soil (Schloss and Handelsman, 2006) and tidal flats (Kim *et al.*, 2008). The limited microbial diversity of the seafood samples may be because of the processes of fermentation and storage. Comparisons of the cluster analyses with the phylogenetic tree and the PCA performed using the UniFrac service (Lozupone *et al.*, 2006) showed similar results (Figures 2 and 3). Samples J3 (*Archaea*) and J2 (*Bacteria*) occupied the most separated positions with the clustering analysis and PCA, as expected from their unique taxonomic classifications. Samples J1, J6, and J7 were clustered each other in both cases of *Archaea* and *Bacteria*, whereas samples J2 and J5 showed quite different clustering profiles depending on *Archaea* and *Bacteria*. In-depth comparison of seafood samples requires additional

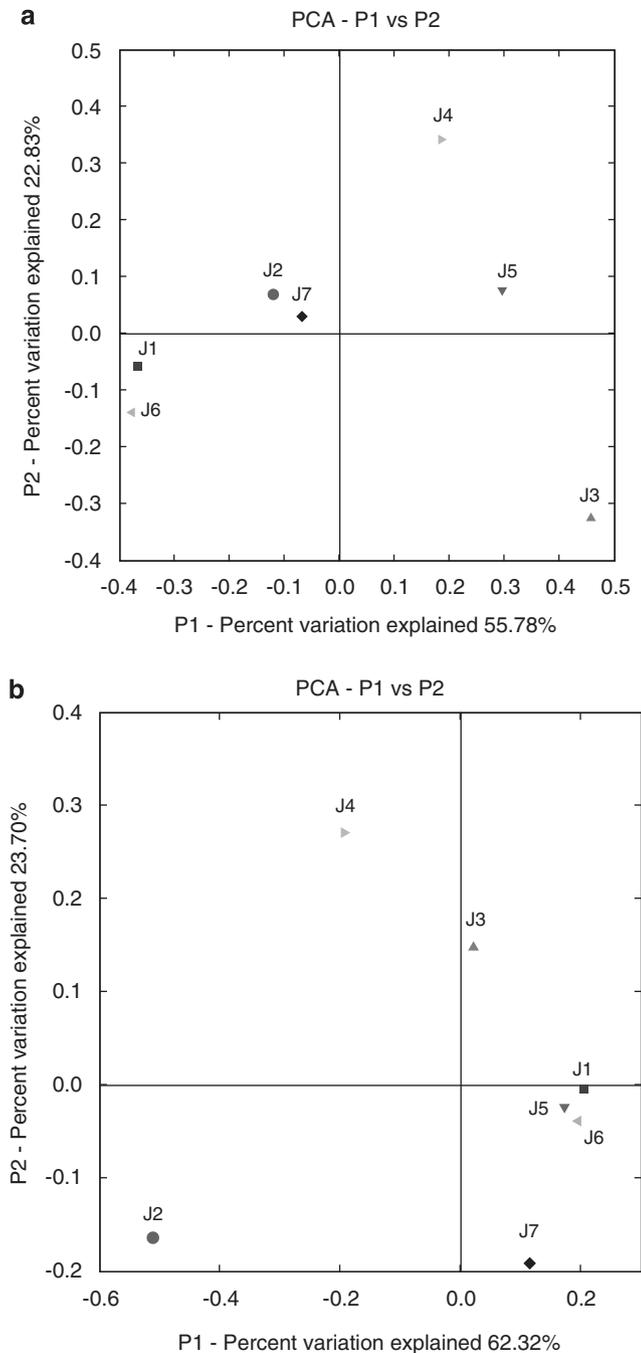


**Figure 2** Clustering analysis for the samples classified as *Archaea* and *Bacteria* using the UniFrac service after pyrosequencing. The scale bar indicates the distance between clusters in UniFrac units. (a) *Archaea*, (b) *Bacteria*.

analysis of the microbial diversity, which depends on the various kinds of seafood samples.

