Genetic identification of the local mukodamashi varieties of foxtail millet (Setaria italica (L.) P. Beauv) in Japan

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ABSTRACT

Different varieties of foxtail millet (Setaria italica (L.) P. Beauv) are grown in Japan; there are several varieties of Mukodamashi, and they are cultivated in more than 10 prefectures. However, while most of Mukodamashi varieties have white grains and waxy endosperms, it is uncertain whether they have the same or different genotypes. In this study, five Mukodamashi varieties, from Gunma, Miyazaki, Ehime, Kochi, and Nara Prefectures, as well as 23 other local varieties of foxtail millet, were examined using simple sequence repeat markers to elucidate their genetic identities. The five Mukodamashi varieties have different genotypes. Therefore, we concluded that different varieties in different areas have been named Mukodamashi. It is possible that local varieties with the same name are actually different varieties, even if they have similar characters.

Introduction

In pre-modern Japan, the foxtail millet (Setaria italica (L.) P. Beauv) is one of the most important crops along with rice, especially in mountainous areas, where it was cultivated by swidden or permanent agriculture as a main crop (Verschuer, 2017; Yokoyama et al., 2014). The cultivation of foxtail millet in Japan predates that of rice. Consequently, there are many local varieties of foxtail millet that have been established by farmer selection and crossing.

Horiuchi et al. (1980) found that farmers selected and cultivated different foxtail millet varieties according to their thermosensitivities, depending on the elevation of fields in the same prefecture. However, Takei and Sakamoto (1987) pointed out that local farmer selections of varieties were related to the establishment of various photosensitivities in the local foxtail millet varieties. As Japan is a long archipelago extending from north to south and has various climates and photoperiods, the local conditions have also contributed to the establishment of many local varieties of foxtail millet. Currently, the Genebank Project of the National Agriculture and Food Research Organization in Japan (https://www.gene.affrc.go.jp/databases-plant_search.php) maintains more than 500 local varieties of foxtail millet that have been collected from across Japan.

In the list of local varieties of foxtail millet, identically named varieties have been cultivated in many different prefectures. Specifically, Mukodamashi had been cultivated in Gunma, Chiba, Tokyo, Kanagawa,
Niigata, Ishikawa, Nara, Tottori, Hiroshima, Kochi, Ehime, and Miyazaki Prefectures (Kato et al., 2017). There were at least 12 Mukodamashi varieties in Japan. In this study, four Mukodamashi varieties maintained in the gene bank and 1 Mukodamashi variety collected in Nara Prefecture were examined. Mukodamashi means ‘deceiving husband’ in Japanese, and in Nara, the legend is that when a wife makes millet dumplings from Mukodamashi, the husband mistakes them for rice cakes because the grain of Mukodamashi is white and sticky (Kato et al., 2017). However, the genetic identities of Mukodamashi varieties and their distribution are not clear. One possibility is that a specific variety named Mukodamashi was transported to various areas in Japan. Another possibility is that Mukodamashi varieties were named individually for similar reasons as the variety in Nara Prefecture.

If Mukodamashi varieties have different genetic backgrounds, the naming criteria of Mukodamashi varieties are not clear. Previous ethnological and ethnobotanical studies have shown that plant and animal species are sometimes classified according to unique taxonomical rules in traditional societies, and such taxonomical rules do not always relate to genetic distance (Berlin, 1999; Berlin et al., 1973). Previous studies on the classifications and nomenclature of local rice and cassava varieties showed that varieties with different genetic backgrounds sometimes have the same name, whereas those with the same genetic background sometimes have different names (Kizito et al., 2007; Nuijten & Almekinders, 2008). This often occurred because local people tended to classify local varieties by their appearance.

In this study, Mukodamashi from Gunma, Miyazaki, Ehime, Kochi, and Nara Prefectures were examined using simple sequence repeat (SSR) marker analyses to compare their genetic backgrounds. Other local varieties of foxtail millet in Japan were also tested to compare their genetic distances. This study was based on the hypothesis that if different Mukodamashi varieties had the same genotype, then an original Mukodamashi variety could be theorized to have been introduced to other areas. Conversely, if they have different genotypes, it indicates that different local varieties were named Mukodamashi, or each Mukodamashi had been bred from an original Mukodamashi variety that had been introduced to each area. The results of this study might provide useful information regarding relationships between names of local varieties and gene backgrounds.

