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Convenient preparation of fungal genomic DNA templates for polymerase chain reaction and temporal temperature gradient gel electrophoresis analyses of fungal diversity in the Japanese fermented soybean paste *miso*

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Abstract

We have developed a method to extract DNA from a traditional Japanese fermented soybean paste (*miso*) more easily and quickly than by conventional methods. The extracted *miso* DNA was used as a template for the polymerase chain reaction (PCR) to amplify the ITS2 region of ribosomal DNA. We found the PCR sensitivities with the templates extracted by this new method and by the conventional method to be comparable for the detection of fungal communities. We also investigated the fungal diversity in *miso* by using a culture-independent genetic fingerprinting technique known as temporal temperature gradient gel electrophoresis (TTGE), and then compared the TTGE profiles of 18 *miso* samples. The TTGE profile of each *miso* type could be classified according to the fermentation procedure used during manufacture.

Key words: FTA® card, traditional Japanese fermented food *miso*, PCR-TTGE, fungal diversity

1. Introduction

Miso is a traditional Japanese fermented salty-tasting seasoning made from soybeans, rice or barley, and salt. Analysis of the microflora of *miso* is important for efficient quality control in the *miso*-producing industry. *Aspergillus oryzae* digests the grain starch to glucose; halotolerant yeast species such as *Zygosaccharomyces rouxii*, *Candida*

etchellsii, and *C. versatilis* produce the characteristic flavors and alcohols; and lactic acid bacteria produce the lactic acid. The fermentation room of every *miso*-manufacturing facility has its characteristic resident flora that impart the original flavor of the *miso* produced by each manufacturer. In addition to the flavor-producing favorable yeasts, the air can also contain undesirable contaminants such as *Bacillus* species.

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Running title: TTGE analyses of Japanese fermented soybean paste

The conventional method for analyzing *miso* microflora is by the dilution and spread plate method, which requires several days for incubation and much effort. Previously, we reported a rapid and direct method for preparing PCR templates from *miso*¹⁾ by using the FTA® card. In that study, although we successfully amplified the fungal internal transcribed spacer (ITS) region from *miso* samples and observed the ITS DNA fragments by agarose gel electrophoresis, we could not identify the fungal species without cloning and sequencing each DNA fragment, because agarose gel electrophoresis cannot distinguish DNA fragments of different sequence but same length. Culture-independent approaches such as denaturing gradient gel electrophoresis (DGGE) or temporal temperature gradient electrophoresis (TTGE) have been developed for complex matrices such as food²⁾ and soil³⁾. The ability of DGGE or TTGE to separate DNA fragments of the same length depends on the difference of melting temperature applied during electrophoresis. Hosoi reported the analysis of bacterial communities in *miso* by PCR followed by DGGE⁴⁾, and Taiyouji, Ando, and Shima reported the analysis of lactic acid bacterial communities in soybean *koji*⁵⁾. The microflora of several Chinese and Korean soybean pastes were also analyzed by DGGE^{6,7)}. TTGE is a simpler technique than DGGE, in that it does not require a denaturing gradient gel. Instead, the gradually increasing gel temperature denatures the DNA fragment.

Here, we report on the amplification of the ITS2 region of fungal rDNA from 18 kinds of *miso* using the FTA® card and analysis by TTGE.

2. Materials and methods

2.1. Food samples and strains

Eighteen kinds of *miso* samples (A1, C1, C2, C3, C4, D1, D2, E1, E2, E3, E4, E5, E6, E7, E8, F1, H1, and I1) obtained from Chuo *Miso* Kenkyujo (Central *Miso* Research Institute, Tokyo, Japan) were frozen at -20°C and stored. Each *miso* sample name consisted of an alphabet letter and a number. The alphabet letter shows the category of the *miso* (A: white-colored and sweet-tasting type rice *koji miso*; C: ocher-colored and salty-tasting type rice *koji miso*; D: red ocher-colored and salty-tasting type rice *koji miso*; E: brown-colored and salty-tasting type rice *koji miso*; F: soybean *koji miso*; H: barley *koji miso*; and I:

blend of rice and barley *koji miso*.) The number reflects the sample number of this study. Six reference strains of *miso* microorganisms (*A. oryzae* NFRI1599, *A. sojae* NFRI1147, *Z. rouxii* NFRI3471, *C. parapsilosis* NFRI4051, *C. versatilis* NFRI4052, and *C. etchellsii* NFRI4053) were obtained from the gene bank of the National Food Research Institute (Tsukuba, Japan). The fungal species were maintained on potato dextrose agar for 5 days at 25°C. The fungal species were inoculated into 10 mL of YPD medium (1% yeast extract, 2% polypeptone, 2% D-glucose) in an Erlenmeyer flask and cultured at 30°C with 130 rpm rotary shaking for 1 day.

2.2. Preparation of template genomic *miso* DNA for PCR

2.2.1. Conventional DNA extraction

Miso samples (5 g each) were suspended in 20 mL of 10 mM phosphate buffer (pH 6.8) and homogenized for 1 min with a stomacher (MC-D400D; Gunze Sangyou, Tokyo, Japan). Two milliliters of the resulting suspension was centrifuged at 14,000 rpm for 3 min. The pellets were frozen in liquid nitrogen and crushed to a fine powder using an automatic grinding mill (TK-AM4; Tokken, Kashiwa, Japan) and approximately 100 mg of the frozen *miso* powder was suspended in the AP1 buffer of the DNeasy Plant Kit (Qiagen, Tokyo, Japan). DNA extraction from the frozen *miso* powder was carried out according to the manufacturer's protocol.

2.2.2. Template preparation by FTA® card

Template preparation by FTA® card was done according to a modified procedure of our previous report¹⁾. Approximately 50 mL of each *miso* sample was picked up using a disposable plastic inoculating loop and suspended in 300 mL of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and then subjected to filtration through an empty Micro Bio-Spin chromatography column (Bio-Rad, Tokyo, Japan) by centrifugation at 15,000 rpm. A large proportion of the source crop debris is retained on the column filter, while the buffer and cells of the microorganisms are eluted in the flow-through fractions. The cells were pelleted by column centrifugation. The pellets were resuspended in 25 mL of TE buffer and heated three times in a microwave oven for 30 s each time. In order not to evaporate the entire buffer, the lid of the tubes was kept firmly closed. Usually,

a small amount of vaporized water would be observed on the inner wall of the tube after heating. Five microliters of the preheated samples was applied onto the indicated FTA® classic cards (Whatman Plc, Brentford, Middlesex, UK) that were then dried for 10 min at room temperature. Disks of 3 mm diameter were punched out from the FTA® card with a paper punch and were washed according to the manufacturer's protocol.

2.2.3. DNA extraction from pure cultured fungi by the conventional method

The fungal culture in YPD medium was harvested by filtration or centrifugation. Approximately 100 mg of fungal cells was frozen by liquid nitrogen and crushed into a fine powder, as described above. Genomic DNA was extracted from approximately 100 mg of fungal cell powder using the DNeasy Plant Kit (Qiagen) according to the manufacturer's instructions.

2.3. PCR amplification

The forward primer ITS3 (5'-GCA TCG ATG AAG AAC GCA GC-3')⁸⁾ and the reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3')⁸⁾ were used. A GC clamp (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3')⁹⁾ was added to the 5' end of the ITS3 or ITS4 sequence (ITS3GC, ITS4GC). PCR was performed using the GeneAmp PCR System 2700 (Applied Biosystems Japan, Tokyo, Japan) in a final reaction mixture volume of 50 µL containing 10 µL of 5 × GC buffer, 25 µL of Ampdirect (Shimadzu, Kyoto, Japan), 0.375 M concentrations of each primer, 0.375 U of Phusion High-Fidelity DNA Polymerase (Daiichikagaku, Tokyo, Japan), two FTA® card disks or 20 ng of DNA as template, and 7.5% dimethylsulfoxide. The following thermal cycling program was used: 94°C for 5 min (1 cycle), 98°C for 1 min, 48°C for 2 min, and 72°C for 3 min (10 cycles); the denaturing time was decreased by 3 s (i.e., from 1 min in the first cycle to 33 s in the tenth cycle); the annealing temperature was increased by 1°C (i.e., from 48°C in the first cycle to 57°C in the tenth cycle); and the extension time was increased 6 s after each cycle (i.e., from 3 min in the first cycle to 3 min 54 s in the tenth cycle); followed by 98°C for 30 s, 58°C for 2 min 30 s, and 72°C for 4 min (40 cycles). The size and quantity of the PCR products were confirmed by 2% agarose gel electrophoresis.