#### PCR-DGGE and phylogenetic analysis

As the DGGE analysis is now the most widely applied for comparative analysis of parallel samples, results of the barcoded pyrosequencing analysis were compared with the findings with DGGE, for which the bulk community DNAs



**Figure 3** PCA of the samples obtained by pyrosequencing, and classified as (a) *Archaea* and (b) *Bacteria* using the UniFrac service.

extracted from the seafood samples were PCR amplified and analyzed and 49 of the 16S rRNA gene sequences were obtained after the bands of interest were excised from the DGGE lanes, reamplified, and sequenced. Archaeal and bacterial DNA DGGE profiles were significantly different from one another and varied with the seafood samples, as shown in Figure 4. However, based on the DGGE analysis, we could not quantitatively compare the bacterial and archaeal populations in the fermented seafood. Only the sequencing results for the 23

archaeal and 26 bacterial DGGE bands (labeled with a number followed by A or B for *Archaea* or *Bacteria*, respectively) were summarized in Tables 3 and 4; the results were compared with sequences found in the GenBank and REF 16S databases (Chun *et al.*, 2007). Among the retrieved archaeal sequences (Table 3), 14 of the 23 archaeal DGGE bands (61%) were related to extremely halophilic archaea belonging to the family Halobacteriaceae in the phylum Euryarchaeota; 9 bands (39%) were affiliated with the uncultured Crenarchaeota clones that had  $\leq 83.3\%$  similarity of their 16S rRNA gene sequence with valid species of the hyperthermophilic Crenarchaeota that belong to genus *Sulfolobus*, *Thermocodium*, or *Pyrobaculum* in class Thermoprotei. Table 4 shows that 13 of the 26 bacterial bands (50%) corresponded to the LAB group affiliated with genus *Lactobacillus* or *Weissella* in phylum Firmicutes; 11 bands (42%) belonged to the class Gammaproteobacteria, such as genus *Salinivibrio*, *Vibrio*, *Cobetia*, or *Psychrobacter*; and 2 bands (8%) were affiliated with the uncultured environmental clones within the domain of *Bacteria*.

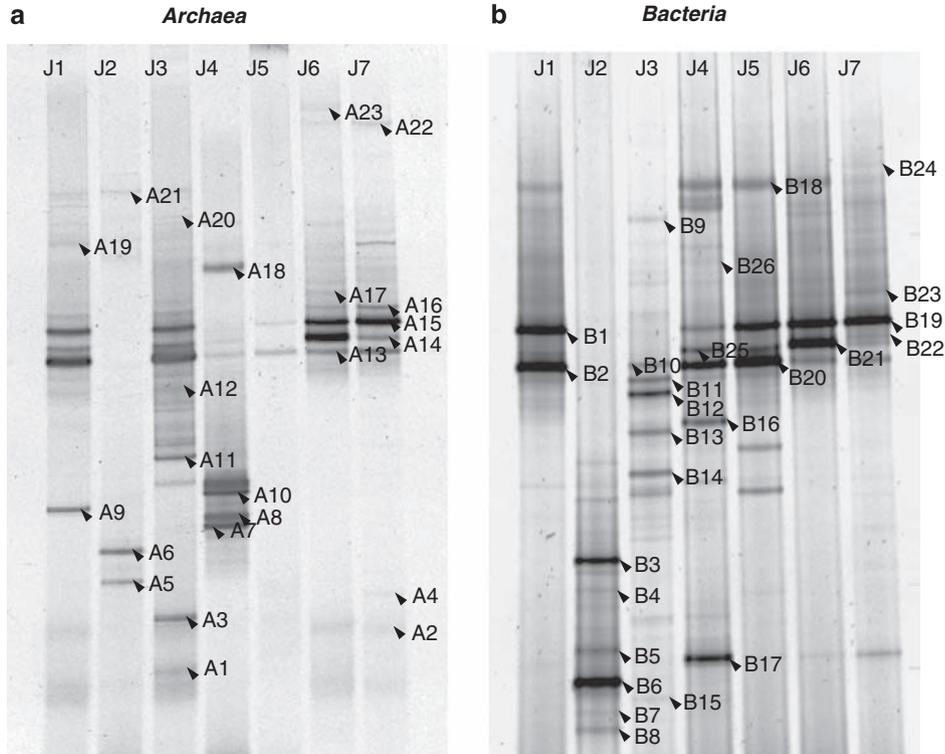
#### Comparison of pyrosequencing with DGGE analysis

The phylotypes detected in the seven samples with the two molecular analyses, pyrosequencing and PCR-DGGE, generally matched each other with respect to non-detection of genera *Halorubrum*, *Lactobacillus*, and *Weissella* and detection of genera *Halalkalicoccus* and *Salinivibrio* in sample J2; and detection of LAB or unclassified bacterial groups in all samples, except sample J2 or sample J4, respectively. However, the archaeal and bacterial communities determined with the pyrosequencing analysis were more diverse than those communities determined with the PCR-DGGE analysis, as there were some phylotypes revealed with the pyrosequencing analysis but not with PCR-DGGE. For example, the sequence reads belonging to uncultured Crenarchaeota, unclassified Halobacteriaceae, several Gammaproteobacteria groups in sample J2 and the minor bacterial groups (excluding LAB) in samples J1, J5, J6, and J7 were shown only with the pyrosequencing analysis. One disadvantage of DGGE fingerprinting is that there is a limit for detecting bands in the DGGE profiles of complex communities (Ercolini, 2004), which may not show minor archaeal and bacterial populations in samples. However, pyrosequencing provides an in-depth analysis of the 16S rRNA gene sequences, which can compensate for the disadvantage with the PCR-DGGE method in detecting minor populations in seafood samples.

