Genetic Diversity among Japanese Cultivated Sorghum Assessed with Simple Sequence Repeats Markers

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Abstract : The objectives of this study were to determine the genetic diversity of Japanese cultivated sorghum (sorghum lines cultivated in Japan), using the simple sequence repeats (SSR) technology and to determine the diversity of sorghum breeding germplasm accessions and their relationship with Japanese cultivated sorghum. The cluster analysis of SSR data showed that Japanese cultivated sorghum showed a wide genetic background, but the lines from the same breeding station tended to have a close genetic background. Sorghum breeding germplasm accessions that included lines from ICRISAT, U.S.A., Japan, and improved lines (elite inbred lines) showed a narrower genetic background than Japanese cultivated sorghum and were distinctly separated from them. An efficient method of using germplasm as a genetic resource is proposed.

Key words : Genetic diversity, Sorghum, SSR.

Simple sequence repeats (SSR) markers are useful indices for studying the genetic diversity of the world sorghum germplasm lines, and were used for the study of Eritrean sorghum landraces, the elite sorghum inbred lines and the sorghum from Southern Africa (Dje et al., 2000; Ghebru et al., 2002; Smith, et al., 2000; Uptmoor et al., 2003); genetic redundancy in the 'Orange' sorghum (Dean et al., 1999); and mapping of sorghum genome (Bowers et al., 2003; Menz et al., 2002). SSR fingerprints are generally highly discriminative and are often used to distinguish varieties, or even individuals, and reveal parentage and identity (Karp et al., 1996) and grouping the maize germplasm based on heterotic groups (Reif et al., 2003). The other advantages of SSR markers include the need of only small quantities of a DNA sample, and technical simplicity.

Sorghum is believed to have been brought to Japan about 700~1100 years ago, probably from China through Korea (Matano, 2000). Recently, sorghum is generally used as feed and green fodder for livestock in Japan. Sorghum has been introduced to Japan from U.S.A. and some sorghum cultivars in Japan are probably derived from U.S.A. germplasm. For effective conservation, selection and use of crop genetic resources in breeding programs, a population should be characterized by the amount and type of genetic variability it contains (Fehr, 1987). However, the detailed information of the pedigree history of sorghum cultivars cultivated in Japan is not known and information of genetic background and genetic diversity of these sorghum cultivars has not been reported yet at least at the DNA level.

We had bred inbred sorghum lines with superior characters, such as early maturity, short plant height, high yield and tolerance to Al toxicity (Anas and Yoshida, 2000; 2002; Can and Yoshida, 1999a; 1999b; Can et al., 1997). Almost all of the sorghum breeding germplasm were from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT)-India, Kansas University-U.S.A. and Chugoku National Agricultural Experiment Station-Japan. However, information about genetic background of these materials using DNA-based techniques and relationship with Japanese cultivated sorghum has not been observed yet.

The objectives of this study were (i) to determine the genetic diversity of Japanese cultivated sorghum (sorghum lines cultivated in Japan), (ii) to know the diversity of sorghum breeding germplasm and their relationship with Japanese cultivated sorghum, using high-throughput SSR technology, (iii) to propose adequate information for the assembly of a wide assortment of sorghum germplasm.

Materials and Methods

1. Plant materials

The sorghum (*Sorghum bicolor* (L.) Moench) lines evaluated in this study, their origins and line grouping are listed in Table 1. Dr. H. Nakagawa (National Institute of Livestock and Grassland Science-Japan), kindly provided the seeds of Japanese cultivated sorghum, which are the hybrid sorghum cultivars in Japan (No. 13~31). Some of Japanese cultivated

Table 1.   List of sorghum genotypes used in this study.