**Materials and methods**

**Varieties of foxtail millet**

Varieties of foxtail millet named Mukodamashi, in Gunma, Miyazaki, Ehime, Kochi, and Nara Prefectures, were utilized in this study. Other Mukodamashi varieties were not examined because they could not be obtained. To distinguish the varieties from each other, they were labelled; hereinafter, the varieties will be referred to as follows: Mukodamashi (Gunma), Mukodamashi (Miyazaki), Mukodamashi (Ehime), Mukodamashi (Kochi), and Mukodamashi (Nara), respectively. Furthermore, 23 other local varieties of foxtail millet from various regions of Japan were also examined.

The local varieties used in this investigation and their origins are summarized in Supplementary Table S1. The four Mukodamashi varieties and the other 19 varieties were distributed from Genetic Resources Center, National Agriculture and Food Research Organization (NARO). Mukodamashi (Nara), Kamimurazairai (1), (2), (3), and Mochiwa were collected in their places of origin.

The selection criteria for the varieties were that they were local and not improved varieties. The collection areas for the local varieties covered a widespread area from the northern to southern parts of Japan (Figure 1). Especially, the local varieties in Honshu, Shikoku, and Kyushu Islands were selected, because the five Mukodamashi varieties also originated in these three Islands in Japan.

If the name of the local variety was uncertain, we renamed them based on the name of the collection area and added the word of ‘zairai,’ which means local variety in Japanese. Moreover, in case same locally named varieties already existed, we also used numbers after the variety names to distinguish them, for example, Kamimura zairai (1) and Awa (1).

**DNA extraction**

For DNA extraction, 10 seeds of each variety were germinated in plant boxes filled with vermiculite. After 3 days of germination, three to five seedlings of each variety were selected. Entire plant bodies containing leaves, stems, and roots were homogenized after freezing in liquid nitrogen. DNA extraction was performed using the DNeasy Plant Mini kit (Qiagen, Germany) according to the manufacturer’s instructions.

**SSR marker analysis**

Thirty-seven foxtail millet SSR marker primer sequences (Jia et al., 2009) were used in this investigation. The SSR-PCR amplifications were performed in 10 µL reaction
mixtures, containing 5 μL of GoTaq Master Mix including GoTaq DNA Polymerase (Promega, USA), 5 pmol FAM-labeled universal primer (5′-FAM-gctacggactgacctcggac-3′), 2.5 pmol forward and reverse primers (unlabeled), and 5 ng of template DNA. The ‘gctacggactgacctcggac’ nucleotide sequence was added to the 5′ ends of forward primers as a universal label to obtain FAM-labeled PCR products. For the reverse primers, the ‘gtttctt’ nucleotide sequence was added to the 5′ end as a means of pig-tailing (Brownstein et al., 1996) to enhance the adenylation and facilitate accurate genotyping. SSR marker sequences and their motifs of these 19 SSR markers are shown in Supplementary Table S2. The DNA amplification program was as follows: 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 5 min. The amplified PCR products were separated and detected using a PRISM 3130xl DNA sequencer (Applied Biosystems, USA). The size of the amplified bands was scored against the internal-standard DNA (400HD-ROX; Applied Biosystems), using GeneMapper (Applied Biosystems, USA). SSR markers with one or two distinct and stable peaks for all tested varieties were used as valid SSR markers. Genotypes of the valid SSR markers were used for phylogenetic analysis.

**Phylogenetic analysis**

DARWIN phylogenetic analysis software (Perrier & Jacquemoud-Collet, 2006; http://darwin.cirad.fr/darwin) was used to estimate dissimilarity from the allelic matrices, using simple matching coefficients with 1000 repetitions of bootstrap analysis. Varieties were clustered using the unweighted neighbor-joining (NJ) method according to the estimated dissimilarity.