## Discussion

#### Application of barcoded pyrosequencing to evaluate food microbial diversity

As the costs are much lower than for conventional methods, such as PCR-cloning, DGGE, so on, and the



**Figure 4** DGGE profiles of the PCR-amplified 16S rDNA segments from the archaeal and bacterial samples. The band labels in this figure correspond to the band labels indicated in Tables 3 and 4. **(a) Archaea, (b) Bacteria.**

**Table 3** Identification of the archaeal 16S rRNA sequences of the DGGE bands obtained from the seven kinds of fermented seafood

Band <sup>a</sup>	Closest GenBank match	Identity	Closest type strain <sup>b</sup>	Identity
A1	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.1	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.1
A2	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.1	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.1
A3	Uncultured archaeon clone SA93 (EU722674)	100.0	<i>Halomicrobium mukohataei</i> JCM 9738 <sup>T</sup> (EF645691)	99.1
A4	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	100.0	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.0
A5	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	100.0	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.0
A6	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.1	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.1
A7	Uncultured crenarchaeote clone b11 (EU123492)	97.1	<i>Thermocladium modestius</i> IC-125 <sup>T</sup> (AB005296)	74.3
A8	Uncultured crenarchaeote clone A1RNA (EU283416)	98.9	<i>Pyrobaculum aerophilum</i> IM2 <sup>T</sup> (L07510)	74.4
A9	Uncultured archaeon clone CEHLW-A36 (FJ155652)	99.0	<i>Halorhabdus utahensis</i> AX-2 <sup>T</sup> (AF071880)	90.8
A10	Uncultured crenarchaeote clone b11 (EU123492)	98.0	<i>Thermocladium modestius</i> IC-125 <sup>T</sup> (AB005296)	74.8
A11	Uncultured archaeon clone CEHLW-A64 (FJ155656)	99.0	<i>Sulfolobus shibatae</i> DSM 5389 <sup>T</sup> (M32504)	79.8
A12	Uncultured archaeon clone CEHLW-A64 (FJ155656)	99.0	<i>Sulfolobus shibatae</i> DSM 5389 <sup>T</sup> (M32504)	79.6
A13	Uncultured archaeon clone CEHLW-A64 (FJ155656)	100.0	<i>Sulfolobus shibatae</i> DSM 5389 <sup>T</sup> (M32504)	83.3
A14	<i>Halorubrum luteum</i> CGSA15 <sup>T</sup> (DQ987877)	98.3	<i>Halorubrum luteum</i> CGSA15 <sup>T</sup> (DQ987877)	98.3
A15	Uncultured archaeon clone CEHLW-A64 (FJ155656)	100.0	<i>Sulfolobus yangmingensis</i> IFO 15161 <sup>T</sup> (AB010957)	79.5
A16	<i>Halorubrum lipolyticum</i> 9-3 <sup>T</sup> (DQ355814)	99.0	<i>Halorubrum lipolyticum</i> 9-3 <sup>T</sup> (DQ355814)	99.0
A17	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	100.0	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.0
A18	Uncultured crenarchaeote clone b11 (EU123492)	100.0	<i>Thermocladium modestius</i> IC-125 <sup>T</sup> (AB005296)	73.0
A19	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	100.0	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.0
A20	Uncultured archaeon clone CEHLW-A64 (FJ155656)	99.0	<i>Sulfolobus shibatae</i> DSM 5389 <sup>T</sup> (M32504)	77.8
A21	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	100.0	<i>Halalkalicoccus jeotgali</i> B3 <sup>T</sup> (EF077632)	99.0
A22	<i>Halorubrum</i> sp. YYJ21 (EU661608)	98.9	<i>Halorubrum saccharovororum</i> JCM 8865 <sup>T</sup> (U17364)	97.8
A23	<i>Halovivax ruber</i> XH-70 <sup>T</sup> (AM269467)	93.5	<i>Halovivax ruber</i> XH-70 <sup>T</sup> (AM269467)	92.6

<sup>a</sup>The position of each band is shown in Figure 4.