Line No.	Name	Origin/donated	Line grouping	Agronomic groups and morphological note
1	Can 1	Utsunomiya University	Japan-U.S.A. (JU)	grain sorghum, early maturity, Al - susceptible
2	Can 2	Utsunomiya University	Japan-U.S.A. (JU)	grain sorghum, tall, late maturity, Al - susceptible
3	H11	Kansas University	U.S.A. (U)	grain sorghum
4	H13	Kansas University	U.S.A. (U)	grain sorghum
5	C9	Chugoku Natl. Agric. Exp. Stn.	Japan (J)	grain sorghum, early maturity
6	C8	Chugoku Natl. Agric. Exp. Stn.	Japan (J)	grain sorghum
7	ICR3	ICRISAT-India	ICRISAT (I)	grain sorghum, dwarf, late maturity, Al tolerance
8	SPA2	ICRISAT-India	ICRISAT (I)	grain sorghum, Al tolerance
9	Real 60	ICRISAT-India	ICRISAT (I)	Al tolerance
10	D12	ICRISAT-India	ICRISAT (I)	grain sorghum
11	SPAD	ICRISAT-India	ICRISAT (I)	grain sorghum, Al tolerance
12	TX403	Kansas	U.S.A. (U)	
13	Haysudan	U.S.A.	U.S.A. (U)	sudangrass
14	Green A	Hiroshima Pref. Agric. Res. Cent.	Japan (J)	sudangrass
15	Tentaka	Nagano Animal Exp. Stn.	Japan (J)	very late
16	High Sugar Sorgo	U.S.A.	U.S.A. (U)	sorgo
17	Piper	U.S.A.	U.S.A. (U)	sudangrass
18	Suzuho	Nagano Animal Exp. Stn.	Japan (J)	sorgo, silage, fodder
19	Touzan Kou 20	Nagano Animal Exp. Stn.	Japan (J)	
20	Sendachi	Hiroshima Pref. Agric. Res. Cent.	Japan (J)	sudangrass
21	Dry Sudan II	U.S.A.	U.S.A. (U)	sudangrass
22	Green Hope	Hiroshima Pref. Agric. Res. Cent.	Japan (J)	sudangrass, early green
23	Ryuujin-wase	Nagano Animal Exp. Stn.	Japan (J)	sorgo, early green, silage, fodder
24	Koutobun	Snow Brand Seed Co. Ltd.	U.S.A. (U)	sorgo, high sugar
25	Hybrid Sorgo NK326	Takii & Co. Ltd.	U.S.A. (U)	
26	Green A (010134)	U.S.A.	U.S.A. (U)	
27	Cyrup Sorgo No.2	U.S.A.	U.S.A. (U)	sorgo
28	MTCI-Sunrise	U.S.A.	U.S.A. (U)	sudangrass
29	Kazetachi	Nagano Animal Exp. Stn.	Japan (J)	very late, dwarf
30	Sugar Grace	U.S.A.	U.S.A. (U)	sorgo, high sugar
31	Natsuibuki	Nagano Animal Exp. Stn.	Japan (J)	sorgo, silage, fodder

Note : Line No.1 to 12 are sorghum breeding germplasm and No.13 to 31 are Japanese cultivated sorghum (sorghum lines cultivated in Japan).

Table 2.   Characteristic of the nine SSR loci analyzed.

SSR locus	Linkage group[1]	Fragment size (bp)	Number of observed alleles	Diversity index[2]
Sb5-236	G	165-229	10	0.82
Sb6-342	A	265-304	5	0.75
Sb1-1	H	227-295	9	0.82
Sb1-10	D	225-492	20	0.94
Sb5-256	C	154-188	4	0.87
Sb6-84	F	164-218	6	0.80
Sb4-72	B	181-209	7	0.74
Sb5-206	E	101-149	5	0.76
Sb6-34	I	180-202	4	0.70

[1]Dean et al. (1999); [2]Saghai-Maroof et al. (1994).

sorghums were introduced from U.S.A., directly or through seed companies in Japan (line grouping U). Some of them were developed by Nagano Animal Experiment Station or Hiroshima Prefectural Agricultural Research Center - Japan (line grouping J). Seed sources of sorghum breeding germplasm (No. 1~12) that were developed by ICRISAT-India, Kansas-U.S.A. and Chugoku National Agricultural Experiment Station-Japan were obtained as previously described by Anas and Yoshida (2000) and Can and Yoshida (1999a).

Lines No. 1 and 2 are improved lines (elite inbred line) from Utsunomiya University that were pedigree-selected from crossing between Japanese inbred lines (C9) and U.S.A. pure lines (H11 and H13). These improved lines showed superior characters in early maturity, plant height and high grain yield (Can and Yoshida, 1999a) and were grouped as line grouping JU in Table 1.