2.4. Temporal temperature gradient gel electrophoresis

TTGE was carried out using the Dcode Universal Mutation Detection System (Bio-Rad). Polyacrylamide gels (8%) containing 7 M urea (16 cm × 16 cm × 1 mm) were prepared and run with 1.25 × TAE buffer. Electrophoresis was performed at a constant voltage of 140 V for 6 h with an increasing temperature gradient of 2°C /h from an initial temperature of 48°C to a final temperature of 60°C. The gels were stained for 30 min with either SYBR Safe (Invitrogen Japan, Tokyo, Japan) and scanned by the Typhoon9400 scanner (GE Healthcare Japan, Tokyo, Japan), or with ethidium bromide and observed by an ultraviolet illuminator.

2.5. Sequencing

TTGE fragments of the *miso* samples were compared with the fragments of pure culture reference strains, and bands unique to the *miso* samples were selected for sequence analysis. Materials from the selected bands were excised using a razor blade, and the DNA fragments were eluted into 20 mL of sterile water at 4°C overnight. From the eluate, 5 mL was used as a template and re-amplified by PCR as described above. The PCR products were ligated into the pCR-Blunt vector (Invitrogen Japan) according to the manufacturer's instructions and subjected to the cycle sequencing reaction using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Japan). Sequence analysis was performed with the ABI PRISM 310 Genetic Analyzer. The sequencing results were compared with sequences in the NCBI nucleotide database.

3. Results

In this study, we first confirmed the validity of the FTA® DNA extraction method, and then investigated the fungal diversity of *miso* by the PCR-based method using the fungal genomic DNA template prepared by the FTA® DNA extraction method.

3.1. Comparison of the FTA® DNA extraction method with the conventional method

To confirm the validity of the FTA® DNA extraction method, we compared the TTGE profiles of the ITS2 region (from four *miso* samples) amplified from genomic DNA extracted by the FTA® method with those extracted

by the conventional method. The selected *miso* samples represented the typical types: white-colored and sweet-tasting type rice *koji miso*, brown-colored and salty-tasting type rice *koji miso*, soybean *koji miso*, and barley *koji miso*. To amplify the ITS2 region, the universal fungus-specific primers ITS3 and ITS4 were used. The GC clamp was added to the 5' end of these primer sequences. We compared the result of PCR amplification using ITS3GC and ITS4 with that of ITS3 and ITS4GC. ITS3GC and ITS4 amplified PCR products with better intensity than did ITS3 and ITS4GC (data not shown). We subsequently chose ITS3GC and ITS4 for further experiments. Fig. 1 shows a TTGE gel image, in which Fig. 1A displays results with the reference strains (see next paragraph) and Fig. 1B displays results of the conventional extraction. The *miso* genomic DNA prepared by the FTA® DNA extraction method was subjected to PCR amplification of the ITS2 region of rDNA

and separated by TTGE (Fig. 1C). Comparison with the results of conventional extraction (Fig. 1B) revealed nearly identical numbers and mobility of bands, indicative of similar sensitivities. It was noted that in the TTGE profiles of *miso* by the FTA® DNA extraction method, some bands were fainter and others were thicker, relative to the profile from conventional DNA extraction.