<sup>b</sup>Data from the REF 16S database, EzTaxon (www.eztaxon.org) (Chun et al., 2007).

comprehensive analysis of the microbial diversity of foods is needed, barcoded pyrosequencing could be applied to routine analysis of the microbial diversity of various foods and replace conventional cloning

analyses that are conducted to acquire hundreds of sequences. Although the pyrosequencing read lengths of the sequences were significantly short compared with the sequences obtained with

**Table 4** Identification of the bacterial 16S rRNA sequences of the DGGE bands obtained from seven kinds of fermented seafood

Band <sup>a</sup>	Closest GenBank match	Identity	Closest type strain <sup>b</sup>	Identity
B1	<i>Weissella paramesenteroides</i> NRIC 1542 <sup>T</sup> (AB023238)	99.3	<i>Weissella paramesenteroides</i> NRIC 1542 <sup>T</sup> (AB023238)	99.3
B2	<i>Lactobacillus graminis</i> DSM 20719 <sup>T</sup> (AM113778)	100.0	<i>Lactobacillus graminis</i> DSM 20719 <sup>T</sup> (AM113778)	100.0
B3	<i>Cobetia</i> sp. JO1 (EU909460)	99.3	<i>Cobetia marina</i> DSM 4741 <sup>T</sup> (AJ306890)	96.5
B4	<i>Salinivibrio costicola</i> subsp. <i>Costicola</i> NCIMB 701 <sup>T</sup> (X95527)	100.0	<i>Salinivibrio costicola</i> subsp. <i>Costicola</i> NCIMB 701 <sup>T</sup> (X95527)	99.3
B5	<i>Salinivibrio costicola</i> subsp. <i>Costicola</i> NCIMB 701 <sup>T</sup> (X95527)	100.0	<i>Salinivibrio costicola</i> subsp. <i>Costicola</i> NCIMB 701 <sup>T</sup> (X95527)	99.3
B6	<i>Salinivibrio costicola</i> subsp. <i>Costicola</i> NCIMB 701 <sup>T</sup> (X95527)	100.0	<i>Salinivibrio costicola</i> subsp. <i>Costicola</i> NCIMB 701 <sup>T</sup> (X95527)	99.3
B7	<i>Salinivibrio costicola</i> subsp. <i>Costicola</i> NCIMB 701 <sup>T</sup> (X95527)	98.0	<i>Salinivibrio costicola</i> subsp. <i>Costicola</i> NCIMB 701 <sup>T</sup> (X95527)	99.3
B8	<i>Salinivibrio costicola</i> subsp. <i>Costicola</i> NCIMB 701 <sup>T</sup> (X95527)	97.4	<i>Salinivibrio costicola</i> subsp. <i>Costicola</i> NCIMB 701 <sup>T</sup> (X95527)	98.7
B9	<i>Psychrobacter</i> sp. OTUC8 (FJ210849)	98.6	<i>Psychrobacter immobilis</i> DSM 7229 <sup>T</sup> (AJ309942)	99.3
B10	<i>Lactobacillus delbrueckii</i> subsp. <i>Indicus</i> NCC725 <sup>T</sup> (AY421720)	99.3	<i>Lactobacillus delbrueckii</i> subsp. <i>Indicus</i> NCC725 <sup>T</sup> (AY421720)	99.3
B11	<i>Lactobacillus delbrueckii</i> subsp. <i>Delbrueckii</i> ATCC 9649 <sup>T</sup> (AY050172)	100.0	<i>Lactobacillus delbrueckii</i> subsp. <i>Delbrueckii</i> ATCC 9649 <sup>T</sup> (AY050172)	98.1
B12	<i>Psychrobacter</i> sp. OTUC8 (FJ210849)	99.3	<i>Psychrobacter cibarius</i> JG-219 <sup>T</sup> (AY639871)	100.0
B13	Uncultured bacterium clone 1103200832100 (EU845582)	98.7	<i>Psychrobacter cibarius</i> JG-219 <sup>T</sup> (AY639871)	99.3
B14	<i>Psychrobacter okhotskensis</i> MD17 <sup>T</sup> (AB094794)	100.0	<i>Psychrobacter okhotskensis</i> MD17 <sup>T</sup> (AB094794)	100.0
B15	<i>Vibrio</i> sp. JG 07 (EU937751)	100.0	<i>Photobacterium profundum</i> DSJ4 <sup>T</sup> (D21226)	94.5
B16	Uncultured spirochetes clone LH020 (AY605168)	91.3	<i>Oceanicaulis alexandrii</i> C116-18 <sup>T</sup> (AJ309862)	86.2
B17	Uncultured organism clone SIPclo_KH44 (EU450408)	99.2	<i>Anabaena cylindrica</i> NIES19 (PCC7122) <sup>T</sup> (AF091150)	83.5
B18	Uncultured bacterium DGGE gel band C-3_DFM_f (EF622465)	98.7	<i>Lactobacillus sakei</i> subsp. <i>Carnosus</i> CCUG 31331 <sup>T</sup> (AY204892)	99.3
B19	<i>Weissella paramesenteroides</i> NRIC 1542 <sup>T</sup> (AB023238)	99.3	<i>Weissella paramesenteroides</i> NRIC 1542 <sup>T</sup> (AB023238)	99.3
B20	Uncultured bacterium DGGE gel band C-3_DFM_f (EF622465)	100.0	<i>Lactobacillus graminis</i> DSM 20719 <sup>T</sup> (AM113778)	99.3
B21	<i>Lactobacillus sakei</i> strain FC99 (EU161990)	99.3	<i>Lactobacillus sakei</i> subsp. <i>Carnosus</i> CCUG 31331 <sup>T</sup> (AY204892)	100.0
B22	<i>Weissella paramesenteroides</i> NRIC 1542 <sup>T</sup> (AB023238)	99.3	<i>Weissella paramesenteroides</i> NRIC 1542 <sup>T</sup> (AB023238)	100.0
B23	<i>Weissella paramesenteroides</i> NRIC 1542 <sup>T</sup> (AB023238)	100.0	<i>Weissella paramesenteroides</i> NRIC 1542 <sup>T</sup> (AB023238)	99.4
B24	<i>Weissella paramesenteroides</i> NRIC 1542 <sup>T</sup> (AB023238)	100.0	<i>Weissella paramesenteroides</i> NRIC 1542 <sup>T</sup> (AB023238)	100.0
B25	Uncultured bacterium DGGE gel band C-3_DFM_f (EF622465)	100.0	<i>Lactobacillus graminis</i> DSM 20719 <sup>T</sup> (AM113778)	100.0
B26	Uncultured bacterium DGGE gel band C-3_DFM_f (EF622465)	95.8	<i>Lactobacillus graminis</i> DSM 20719 <sup>T</sup> (AM113778)	99.3