Lines No. 3, 4, 12 are pure lines from Kansas University-U.S.A. and lines No. 5, 6 are inbred lines from Chugoku National Agricultural Experiment Station-Japan. Inbred lines from Japan (No. 5 and 6) showed superior character in short plant height (Can and Yoshida, 1999c). Lines No. 7~11 are pure lines from ICRISAT-India and were grouped as line grouping I (Table 1). Lines No. 8, 9 and 11 are lines tolerant to Al-toxicity (Anas and Yoshida, 2000).

2.   DNA preparation

Seeds were grown in a growth chamber at $27^{\circ}C$ with 12d light. Genomic sorghum DNA was isolated from leaves collected from 10- to 15-day old seedlings using Nucleon Phytopure (Amersham). Total DNA was extracted from 0.1g fresh weight of leaves following supplied instructions that yielded about 20 to 100ng total DNA. A 100 dilution of these total DNA extracts was consecutively used for PCR reactions.

3.   SSR primers

Nine primer pairs were used for genotyping (Table 2). These oligonucleotide sequences were derived from SSR-containing clones of *Sorghum bicolor* cultivar RTx430 (Brown et al., 1996). The SSR markers used are distributed widely across the sorghum linkage groups (A-I), thus giving the comprehensive coverage of the sorghum genome.

4.   PCR amplification

PCR amplification was performed in a volume of 20 μL containing approximately 20ng of templete DNA solution (1μL), 1.23μg to 4.55μg of each primer, 1 U Qiagen PCR buffer, 200μM of each dNTP, 15 mM $MgCl_2$, and 2.5 U *Taq* DNA Polymerase (Core Kit-Qiagen). For maximum yield and specificity, annealing temperature (T_m) and cyling times were optimized for each primer pair. Annealing temperature was generally set $2^{\circ}C$ below the highest T_m of each primer pair.

Reactions were run in a Takara PCR Thermocycler MP TP3000 (Takara, Biomedicals) with an initial denaturation step for 4 min at 94oC; followed by 30 cycles of $94^{\circ}C$ for 1 min, $60^{\circ}C$ to $70^{\circ}C$ (depend on primers, Table 2) for 2 min, and $72^{\circ}C$ for 1 min; followed by a final extension at $72^{\circ}C$ for 10 min. Samples were not diluted prior to electrophoresis.

5.   Electrophoresis and Detection

In order to obtain a precise estimate of fragment size and to identify small size differences between fragments, polymorphic PCR products were electrophoresed on 8% non-denaturing polyacrylamide gels (10 cm length) running vertically. After ethidium bromide staining, amplified fragments were visualized using LAS-1000UV mini (Fuji Photo Film Co., LTD) connected with Image Reader Software Version 1.01 (Fuji Photo Film Co., LTD) and size of DNA fragments was determined using Science Lab. 2001-Image Gauge Ver.4.0 software (Fuji Photo Film Co., LTD), which could automatically recognize
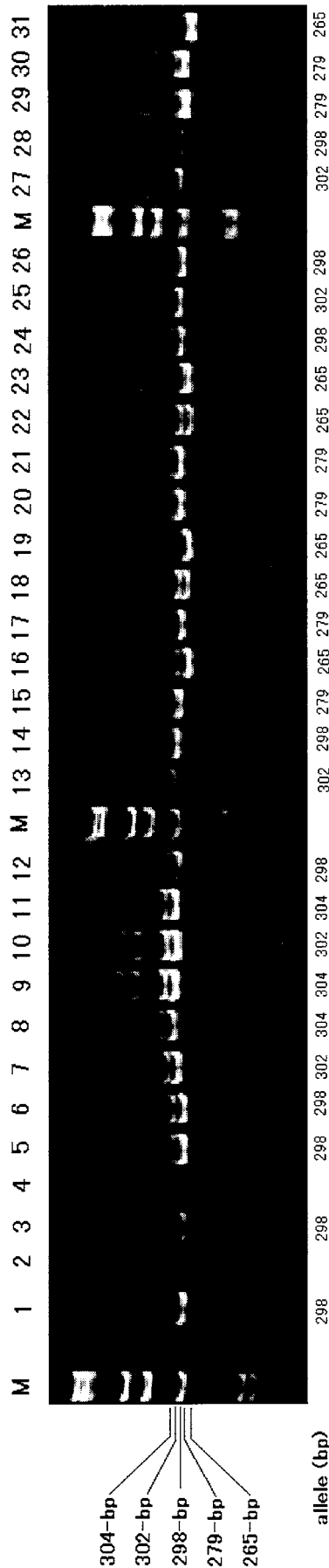
Fig. 1. PCR products from 31 sorghum lines amplified with SSR-specific sorghum primer pair Sb6-342. Line No. is shown above the photograph. M=1-kb size marker. Note : diagnostic shadow bands (DSBs) were observed in some lines.