![Figure 1. Origin locations of the foxtail millet varieties examined in this study. The numbers correlate to the variety numbers given in supplementary table S1.](image-url)
Analysis of grain and stem characters of foxtail millet varieties

To compare the genotypes and phenotypes of the examined foxtail millet varieties, grain characters, that is, endosperm color reaction to iodine-potassium iodide solution, dehusked grain color, glume color, grain shape, and basal leaf sheath color were examined. Dehusked grain color, glume color, and grain shape were determined by observing 30 grains of each variety. The color of the basal leaf sheath was determined by observing 10 plants of each variety in the five-leaf stage. To determine the characters of endosperm of foxtail millet, a simple starch test of color reaction to iodine-potassium iodide solution was performed. To prepare iodine-potassium iodide solution, we dissolved 5 g of potassium iodide in 100 mL of distilled water and added 1 g of iodine; the solution was diluted three times with distilled water. Thirty grains of each variety were cut in half, and the solution was added on the section. After 1 min, color reaction of the grain section was observed with a visual assessment. In case the grain has nonwaxy starch, the section turns dark purple. In case the grain has waxy starch, the section turns reddish brown (Fukunaga, 2019).

Results

SSR marker analysis

For genotyping the 28 foxtail millet varieties, 37 SSR markers (Jia et al., 2009) were tested. Of these SSR markers, 19 presented stable and distinct fragment amplification in all tested varieties (Supplementary Table S3). Therefore, these 19 SSR markers were considered sufficiently reliable to assess genetic diversity. While most varieties showed homozygous genotypes for the 19 SSR markers, 2 SSR markers in 27. Yoshitoshi and 1 in 24. Awa (2) were heterozygous (Supplementary Table S3).

Genetic relationships among the different foxtail millet varieties

We constructed a phenogram of the 28 foxtail millet varieties, based on their SSR marker genotypes. The varieties were divided into four unspecific groups (Figure 2). The phenogram showed that 26 of the 28 varieties could be differentiated from each other, whereas 25. Shinano No. 1. and 8. Kamimura zairai (3) had identical genotypes. The five Mukodamashi varieties had different genotypes.

Several combinations showed close relationships, as they shared more than half of their SSR marker alleles. Varieties 26. Nekoashi and 16. Sarude shared 18 out of their 19 SSR marker alleles, and they also shared 15 SSR marker alleles with 7. Kamimura zairai (2). Whereas, 11. Shiromochi and 12. Mushiwa shared 10 SSR marker alleles and 4. Mukodamashi (Kochi), 3. Mukodamashi (Ehime), and 19. Ikedakei shared 12 or 10 SSR marker alleles.

Figure 3 shows the geographic distributions of these groups. Groups I, II, III, and IV did not show distribution tendencies. Groups I, II, and III were distributed from the northern area to the southern area in Japan. Group IV was cultivated in the southern area, and there were only two samples. Thus, geographic distribution tendencies were not clear from the results of this investigation.

Grain and stem characters of foxtail millet varieties

Table 1 summarizes the grain and stem characters of the foxtail millet varieties. In the simple starch test, the sections of the five Mukodamashi varieties were reddish brown. Moreover, the dehusked grain of the five Mukodamashi varieties were white or yellow white. However, Mukodamashi varieties had different stem characters in terms of the color of the basal leaf sheath. Mukodamashi (Gunma), Mukodamashi (Miyazaki), and Mukodamashi (Ehime) were red; Mukodamashi (Kochi) was light red; and Mukodamashi (Nara) was green.

Varieties 8. Kamimura zairai (3) and 25. Shinano No. 1 had the same characters in this study. Varieties 26. Nekoashi and 16. Sarude also had same characters, except dehusked grain color. Furthermore, both 26. Nekoashi and 16. Sarude were similar to 7. Kamimura zairai (2). However, in the easy starch test, the endosperm of 26. Nekoashi and 16. Sarude was dark purple, and that of 7. Kamimura zairai (2) was reddish brown.

Discussion

Japanese famers who cultivate foxtail millet inherit cultivation techniques and the seed of local varieties from their predecessors. This means that famers often keep local varieties via in-house seed production. Furthermore, introducing breeding or selective breeding is common for local varieties. In this study, 27. Yoshitoshi and 24. Awa (2) showed heterozygous genotypes in 1 and 2 SSR markers, respectively. This indicates that some of the local varieties are hereditarily unfixed, and these phenomena were caused by crossing other varieties during in-house seed production.

Some varieties that were cultivated in close geographical distances, were also close genetically. For example, 11. Shiromochi and 12. Mushiwa were genetically close, and both varieties were cultivated in Akita Prefecture. Similarly, 3. Mukodamashi (Ehime), 4. Mukodamashi (Kochi), and 19. Ikedakei had a close genetic
genotype analyses enabled varieties with different names but the same genotype or the same name but different genotypes to be clarified.