<sup>a</sup>The position of each band is shown in Figure 4.

<sup>b</sup>Data from the REF 16S database, EzTaxon ([www.eztaxon.org](http://www.eztaxon.org)) (Chun et al., 2007).

traditional Sanger sequencing methods (600–800 bp), these short sequences were long enough to enable classification of the bacterial genera. A systematic evaluation of the accuracy of the classifications, according to the sequence lengths, was carried out earlier and showed that the average read lengths of 80–100 bases provided not only excellent coverage, but also excellent recovery for classification at the genus level (Liu et al., 2008). In addition, 59 base pairs in highly variable region V6 of the 16S rRNA gene sequences were sufficient for phylogenetic affiliation (Andersson et al., 2008). Therefore, the average lengths of the pyrosequencing reads in this study were also thought to be sufficient for assigning the taxa, provided that a 1-bp difference in the 16S rRNA gene sequences differentiated the

reads by 0.55% for *Bacteria* and 0.68% for *Archaea*. This resolution was at the subspecies level because archaeal and bacterial species are demarcated by Stackebrandt and Goebel's 97% rule (different species share more than 3% of their 16S rRNA identity) (Stackebrandt and Goebel, 1994).

#### Determination of richness estimators and diversity indices

From the results of *in silico* modeling, 1% dissimilarity of short (100–140 bp) sequences was corresponding to 3% similarity to full-length sequences, in which diversity indices showed similar values to each other (Supplementary Table 1SM, 2SM, and 3SM). As dissimilarity criterion was changed according to the length of pyrosequencing reads (our data)

and the region within the 16S rDNA gene (Andersson *et al.*, 2008), the dissimilarity criterion must be determined before calculation of diversity indices.

Most rarefaction curves of the samples were not saturated. The curves were not saturated even if more 3000 sequences were retrieved (J3 and J6; Supplementary Figure 1SM). Roesch *et al.* (2007) reported that bacterial rarefaction curves were not saturated up to 5000–10 000 sequences in some soil samples. It needs more sequencing effort to obtain the saturation of rarefaction curves. We performed re-sampling analyses adjusting the sample sizes as same as that of the smallest sample to compare the diversity indices in same condition as described in the next section.

