

Analysis of bacterial community structure in *Saba-Narezushi* (*Narezushi* of Mackerel) by 16S rRNA gene clone library

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Abstract *Narezushi*, a derivation of *sushi*, is a traditional Japanese food made by fermenting salted fish meat and cooked rice together. In this study, the microbial diversity of *saba-narezushi* (*narezushi* of mackerel, *Scomber japonicus*) was analyzed by the 16S ribosomal RNA gene clone library method. Chemical composition was also analyzed to compare with different kinds of *narezushi*. The chemical composition of the *narezushi* was similar to those obtained from *samma-narezushi*. Ninety-four clones were randomly selected and DNA sequences of cloned fragments (approx. 890 bp) were analyzed. The DNA sequences obtained were phylogenetically analyzed. The expected operational taxonomy units (OTUs) by Chao1 estimates and Shannon-Wiener index (H') at 97% identity threshold were 48 and 1.822, respectively. The sequence similarity of the cloned fragment was equal to or higher than 98% of the sequence of cultivated bacterial species in the public database. Most of the clones (85%) belonged to lactic acid bacteria (LAB). *Lactobacillus curvatus* was the most abundant species followed by *Lactococcus piscium* and *Leuconostoc gasicomitatum*, suggesting that these bacteria play important roles in the fermentation of *saba-narezushi*.

Keywords 16S rDNA clone library · Fermented food · Lactic acid bacteria (LAB) · Mackerel (*Scomber japonicus*) · *Narezushi*

Introduction

Fish proteins have an excellent essential amino acid composition, which is widely recommended for a well balanced and healthy diet (Zhang et al. 2010). Fermented fish foods are popular in many regions of Southeast and Northeast Asia (Ishige 1993). In this preparation, fish is processed as a preserved food mainly by lactic acid fermentation. This process allows a longer preservation period for perishable proteinaceous food by lowering the pH, and also allows transporting the product to large distances (Cooke et al. 1993). This type of food is also found in Japan and is called *narezushi*. *Narezushi* is made by fermenting salted fish meat with cooked rice for a certain period. The prototypical *narezushi* has long fermentation periods. In Japan, this type of *narezushi* is fermented for several months (Isobe et al. 2002; Itou et al. 2006). The fermentation period of *narezushi* was reduced during the history of the development of this food product. Currently, *narezushi* is fermented from 5 d to 2 months in various regions of Japan. In Mie Prefecture, Japan, several *narezushi* of various kinds of fish, such as *narezushi* of *samma* (saury, *Cololabis saira*) (Matsui et al. 2008), *ayu* (sweetfish, *Plecoglossus altivelis altivelis*), *konoshiro* (spotted sardine, *Konosirus punctatus*), and *saba* (mackerel, *Scomber japonicus*), are produced as ritual or celebratory foods. Since *narezushi* is empirically known to be an intestinal regulator, it is recognized as a functional food as well as culturally important food in the region where it is consumed.

Analysis of microbial ecology by the culture-dependent method is time-consuming and laborious. Because vast majority of microorganisms are uncultivable, this has been a considerable handicap to the microbial ecology (Head et al. 1998). Recent advances in molecular biology enable us

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to use genetic information for the analysis of microbial communities. The PCR-derived clone library method using 16S ribosomal RNA gene (16S rDNA) is often used as the first step in the analysis of unknown microbial communities in various environments, such as the digestive tract of humans (Hold et al. 2002), aquatic and terrestrial biospheres (Bernhard et al. 2005; Chan et al. 2006), and fermented foods (Abriouel et al. 2008; Kim and Chun 2005). The advantage of the PCR-derived clone library method over the culture dependent method is ability to uncultivated microorganisms. The exhaustive analysis of microbial diversity can be achieved by the PCR-derived clone library method. This is advantageous to other culture-independent methods such as denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP) or fluorescent in situ hybridization (FISH). To date, studies on the diversity of microorganisms involved in the fermentation of *narezushi* have been done using the culture-dependent technique (Fujii et al. 1992; Isobe et al. 2002). Recently, we have published a microbial diversity analysis of *samma-narezushi* (*narezushi* of saury) by using the 16S rDNA clone library technique. We also demonstrated the usefulness of this technique in analyzing microbial diversity in *narezushi* (Matsui et al. 2008). Our results showed that *Lactobacillus sakei* is the main organism involved in the fermentation of *samma-narezushi*. Furthermore, we have detected uncultured LAB in *ayu narezushi* by using the 16S rDNA clone library technique (Matsui et al. 2010). Thus the advantage of the 16S rDNA clone library technique is obvious.