Although the examined varieties were divided into four groups during the analyses, there were no geographical tendencies to the groups. Moreover, there was no tendency between the examined phenotypes and these four groups. Studies have classified foxtail millet varieties. For example, Ochiai (1996) classified foxtail millet varieties into four groups by considering their tillering type. Takei and Sakamoto (1989) examined the photosensitivity of foxtail millet varieties and divided the varieties into three groups. Studies have previously used genetic analysis to classify foxtail millet varieties. Kawase and Sakamoto (1987) classified foxtail millet varieties into four groups, using the pollen and seed fertilities of F1 hybrids of the foxtail millet. Fukunaga et al. (2002) classified varieties into five groups using the RFLP analysis. These studies dealt with varieties from all over the world, and Japanese foxtail millet varieties have often been classified into just one or two groups. In contrast, here, we dealt with only Japanese varieties. Moreover, there were no tendencies between

Figure 2. The genealogical tree of foxtail millet. The DARWIN phylogenetic analysis software (http://darwin.cirad.fr/darwin) was used to estimate the genetic distances from the allelic matrices using simple matching coefficients; bootstrap analysis was computed for 1000 repetitions. Varieties were clustered using the unweighted neighbor-joining (NJ) method.

relationship and were collected from neighboring prefectures. These findings suggest that these varieties had the same or closely related ancestors.

Conversely, 6. Kamimura zairai (1), 7. Kamimura zairai (2), and 8. Kamimura zairai (3) were collected from geographically close areas, but they had distant genetic relationships. These results indicate that some local varieties that were cultivated in the same prefectures had genetic diversity, and they were derived by farmers intentionally or unintentionally by repeatedly introducing different foxtail millet varieties.

Here, we found that gene distances are related to the phenotypes of the foxtail millet. For example, the results for 26. Nekoashi and 16. Sarude showed that they were close genetically. Furthermore, both varieties had the same grain and stem characters. The results of 25. Shinano No. 1. and 8. Kamimura zairai (3) showed that they have the same genotype, as all examined SSR markers showed identical genotypes. The results of phenotype examination showed that both varieties had the same grain and stem characters. As 25. Shinano No. 1. grows widely in Nagano Prefecture, it is possible that the local farmers gave it a different name. The SSR marker
the examined phenotypes and these four groups. Therefore, we cannot discuss the results of these previous studies in relation to the results presented here. The discussion of the characteristics of our four groups is a subject for future analysis.

The grain and stem characters of Mukodamashi (Gunma), Mukodamashi (Miyazaki), Mukodamashi (Ehime), Mukodamashi (Kochi), and Mukodamashi (Nara) were compared. Of course, the color of the unhusked grain of foxtail millet correlates with the xanthophyll content, and they are affected not only by genotype, but also growing environment (Yano et al., 2017). The results showed that the five varieties had white or yellow grains in this study. The results also showed that the endosperm of the five Mukodamashi varieties turned reddish brown in the simple starch test. According to Fukunaga (2019), the endosperm of foxtail millet is of three types. One is nonwaxy type, the others are waxy type and low-amylose type. Here, it was difficult to determine the endosperm type using the simple starch test. All Mukodamashi varieties turned reddish brown in the simple starch test. This result indicated that the grain of all Mukodamashi varieties had high amylopectin starch. Therefore, all Mukodamashi varieties had some common features, such as white or yellow white grains and not nonwaxy type. In other words, the five Mukodamashi varieties turn white and sticky when cooked.

On the contrary, the basal leaf sheaths of the five Mukodamashi varieties were of three different colors. The results of grain and stem characters suggested that these varieties have different genotypes. The results of the SSR marker analysis support this suggestion.