#### *Comparison of diversities of Archaea and Bacteria in seven kinds of fermented seafood*

Generally, most of archaeal diversities have been reported to be lower than bacterial diversities. In only 5 libraries among 173 libraries surveyed from published reports, archaeal diversity is higher than bacterial diversity in the same environment (Aller and Kemp, 2008). In the case of this study, archaeal diversity was always higher than bacterial diversity in the same food sample based on the diversity indices and richness estimators except for Good and Simpson index (Table 2). Chao1, ACE, Jackknife richness estimator, and Shannon indices of archaea were always higher than those of bacteria in the same samples. As richness estimators such as Chao1 and ACE were correlated positively with the number of sequences analyzed (Schloss and Handelsman, 2005), we performed a re-sampling analysis. We selected same number of sequences (as same as the number of the bacterial sample J4 that have the smallest number of sequences) randomly 100 times per each jeotgal sample and calculated the diversity indices of the 100 sub-samples. Average and standard deviation values of the estimators and indices according to jeotgal samples were calculated. Although the richness estimators and Shannon index became lower than compared with original samples with large number of sequences, the values of archaeal samples were still higher than those of bacterial samples (Supplementary Figure 2SM).

It is not surprising that the dominant bacterial populations in the seafood samples contained LAB because the food fermentation process is largely dependent on LAB (Caplice and Fitzgerald, 1999).

Also expected was the finding that the *Archaea* in the seafood samples were characterized by extremely halophilic archaea that are phylogenetically affiliated to the family Halobacteriaceae, which inhabit environments saturated or nearly saturated with salt. As seafood samples have high salt concentrations, several extremely halophilic *Archaea* (GenBank accession number EF077631-EF077641) had already been isolated and two novel species, *Halalkalicoccus jeotgali* and *Natronococcus jeotgali*, were validated in earlier studies through culture-dependent methods with another seafood sample (Roh *et al.*, 2007a,b).

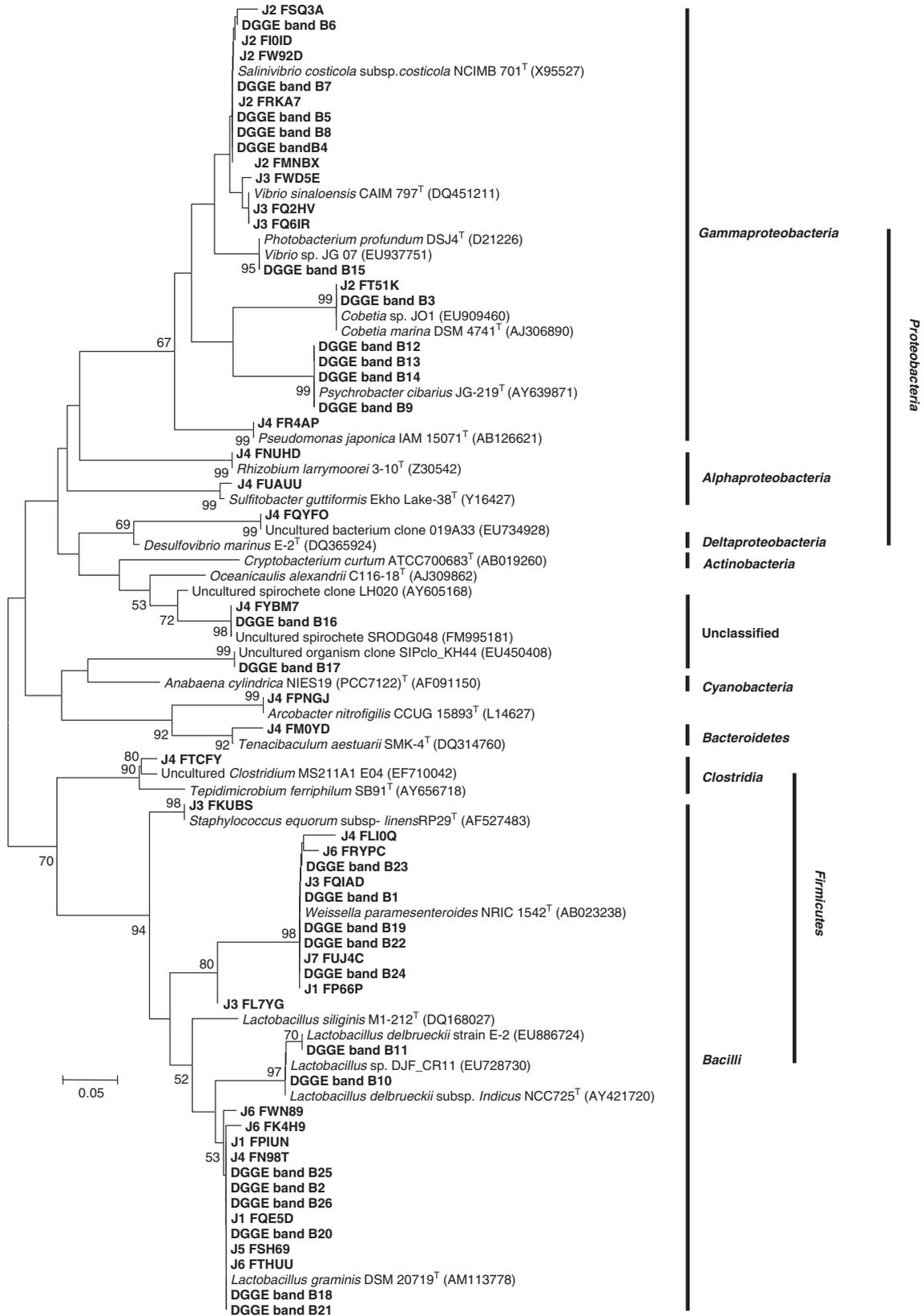
Samples J2 and J3 had significantly different bacterial and archaeal communities, respectively. Although raw materials of food samples analyzed were quite different: for example, shellfish for sample 2 and cuttlefish for sample J3, there was nothing peculiar on manufacturing process and fermentation conditions. The significant differences of archaeal and bacterial communities can be considered owing only to the raw materials of food samples.