The purpose of this study was to analyze bacterial diversity in *saba-narezushi* (*narezushi* of mackerel) produced in Mie Prefecture. Bacteria involved in the fermentation of *narezushi* were estimated by the culture-independent 16S rDNA clone library technique.

Materials and methods

Narezushi sample *Saba-narezushi* was obtained from a local manufacturer in Kiho town, Mie Prefecture, Japan, where it was produced as follows. After removing the head and internal organs, the mackerel was salted for 1 month and then pickled in a tub together with cooked rice for about 3 weeks in winter.

Analysis of chemical composition Fish meat and rice were separately homogenized with a food processor. The proximate composition of the sample was measured by the general method of food analysis. Moisture content was determined by dryness at 105 °C. Protein content was calculated from the nitrogen content by the micro-Kjeldahl method, using a conversion factor of 6.24. Lipid content

was determined using the Soxhlet method. Ash content was determined by incineration at 450 °C. Carbohydrate content was calculated by subtracting the moisture, ash, protein, and lipid content from the total weight. These determinations were carried out using 6 replicates. Lactic acid contents were determined by HPLC analysis with post-column detection. Triplicate samples were used for this determination.

DNA extraction, cloning, and sequencing The sample was homogenized with a sterilized food processor, immediately suspended in acetone at a final concentration of 70% to avoid DNA degradation (Fukatsu 1999), and stored at –80 °C until analysis.

The stored samples were washed twice with sterilized physiological saline by centrifugation before DNA extraction. DNA was extracted by the bead-beating method, using the FastPrep instrument (Bio 101, Vista, CA, USA) as described by Godon et al. (1997). The crude DNA was purified with Genomic-tip 100/G (QIAGEN, Hilden, Germany) and dissolved in TE buffer. DNA concentration was adjusted to 10 ng μL^{-1} .

The 16S ribosomal RNA gene fragments were amplified by PCR with a set of universal primers (530f and 1392r) (Matsui et al. 2008). PCR was performed using TaKaRa Ex Taq (TaKaRa, Otsu, Japan) with the PCR Thermal Cycler Dice TP600 (TaKaRa, Otsu, Japan). A total of 20 μL of the PCR reaction mixture contained 1 μL of template DNA, 1 \times Ex Taq reaction buffer, 200 μM each deoxynucleoside triphosphate (dNTP mixture), 0.5 unit of Ex Taq DNA polymerase, 0.5 μM of each primer, and 0.5 g L^{-1} bovine serum albumin. The following PCR cycle was used: an initial denaturation at 95 °C for 3 min, followed by 15 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min with a final extension step at 72 °C for 10 min. After separation by using 1.0% agarose gel electrophoresis (in TAE buffer), the PCR products were confirmed by visualization with ethidium bromide staining and then cloned into TOP10 competent *Escherichia coli* by using the TA cloning kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. Positive clones were randomly selected from the clone library. The cloned DNA fragments were commercially sequenced from both directions with M13 reverse primer (5'-CAGGAAACAGCTATGAC-3') and M13 forward (–20) primer (5'-GTAAAACGACGGC CAG-3') by Shimadzu Corporation (Kyoto, Japan).

Homology search, phylogenetic analysis, and diversity analysis of nucleotide sequence DNA sequences were examined for homology with the BLAST program (Altschul et al. 1997). Chimeric artifacts of PCR were checked with the CHECK_CHIMERA online program of the Ribosomal Database Project (RDP-II) and omitted from analysis (Cole

et al. 2003). Operational taxonomy unit (OTU) assignment and calculation of Chao1 and Shannon-Wiener index (H') were carried out with the FastGroupII online program (Yu et al. 2006). The DNA sequences of the cloned fragments were aligned with CLUSTAL X ver. 2.0 (Larkin et al. 2007), and phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987). The stability of branches was checked by bootstrapping (1,000 resampling). Njplot ver. 2.3 was used to draw phylogenetic tree.

Nucleotide sequence accession numbers All nucleic acid sequences obtained in this study were deposited in the DDBJ, EMBL, and GenBank databases under accession numbers AB326516–AB326609.

Results and discussion

In Japan, a wide variety of fermented foods such as pickles, rice wine (*sake*), soy sauce (*shoyu*), fermented soybean paste (*miso*), and *natto* are quite popular. These fermented products have attracted worldwide attention as foods that might promote longevity (Murooka and Yamashita 2008). *Narezushi* is a traditional fermented food containing live lactic acid bacteria (LAB). The LAB plays important roles in fermentation and possibly in human health. Therefore, it is important to clarify LAB in the *narezushi* to study their functionality.