3.2. Fungal diversity of 18 kinds of *miso*

To determine the standard mobility of the reference strains of *miso* microorganisms, the ITS2 region of pure cultures of *miso* microorganisms was separated by TTGE. To investigate the fungal diversity of 18 kinds of *miso*, the ITS2 sequences amplified from the *miso* DNA templates prepared by the FTA® DNA extraction method were separated by TTGE. The results are shown in Fig. 2. The TTGE profiles of ITS2 PCR fragments amplified from

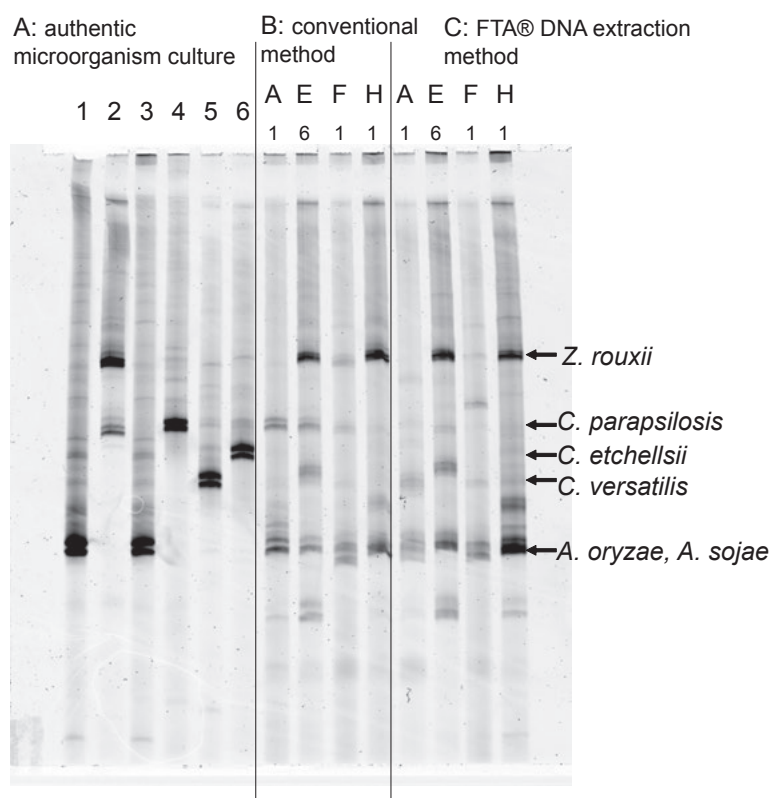


Fig. 1 Temporal temperature gradient electrophoresis (TTGE) profiles.

(A) TTGE profiles of the six *miso* fungal reference strains used in this study. ITS2 sequences were separated by TTGE. Lane 1: *Aspergillus oryzae*; lane 2: *Zygosaccharomyces rouxii*; lane 3: *Aspergillus sojae*; lane 4: *Candida parapsilosis*; lane 5: *Candida versatilis*; lane 6: *Candida etchellsii*. (B) TTGE profiles of the ITS2 region resulting from amplification of DNA extracted from four kinds of *miso* by the conventional method. Lane A1: white-colored and sweet-tasting type rice *koji miso*; lane E6: brown-colored and salty-tasting type rice *koji miso*; lane F1: soybean *koji miso*; lane H1: barley *koji miso*. (C) TTGE profiles of the ITS2 region resulting from amplification of DNA extracted from four kinds of *miso* by the FTA® DNA extraction method. Lane A1: white-colored and sweet-tasting type rice *koji miso*; lane E6: brown-colored and salty-tasting type rice *koji miso*; lane F1: soybean *koji miso*; lane H1: barley *koji miso*.

the reference strains of *miso* microorganisms are shown in Fig. 1A. The major bands of each *miso* sample showed mobility identical to that of the reference strains; bands 1, 4, 5, and 7 displayed mobility identical to that of *Z. rouxii*, *C. parapsilosis*, *C. versatilis*, and both *A. oryzae* and *A. sojae*, respectively.