the lane and band (the peaks region) and measure the fragment size. Only the reported allele size of loci (Brown, et al., 1996; Ghebru et al., 2002) as an amplification product of SSR was used for genotyping (Table 2). The size of the DNA fragments was determined relative to the size standard from the 1-kb size marker GIBCO/BRL.

6.   Statistical analyses

Genotypes of individual plants were represented by the allele size at all SSR loci. Genetic distance was estimated by the pairwise difference method and was calculated for each haplotype pair using the parameters F_{ST} in Arlequin ver. 2.000 software program (Schneider et al., 2000). Distances (d_{xy}) were estimated by simply counting the number of different alleles between two haplotypes :

$$\hat{d}_{xy} = \sum_{i=1}^{L} \delta_{xy}(i)$$

where $\delta_{xy}(i)$ is the Kronecker function, and is equal to 1 if the alleles of the ith locus are identical for both haplotypes and equal to 0 otherwise.

The resulting dissimilarity matrix was employed to construct dendograms by the neighbor-joining method of Saitou and Nei (1987) and the UPGMA method of clustering using the 'Neighbor' program in the Phylip package programs (Felsenstein, 1993). Performance of tree was optimized by 'Treeview' application software (Page, 1996).

The genetic variation of each locus which shows the utility and general information of the informativeness of each SSR as a tool was measured in terms of the number of observed alleles and diversity index (Saghai-Maroof et al., 1994) as DI=1-$\sum p_i^2$, where p_i is the frequency of the ith SSR allele.

Results

1.   Amplification product and estimation of genetic diversity

In the present study, null alleles were consistently observed for Sb6-342 in line No. 4 (inbred line) and its progeny (No. 2-improved line). Some amplification products had a higher molecular weight (shadow, heteroduplex and faint minor band) than the SSR allele band. However, nine SSR markers produced all the expected SSR allele size reported (Brown, et al., 1996; Ghebru et al., 2002).