Chemical composition The chemical composition of *saba-narezushi* was analyzed to compare nutrient composition and degree of fermentation with different kinds of *narezushi* which have been previously reported (Matsui et al. 2008, 2010). In both fish meat and rice, the moisture content was more than 70%. The fish meat contained 20% proteins and 7.6% lipids, whereas carbohydrate was not detected (Table 1). On the other hand, rice contained approximately 18% carbohydrate. The rice portion has a slightly higher lactic acid composition than fish meat. We have reported the chemical composition of *narezushi* in the meat of saury fish (*samma-narezushi*) (Matsui et al. 2008). The nutrient composition of *samma-narezushi* was as

follows: 19.7% protein, 3.9% lipid, and 3.5% carbohydrate in fish meat, and 2.5% protein, 0.1% lipid, and 18.1% carbohydrate in rice. The amount of lactic acid, a component that provides taste, in *samma-narezushi* was 0.6% in fish meat and 1.0% in rice. The values obtained in the present study were quite similar to those found in *samma-narezushi* (Table 1). The results suggest that significant growth of LAB and lactic acid fermentation occurred in *saba-narezushi*. The lactic acid would contribute sour taste of *saba-narezushi*. Fujii et al. (1992) analyzed the chemical composition of *saba-narezushi* produced in Wakayama Prefecture in Japan. The concentration of lactic acid in the fish meat and rice portions of the *saba-narezushi* fermented for 20 d in January was 0.37% and 0.49%, respectively. Although the fermentation period of *saba-narezushi* reported by Fujii et al. (1992) and that used in this study was similar, the lactic acid concentration was 2 times higher in the present report. This may be due to the differences in the composition of LAB. Itou et al. (2006) reported the chemical composition of mackerel *narezushi*, which uses the same kind of fish but is fermented for a much longer period (4 months) during summer, in Fukui Prefecture in Japan. The concentration of lactic acid in mackerel *narezushi* was 5% in fish meat and 3.3% in rice. The difference may be due to the variation in preparation: mackerel *narezushi* (another type of *saba-narezushi*) was pickled under high temperatures in summer (from May to September) for a longer period.

Diversity analysis by 16S ribosomal RNA clone library A total of 94 clones were randomly selected from the 16S rDNA clone library of *saba-narezushi*, and DNA sequences of cloned fragments (approx. 890 bp) were analyzed. Homology search, phylogenetic analysis, and calculation of Chao-1 and Shannon-Wiener index (H') were carried out. To the best of our knowledge, this is the first report on microbial diversity analysis of *saba-narezushi* by the PCR-derived clone library method. No chimeric sequence was found using the CHECK_CHIMERA program. Cloned sequences were classified into 16 OTUs at the 97% identity threshold and summarized in Table 2. All sequences in the library were eubacterial 16S rDNA sequences, and no archaeal or eukaryotic sequence was recovered. The sequence similarity of the cloned fragment was equal to

Table 1 Chemical composition (%) of *saba-narezushi* (*narezushi* of mackerel). Values are mean \pm standard deviation ($n=5$)

	Moisture	Protein	Lipid	Ash	Carbohydrate	Lactic acid
Fish meat	72.6 \pm 0.08	19.7 \pm 0.89	7.6 \pm 0.07	1.3 \pm 0.06	nd ¹	0.77 \pm 0.22
Rice	78.2 \pm 0.27	1.3 \pm 0.40	0.12 \pm 0.07	1.8 \pm 0.05	18.7	0.99 \pm 0.01

¹ Not detected

Since the carbohydrate content was less than the detectable level, the sum of moisture, protein, lipid, and ash was greater than 100% in fish meat

Table 2 Abundance and similarity of OTU to known species of bacteria in *saba-narezushi* (*narezushi* of mackerel)

OTU (number of clone)	Nearest known species (Accession Number)	Similarity (%)	Composition (%)
	Lactobacillales		
OTU-01 (47), OTU-02 (1)	<i>Lactobacillus curvatus</i> (AJ621550)	99–100	51.1
OTU-03 (13)	<i>Lactococcus piscium</i> (DQ343754)	99	13.8
OTU-04 (8)	<i>Leuconostoc gasicomitatum</i> (AF231131)	100	8.5
OTU-05 (3)	<i>Lactococcus lactis</i> subsp. <i>lactis</i> (X64887)	99	3.2
OTU-06 (3)	<i>Vagococcus carniphilus</i> (AY179329)	98	3.2
OTU-07 (2)	<i>Carnobacterium divergens</i> (AY543037)	99	2.1
OTU-08 (1), OTU-09 (1)	<i>Lactobacillus fuchuensis</i> (AB370875)	99	2.1
OTU-10 (1)	<i>Lactobacillus sakei</i> (EU626014)	99	1.1
	Subtotal		85.1
	Bacillales		
OTU-11 (1), OTU-12 (1)	<i>Brochothrix thermosphacta</i> (AY543029)	99–100	2.2
	Subtotal		2.2
	γ -Proteobacteria		
OTU-13 (7)	<i>Hafnia alvei</i> (AB244471)	99	7.5
OTU-14 (3)	<i>Buttiauxella gaviniae</i> (AJ233403)	100	3.2
OTU-15 (1)	<i>Pseudomonas psychrophila</i> (FJ950690)	100	1.1
OTU-16 (1)	<i>Shewanella baltica</i> (CP000891)	99	1.1
	Subtotal		12.9
	Total		100