To determine the species of the bands of *miso* that were not identical to any reference strain, we excised the material containing the particular DNA fragment from the gel and determined the sequence of the DNA fragment contained in the band. Only the DNA sequence of band 8 was determined, because the ethidium bromide staining of bands 2, 3, and 6 was too faint to distinguish flanking bands from each other. We therefore could not separately excise these bands upon ultraviolet illumination. The DNA fragments of the excised band 8 and the smear containing bands 2 or 6 were re-amplified by PCR and cloned into the pCR-Blunt vector. We failed to amplify the DNA fragment

in band 3. The sequences of the cloned DNA fragments were determined and compared with sequences in the NCBI DNA database. The sequence of band 8 was 100% identity to *Clavispora lusitaniae*. The DNA sequences from the faint smear containing bands 2 or 6 contained several clones of DNA fragments. Band 2 contained a DNA fragment that was 99% identical to *Z. pseudorouxii*. Band 6 contained several DNA fragments that showed low identity to uncultured mycorrhizal fungi. Band 6 was detected mainly in the *miso* of categories C and D. Band 8 was detected mainly in brown-colored *miso* (E category). The TTGE profiles of A-type and F-type *miso* displayed a few faint bands of yeast and *Aspergillus*, that of H-type *miso* displayed intense bands of *Z. rouxii* and *Aspergillus* and faint band 6, and that of I-type *miso* indicated that bands 4 and 5 were appropriately included in the profile of H-type *miso*.

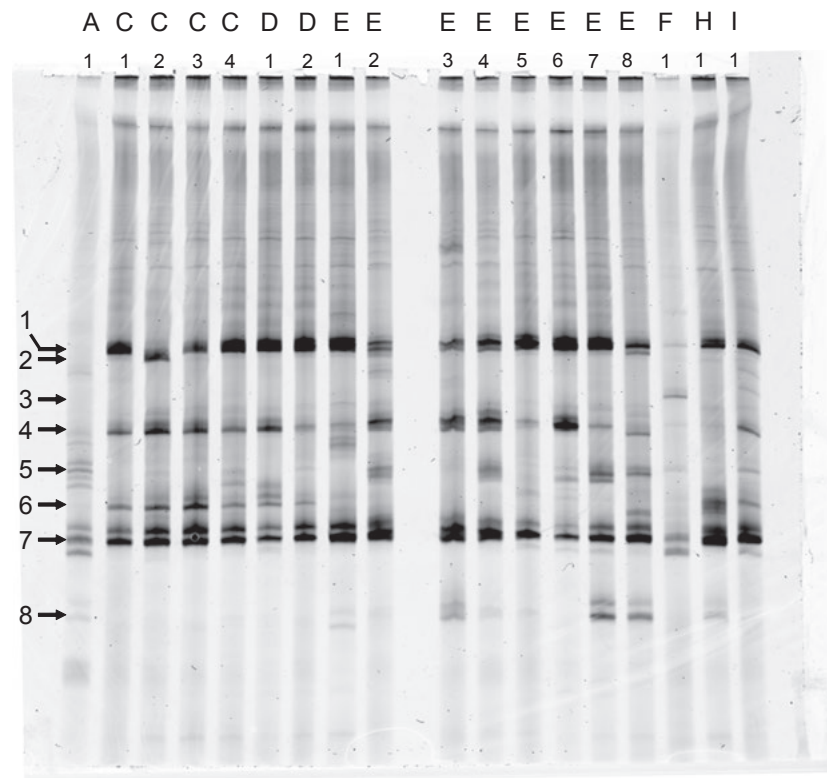


Fig. 2 Temporal temperature gradient electrophoresis (TTGE) profiles of the ITS2 region representing the fungal community present in 18 kinds of miso.

A: white-colored and sweet-tasting type rice *koji miso*; C: ocher-colored and salty-tasting type rice *koji miso*; D: red ocher-colored, salty-tasting type rice *koji miso*; E: brown-colored and salty-tasting type rice *koji miso*; F: soybean *koji miso*; H: barley *koji miso*; I: blend of rice and barley *koji miso*.

4. Discussion

Traditional Japanese fermented foods characteristically use *koji*, grain particles that are fermented by *koji* mold and used as an enzyme source. Fungi are the key microorganisms in traditional Japanese fermented foods and study of the fungal diversity in fermented Japanese foods is important.