In total, 70 SSR alleles (different fragment size) were observed in this study (Table 2). All nine loci were polymorphic. Fig. 1 shows polymorphic PCR products from 31 sorghum lines amplified with primer pair Sb6-342. A summary of the number of alleles and diversity indices for each locus is presented in Table 2. The number of alleles per locus ranged from four (Sb5-256 and Sb6-34) to 20 (Sb1-10), and the average number of alleles per locus was 7.2. Diversity indices of
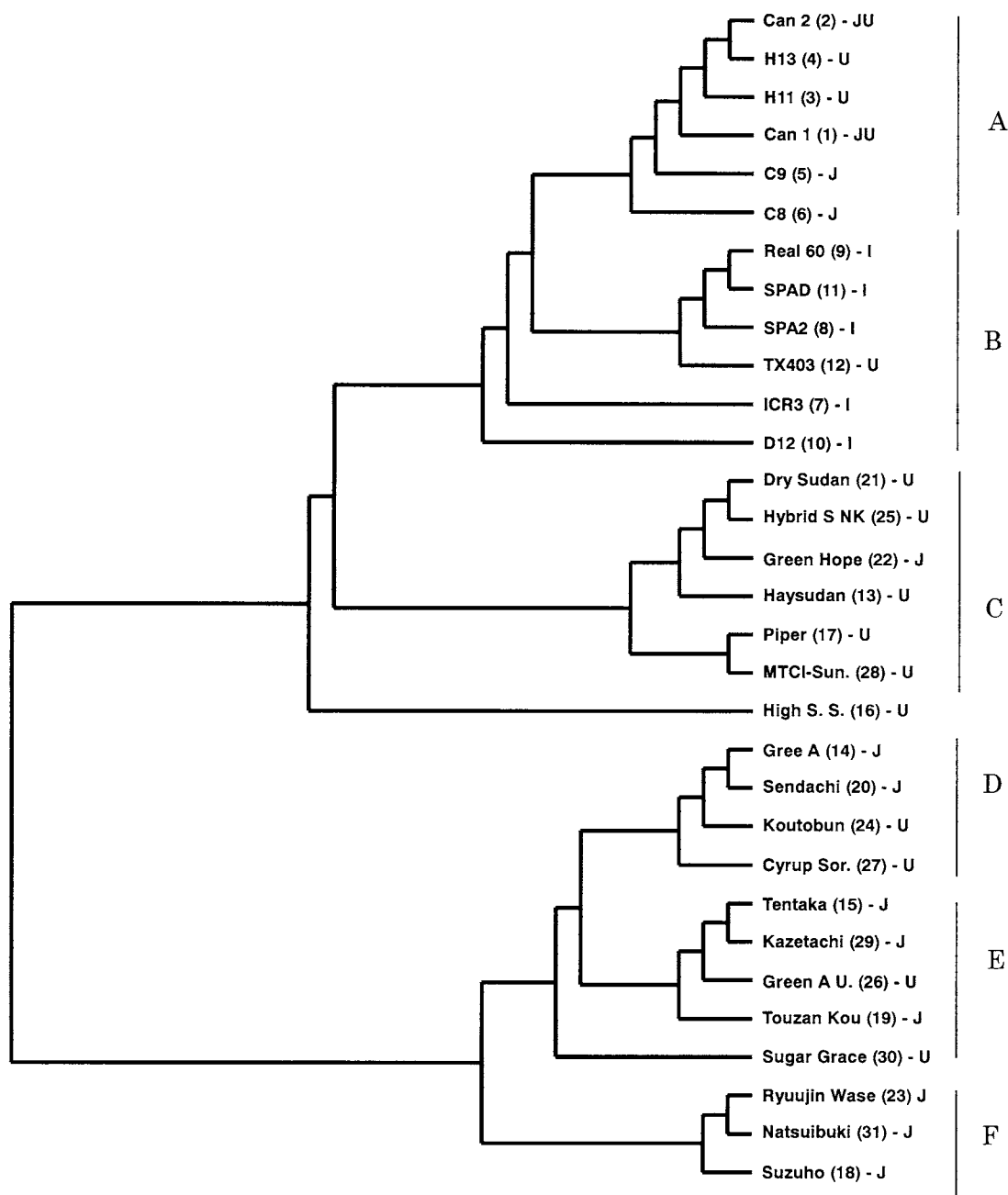
Fig 2.   Dendogram showing genetic relationship among Japanese cultivated sorghum and sorghum breeding germplasm. The numbers in parentheses indicate the line number shown in Table 1. Letters on the right of line name indicate line grouping shown in Table 1 (J=Japan; U=U.S.A.; JU=Japan-U.S.A.; I=ICRISAT). Letters on the right indicate cluster group. Note that branch lengths are not proportional to genetic distance.

each locus ranged from 0.70 (Sb6-34) to 0.94 (Sb1-10).

2.   Genetic relationship among lines

All of the sorghum lines included in this study could be uniquely identified by nine SSR loci. The clustering of the sorghum lines is shown in Fig. 2. All lines were distinctly placed in this dendogram, and showed clustering into six groups (A - F).

All lines from ICRISAT (I line) were clustered into a

cluster group B. Lines from Japan (J line) were widely scattered into five cluster groups (A, C, D, E and F) and lines from U.S.A. (U line) scattered into five cluster groups (A, B, C, D, and E).

All lines from Nagano Animal Experiment Station or Hiroshima Prefectural Agricultural Research Center or Chugoku National Agricultural Experiment Station tended to be placed in the same cluster group. Lines from Nagano Animal Experiment Station were placed in cluster groups E and F, while lines from

Hiroshima Prefectural Agricultural Research Center were clustered within groups C and D. Lines from the Chugoku National Agricultural Experiment Station were clustered within group A.

Cluster group A had two lines from U.S.A. and cluster group C included five lines from U.S.A. Two lines and one line from U.S.A. (U line) were clustered within groups D and E, respectively.

Sorghum breeding germplasm lines that included inbred lines from Japan, pure lines from U.S.A., ICRISAT and improved lines were clustered into cluster groups A and B, and they were distinctly separated from Japanese cultivated sorghum (cluster groups C, D, E and F). Among sorghum breeding germplasm lines, all pure lines from ICRISAT (I line) were distinctly placed in a different cluster group from the two improved lines from Utsunomiya University (JU line) and inbred lines from Japan (J line).