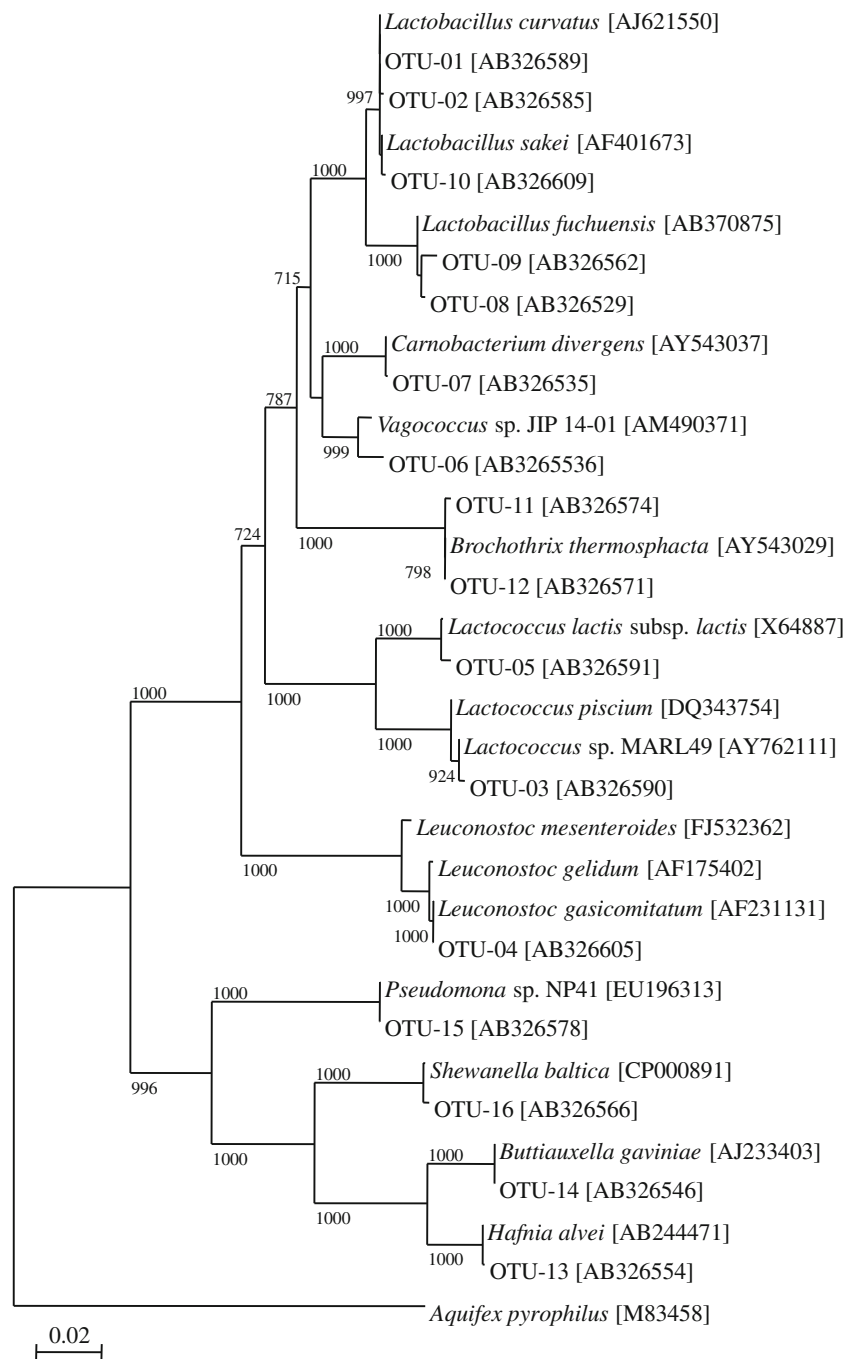
or higher than 98% of the sequence of cultivated bacterial species in the public database. The expected OTU by Chao1 estimates and Shannon-Wiener index (H') of *saba-narezushi* were 48 and 1.822, respectively. The Chao1 estimate and Shannon-Wiener index (H') observed in *saba-narezushi* was higher than those in *samma-narezushi* (Matsui et al. 2008).