We developed an easy and quick method to extract fungal DNA from *miso*. Generally, DNA extraction from food samples is laborious and time consuming. Our novel method does not require any organic solvent, protein denaturant, or specialized equipment. This facilitates quality control by DNA analysis in the food-producing industry. In this study, we compared the FTA® DNA extraction method with the conventional method and showed that both methods display almost equal sensitivity with respect to the number of DNA bands separated. Furthermore, we tried another two commercial DNA extraction kits that are widely used in the research of soil microorganism communities. One method is based on beads beading, and the other method uses benzyl chloride. The DNA extraction results from using these kits were low yielding and of poor quality (data not shown). Generally, in the TTGE or DGGE methods, biases resulting from the method of DNA extraction and PCR amplification cannot be completely ruled out. The simplicity and sensitivity of the method are inconsistent. In this study, we could detect DNA bands with mobility identical to that of the relevant reference strain, indicating that our method is valid as well as sufficient for the rapid analysis of multiple samples of *miso*. However, we must note that TTGE profiles of *miso* DNA extracted by the FTA® card method produced some bands that were fainter or thicker than the corresponding bands obtained by the conventional method. The significance of these dichotomies is not presently known.

The TTGE profile of each *miso* sample reasonably reflected the fermentation procedure used in its manufacture. Band 6 was detected mainly in the *miso* aged for only a short time (in most cases, the aging process lasts less than a few months) and in the C and D types. In contrast, band 8 was detected mainly in E-type *miso* that was older (typically aged for more than four months). The *miso* products of types C, D, and E are made from similar source materials

and using essentially the same procedure, except for the duration of aging. We have no explanation for the impact of differences in fungal diversity between C, D types of *miso* and E type *miso* in terms of their quality, taste, and flavor. The A type has a very short period of aging (less than two weeks). The aging of A-type *miso* is different from the other long-aged types of *miso*, being aged mainly by enzymes already secreted in *koji* by *koji* mold and not fermented by living microorganisms. To prevent fermentation, the aging of A-type *miso* is carried out at a high temperature (35–50°C). The TTGE profile of the lesser yeast bands, which displayed only a few faint bands of A type, is therefore reasonable. F-type *miso* is produced mainly by anaerobic fermentation by the lactic acid bacteria and fungi growing on the surface of the *miso*. As expected, the TTGE profile of F-type *miso* exhibited a few faint bands of yeast. The TTGE profile of H-type *miso* exhibited intense bands of *Z. rouxii* and *Aspergillus*, and faint band 6. Unlike the C and D types of rice *koji miso*, the H type is made using barley *koji*; however, the aging period is the same as the C and D types. The fungal band 6 is likely characteristic of the short-aged type of *miso*. The TTGE profile of I-type *miso* indicated a similar profile, with the exception of the existence of bands 4 and 5 in the profile of the H type. These latter bands were detected in rice *koji miso* of the C, D, and E types. This result is reasonable, given that the I type is manufactured using a blend of rice and barley *koji miso*. Generally, after aging, *miso* is mashed into smooth paste and then packed. Some consumers prefer intact type of *miso* that contains granules of source crops to mashed type *miso*. The C and E types used in this study contain mashed and intact types. There were no significant differences between mashed and intact types in the results shown in this study.

Conventional studies concerning *miso* microorganisms have demonstrated the presence of *Z. rouxii*, *C. versatilis*, and *C. etchellsii* in the final products, and the frequent contamination of low-quality *miso* products by *Pichia membranaefaciens* and *Hansenula anomala*.¹⁰⁾ RFLP analysis of *miso* revealed *Z. rouxii* to be the dominant species in the final product in the case in which a starter culture of *Z. rouxii* was added, whereas *P. guilliermondii*, *P. triangularis*, and *P. farinosa* were the dominant species in *miso* formed without a starter culture¹¹⁾. These authors mentioned that *miso* fermented without a *Z. rouxii* starter culture contained a lower level of 4-hydroxy-2 (or 5)-ethyl-

5 (or 2)-methyl-3 (2H)-furanone (generally known as HEMF) than those with the starter culture. The record of the manufacturers showed that all 18 kinds of *miso* investigated in this study included a starter culture of *Z. rouxii*. This explains why we could not detect the *Pichia* species. The fungi that were detected in this study have not been reported by conventional studies on *miso* using dilution spread plating. In E-type *miso*, *C. lusitanae* (band 8) was observed.

We expect our novel DNA extraction method to be a useful and efficient quality control tool in the food industry.

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