#### *Unexpected occurrence of uncultivated mesophilic Crenarchaeota groups from fermented seafood*

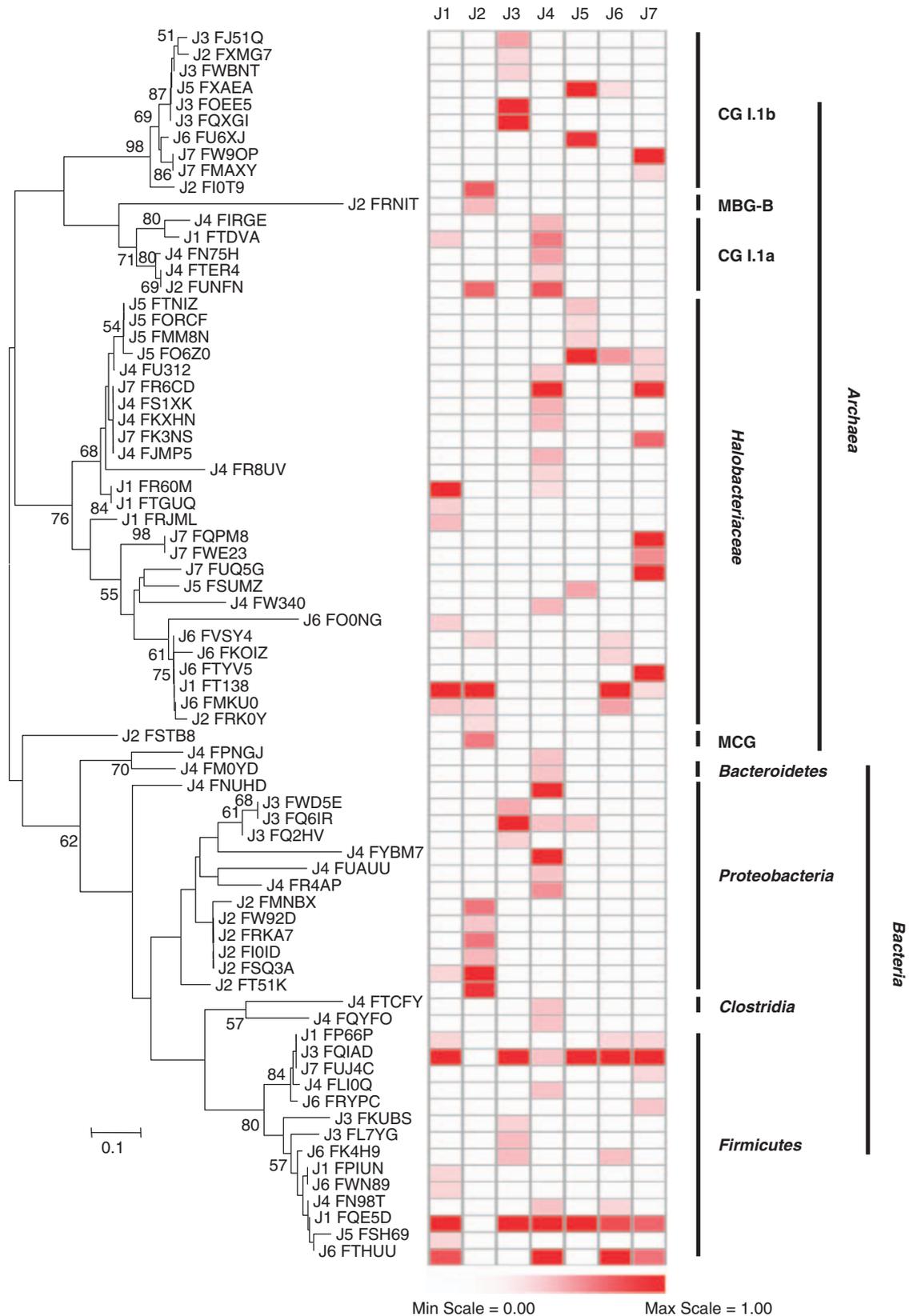
To clearly analyze the archaeal diversity in food samples, phylogenetic trees were constructed with the 16S rRNA gene sequences retrieved from the archaeal DGGE bands and representative pyrosequencing reads selected from each group of operational taxonomic units that were clustered within a DNA distance of 0.03 by the DOTUR program (Figures 5, 6, and 7). It is interesting to note that there are numerous phylogenetic lineages of uncultured crenarchaea in fermented seafoods. The most abundant crenarchaeal sequences obtained from the pyrosequencing and DGGE approaches fell into the clades of the uncultivated mesophilic Crenarchaeota lineages of CG I.1a, CG I.1b, MBG-B, and MCG. This result proved that uncultured mesophilic Crenarchaeota groups are as ubiquitous in fermented seafood ecosystems, as in terrestrial and aquatic niches. Detection of diverse uncultured mesophilic Crenarchaeota groups in food ecosystems was unexpected because the existence of these Crenarchaeota groups has not been reported earlier in any fermented foods. The existence of the Crenarchaeota groups in seafood may be assumed from the fact that the seafood samples were made of fish or shellfish from marine environments in which mesophilic Crenarchaeota groups are enormously