Based on phylogenetic placement and homology results, 85% of clones were assigned as LAB (Fig. 1; Table 2). These clones were classified into 10 OTUs (Table 2). OTU-01 and -02, which constitute 51.1% of the total number of clones, were classified as *Lactobacillus curvatus*. The second most abundant clone was OTU-03 (13.8% of the clones) and was classified as *Lactococcus piscium*. OTU-04 (8.5% of the clones) was classified as *Leuconostoc gasicomitatum*. The results suggest that these LABs play important roles in the fermentation of *saba-narezushi*. Although functional analysis of these LABs was not carried out in the present study, previous reports suggested that *L. curvatus* plays important roles in fermentation of foods (Vogel et al. 1993; Verluyten et al. 2004, Rotsatchakul et al. 2009). *L. curvatus* is best adapted to meat fermentations and dominate the flora during the process in the fermented sausage (Vogel et al. 1993). *L. curvatus* LTH1174 isolated from fermented sausage produces listericidal bacteriocin curvacinA suggesting that possible roles in suppressing *Listeria* growth in the sausage (Verluyten et al. 2004).

Volatile compounds of nham, Thai fermented sausage, are produced by *L. curvatus* (Rotsatchakul et al. 2009). In a previous study, *Lactobacillus plantarum*, *Lactobacillus alimentaria*, and *Lactobacillus coryniformis* were isolated from *saba-narezushi* (Fujii et al. 1992). Another study showed that *Lactobacillus buchneri* was the predominant bacterial species in *funazushi*, which is fermented for 6 months (Isobe et al. 2002). Our results together with those of the previous studies suggest that bacteria belonging to the genus *Lactobacillus* play important roles in the fermentation of various *narezushi*. OTUs affiliated with *Lactococcus lactis* subsp. *lactis*, *Vagococcus carniphilus*, *Carnobacterium divergens*, and *Lactobacillus fuchuensis* were detected in low numbers among the clones (Table 2). In our previous study, 90% of the clones were LABs and most were classified as *L. sakei* in the clone library of *samma-narezushi* (Matsui et al. 2008). In contrast to this, *Lactobacillus sakei* was minor component in the present study. The reason for the small proportion of *L. sakei* in *saba-narezushi* is unclear.

OTU-11 and -12 were classified as *Brochothrix thermosphacta* (Bacillales). These OTUs composed 2.2% of the clones (Table 2). Four OTUs were assigned as γ -proteobacteria constituting 12.9% of the clones (Table 2). OTU-13 (7.5% of the clones) was classified as *Hafnia alvei*. OTU-14, -15, and -16 were classified as *Buttiauxella*, *Pseudomonas psychrophila*, and *Shewanella baltica*, respec-

Fig. 1 Phylogenetic tree of the 16S rDNA fragment recovered from *saba-narezushi* (*narezushi* of mackerel). The scale bar represents 0.1 substitution per nucleotide position. Bootstrap values for 1,000 trees are shown at the branch points. Only values of 70% or above are shown. Numbers in parentheses are GenBank Accession numbers



tively. These OTUs were minor components in the library. Fujii et al. (1992) detected Gram-negative rods in *saba-narezushi*. In the present study, Gram-negative rods such as *H. alvei*, *Buttiauxella gaviniae*, *P. psychrophila*, and *S. baltica* were also detected, suggesting that these organisms are minor but common constituents in *saba-narezushi*.

In our previous result, uncultured LAB was detected in *ayu-narezushi* (Matsui et al. 2010). Therefore, we have used culture-independent method rather than culture-dependent method. The present study shows that the PCR-derived clone library technique can be successfully

applied to diversity analysis of bacteria in *saba-narezushi*. Because the technique is semi-quantitative analysis, the population density of *saba-narezushi* should be determined by quantitative technique such as real-time PCR. *Lactobacillus brevis* FPTLB3 isolated from freshwater fish produced bacteriocin that inhibited *Escherichia coli*, *Enterococcus faecalis*, *L. sakei* and *Staphylococcus aureus* (Banerjee et al. 2011). The isolate can potentially be used for biopreservation of the food. Isolation and phenotypic characterization of bacteria in *saba-narezushi* should be done to clarify their function in the fermentation process.

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