Discussion

1.   SSR diversity and genotyping

Nine of the SSR markers that we applied for the genetic diversity in this study showed polymorphisms with a relatively high diversity index (Table 2). Brown et al. (1996) also observed polymorphisms with a high diversity index. The diversity index of each SSR locus ranged from 0.70~0.94, which allowed us to discriminate each of 31 sorghum lines. This suggests that each SSR marker would be useful as a tool in categorizing sorghum germplasm. We also can use the diversity index value for comparison with other types of molecular markers (Brown et al., 1996). Nine SSR markers listed in this study almost covered all of the sorghum genomes (Dean et al., 1999). Thus, these markers should represent the genetic diversity among these sorghum lines.

Null alleles were observed only in one inbred line from Japan and its progeny. There seemed to be a close relationship between the parents and the progeny in the capability of SSR amplification. Dean et al. (1999) also observed null alleles for one SSR marker in all five individual samples of sorghum 'Orange'. Studies on null alleles in microsatellite amplification in humans and other mammals have revealed that null alleles are generally a result of mutation in the priming region (Callen et al., 1993; Paetkau and Strobeck, 1995).

The minor bands (shadow, heteroduplex and faint bands) have no affect on the occurrence of the SSR allele bands; in fact, they can be useful during gel scoring for genotype verification because the minor bands are generally consistent (Wang et al., 2003; Rodriguez et al., 2001). Shadow bands can arise from misreading by Taq DNA polymerase or from alterations of the secondary structure of the DNA. Faint bands, however, are believed to be PCR artifacts in homozygous individuals, while heteroduplex bands

are caused by the reannealing of non-complementary strands during the later rounds of PCR and occur only in heterozygous individuals (Rodriguez et al., 2001). However in this study, shadow, heteroduplex and faint bands were not considered in scoring and genotyping.

2.   Genetic diversity of sorghum germplasm

Japanese cultivated sorghum that included lines from Japan and U.S.A. (groups J and U) showed a wide genetic background (scattered into groups C, D, E and F). However, the greater degree of gene diversity among Japanese cultivated sorghum was found to be associated with the lines from Japan (J line), which were widely scattered in these cluster groups.

Japanese cultivated sorghum has been developed at various breeding stations in Japan including research centers and private companies resulting in the wide genetic diversity in their background. However, lines from the same breeding station tended to have a close genetic background. For example, three pairs of lines from Japan (J line) that were placed at the same branch in this study were from the same origin : (1) Lines No.14 and 20; (2) Lines No.15 and 29; and (3) Lines No.23 and 31. The similar or limited genetic source in the country or research center for development of sorghum cultivars might have caused the low genetic diversity among breeding lines.

Except for line No. 19 (no maturity information), all lines from Nagano Animal Experiment Station within cluster group E were late or very late maturing (data not shown). This might be why they were distinctly separated from cluster group F. Ghebru et al. (2002) using Eritrean sorghum landraces reported that SSR data reflected their morphological characteristic.

Sorghum breeding germplasm (cluster groups A and B) that included lines from ICRISAT, Japan, U.S.A. and improved lines showed a narrower genetic background than Japanese cultivated sorghum and genetically were different from them.

Except for No. 9, most of the lines from ICRISAT (I line) were selected for resistance to leaf diseases, early vigor, stay-greenness, high yield, and Al tolerance and showed a difference in genetic background from lines from Japan (J line) or improved lines (JU line). Smith et al. (2000) reported that SSR data was in agreement with the pedigree information or performance information.

To our knowledge, this is the first report on the genetic relationship among Japanese cultivated sorghum lines based on microsatellite markers. Japanese cultivated sorghum that included lines from Japan and U.S.A. showed a wide genetic background, but lines from the same breeding station tended to have a close genetic background. Sorghum breeding germplasm that included lines from ICRISAT, Japan, U.S.A. and improved lines showed a narrower genetic background than Japanese cultivated sorghum and

were distinctly separated from them. The efficiency of using germplasm as a genetic resource and its use can be improved if genetic diversity information is available. For example, further crossing between sorghum breeding materials and lines located in cluster groups D~F might give a wide range of ancestors, which could include promising lines with a high yield.

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