**Figure 5** Archaeal phylogenetic tree showing the relationship among the DGGE bands along with representative reads from the pyrosequencing analysis. DGGE bands and representative pyrosequencing reads are indicated by bold-faced type. Representative pyrosequencing reads were selected from each group of the operational taxonomic units clustered within a DNA distance of 0.03 by the DOTUR program. Sequences that appeared more than 1% in a sample were included as representative sequences. Neighbor-joining tree was constructed with the representative and reference sequences. The Juke–Cantor method was used to calculate the distance matrix. Numbers at the nodes indicate bootstrap values calculated from the neighbor-joining probabilities (expressed as a percentage of 1000 replications). Bootstrap values greater than 50% are shown at the branch points. The scale bar represents 0.05 accumulated changes per nucleotide.





**Figure 6** Bacterial phylogenetic tree showing the relationship among the DGGE bands along with representative reads from the pyrosequencing analysis. DGGE bands and representative pyrosequencing reads are indicated by bold-faced type. Representative pyrosequencing reads were selected and neighbor-joining tree was constructed as described in Figure 5. Bootstrap values greater than 50% are shown at the branch points. The scale bar represents 0.05 accumulated changes per nucleotide.



**Figure 7** Phylogenetic tree and the quantities of the representative reads derived from the pyrosequencing analysis for each sample. Construction of the phylogenetic tree was based on a neighbor-joining algorithm. Each row denotes the percentage of the representative reads for each sequence. For each sample, the percentage of the representative reads of each sequence was visualized by ArrayColor.exe (<http://microbecol.khu.ac.kr>), which produces a range of colors, from white to red, to indicate the prevalence of each sequence in each sample. White squares represent low values, whereas red squares represent high values. The scale bar indicates 0.1 accumulated changes per nucleotide.

abundant. Further studies of the uncultured mesophilic Crenarchaeota groups in fermented food will be necessary to understand the ecological role of Crenarchaeota in the food fermentation process. This new insight into the archaeal community structure in fermented food may provide fundamental information for future investigations.

## Conclusions

In this study, the microbial diversity in fermented seafood was evaluated with high-throughput multiplex barcoded pyrosequencing. The results elucidated that archaeal populations are more diverse in food samples than we expected, providing novel insights into the archaeal populations in fermented foods and confirming that the barcoded pyrosequencing approach can be a powerful tool for characterizing the microbiota in fermented food ecosystems compared with classical molecular ecological approaches, such as PCR–DGGE. The barcoded pyrosequencing method will be of value in the area of food microbiology and will allow for a better understanding of food ecosystems, in which fermented food processing can be influenced by diverse archaeal and bacterial microorganisms.

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