The SSR analysis results showed that the five Mukodamashi varieties have different genotypes; we concluded that the five Mukodamashi varieties examined in this study had the same name, but with different genotypes. In other words, we concluded that most of the local varieties were named Mukodamashi.
Table 1. Grain characters of foxtail millet varieties examined in this study.

| No. | Variety name          | Endosperm color reaction to iodine-potassium iodide solution | Dehusked grain color | Glume color | Grain shape | Color of basal leaf sheath |
|-----|-----------------------|---------------------------------------------------------------|----------------------|-------------|-------------|---------------------------|
| 1   | Mukodamashi (Gunma)   | Reddish brown                                                 | White                | Yellow      | Circular    | Red                       |
| 2   | Mukodamashi (Miyazaki)| Reddish brown                                                 | White                | Yellow      | Circular    | Red                       |
| 3   | Mukodamashi (Ehime)   | Reddish brown                                                 | White                | Light yellow| Circular    | Red                       |
| 4   | Mukodamashi (Kochi)   | Reddish brown                                                 | Yellow white         | Light yellow| Circular    | Red                       |
| 5   | Mukodamashi (Nara)    | Reddish brown                                                 | White                | Yellow      | Circular    | Green                     |
| 6   | Kamimura zairai (1)   | Reddish brown                                                 | Yellow white         | Yellowish brown| Circular | Purple                   |
| 7   | Kamimura zairai (2)   | Reddish brown                                                 | Yellow white         | Yellow      | Circular    | Green                     |
| 8   | Kamimura zairai (3)   | Dark purple                                                   | Yellow white         | Orange      | Circular    | Green                     |
| 9   | Fukuka Shimabara      | Dark purple                                                   | Yellow white         | Yellow      | Ovalat      | Purple                   |
| 10  | Tranoo                | Dark purple                                                   | Yellow white         | Brown       | Circular    | Light red                |
| 11  | Shirottomichi        | Dark purple                                                   | Yellowish brown      | Orange      | Ovalat      | Green                     |
| 12  | Mishiawa              | Reddish brown                                                 | Yellow white         | Yellow      | Ovalat      | Purple                   |
| 13  | Kiwa                  | Reddish brown                                                 | Yellow white         | Light yellow| Ovalat      | Light red                |
| 14  | Kariwano zairai       | Reddish brown                                                 | Yellow white         | Light yellow| Ovalat      | Light red                |
| 15  | Toranoo, Shiroawa     | Reddish brown                                                 | Yellow white         | Yellow      | Ovalat      | Green                     |
| 16  | Sarude                | Dark purple                                                   | White                | Yellow      | Ovalat      | Green                     |
| 17  | Izumi zairai          | Reddish brown                                                 | White                | Yellow      | Ovalat      | Light red                |
| 18  | Aoanawa               | Reddish brown                                                 | Yellow white         | Light yellow| Ovalat      | Green                     |
| 19  | Ikedakei              | Reddish brown                                                 | Yellow white         | Yellow      | Ovalat      | Light red                |
| 20  | Iyakei                | Reddish brown                                                 | Yellow white         | Yellowish brown| Ovalat | Light red                |
| 21  | Kizawakei             | Reddish brown                                                 | Yellow white         | Yellow      | Ovalat      | Light red                |
| 22  | Shimokatsugi          | Reddish brown                                                 | Yellow white         | Yellow      | Ovalat      | Green                     |
| 23  | Awa (1)               | Reddish brown                                                 | Yellow white         | Yellowish brown| Ovalat | Light red                |
| 24  | Awa (2)               | Reddish brown                                                 | Yellow white         | Yellow      | Circular    | Green                     |
| 25  | Shinano No. 1         | Dark purple                                                   | Yellow               | Orange      | Ovalat      | Green                     |
| 26  | Nekoashi              | Dark purple                                                   | Yellow white         | Yellow      | Ovalat      | Green                     |
| 27  | Yoshitoshi            | Reddish brown                                                 | Yellow white         | Yellowish brown| Ovalat | Green                     |
| 28  | Mochiwa               | Reddish brown                                                 | Yellow white         | Orange      | Ovalat      | Red                       |

the long genetic distance, each of the Mukodamashi varieties examined in this study had different ancestors, except for Mukodamashi (Ehime) and Mukodamashi (Kochi). It is possible that only these two had a common ancestor and both Mukodamashi had been bred from an original Mukodamashi variety that had been introduced to both areas. Our results showed that local varieties had the same name, even if they share similar characters.

All Mukodamashi varieties examined in this study have white and sticky grains. This suggests that these characters are related to the name Mukodamashi, because dumplings made from white and sticky grains were similar to rice cakes. However, other varieties had different names even if they had white and sticky grains. Future research should focus on why only some varieties are named Mukodamashi.

Acknowledgments

Among of the 28 foxtail millet varieties examined, 23 were obtained from the Genetic Resources Center, National Agriculture and Food Research Organization (NARO), Japan.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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