A Study on 'Genomewide Selection' for Maize (*Zea mays* L.) Breeding in Japanese Public Sectors: Single Nucleotide Polymorphisms Observed among Parental Inbred Lines

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Abstract

Recent rapid decrease of the cost for detecting single nucleotide polymorphisms (SNPs) has enabled maize (*Zea mays* L.) breeders in Japanese public sectors to introduce such molecular breeding techniques as 'genomewide selection (GwS)'. It has been found in the previous simulation study by the authors that GwS can be a powerful tool to accelerate yield improvement if molecular markers can be arranged over the whole genome at intervals of 20 cM or shorter. The purpose of this study was to discover SNPs of maize parental inbred lines developed in Japanese public sectors to conduct GwS in the future. Here 38 individuals from 32 inbred lines, all but one of which had been developed in Japanese public sectors, were genotyped on up to 56110 SNP loci over the whole genome. The results (1) are thought reliable on the whole because they mostly accord with those having been obtained in the past studies and with the breeding records, (2) have shown that sufficient SNP markers can be arranged to conduct GwS, and (3) that some inbred lines have many and/or large unfixed genome regions, suggesting the necessity to revise the assumption having been made in the previous simulation study by the authors that the intra-inbred-line polymorphisms can be negligible.

Key words: genomewide selection, maize breeding, molecular marker, single nucleotide polymorphism

Introduction

There are concerns in Japan on the difficulties in ensuring long-term food supply, which makes the Japanese government promote a policy to raise the food self-sufficiency. A primary measure for this goal is to raise the feed self-sufficiency rate from 26 (in 2008) to 38% [11]. Japanese public sectors are now expected to support the policy through breeding high-yield maize (*Zea mays* L.) varieties for silage use that are highly adapted to the Japanese climates.

Molecular breeding techniques including quantitative trait locus (QTL) analysis [22] and association mapping [25] have widely been applied to maize because of its simple genome construction and economic importance [6]. It has been, however,

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substantially impossible for maize breeders in Japanese public sectors to introduce some of such techniques because they have not had so much information on molecular marker polymorphisms covering the whole genome of their materials as required in these techniques. But this situation has rapidly been changed in these few years; the cost for detecting single nucleotide polymorphisms (SNPs) has drastically been decreased [23], which has enabled maize breeders in Japanese public sectors to explore SNPs over their materials and to introduce some molecular breeding techniques.

The purpose of this study was to discover SNPs over the whole genome of parental inbred lines (hereafter they are simply called inbred lines) developed in Japanese public sectors to conduct 'genomewide selection (GwS)' in the future. GwS is a molecular breeding technique whose details are explained with its concept in Bernardo *et al.* [1] and Meuwissen *et al.* [12]. GwS is thought advantageous for maize breeders in Japanese public sectors interested in accelerating yield improvement, because its focus is on accumulating favorable genes in many minor QTLs whereby yield is thought controlled [24], and because it can be started from a biparental population, i.e. with molecular marker information on a small number of inbred lines. In the previous study by the authors [20], computer simulations have been made on the assumption that a training population for GwS is developed from a three-way cross $(D_1 \times D_2) \times F_T$, where a new inbred line having high combining ability (i.e. yield) toward a specific flint tester inbred line F_T is developed from a biparental crossing between dent inbred lines D_1 and D_2. The simulations have shown that GwS can be a powerful tool to accelerate yield improvement if the following two conditions are fulfilled; i.e. (1) if molecular markers can be arranged over the whole genome at intervals of 20 cM or shorter, and (2) if the heritability in the training population is 25% or more. In this study the authors have focused on the former condition; discussion will be made especially in terms of the feasibility to conduct GwS with sufficient SNP markers detected in this study.

Materials and Methods

Plant materials

Shown in Table 1 are the 38 individuals from 32 inbred lines whose SNPs were explored in this study. All inbred lines but 'B73', a public line of U.S. widely regarded as the standard [17], have been developed in Japanese public sectors; i.e. National Agriculture and Food Research Organization (NARO) Hokkaido Agricultural Research Center (NARO/HARC), Nagano Animal Industry Experiment Station (NAIES), NARO Kyushu Okinawa Agricultural Research Center (NARO/KARC) or NARO Institute of Livestock and Grassland Science (NARO-ILGS). Their seeds were provided from the stocks in NARO-ILGS for breeding experiments. The 31 lines can be classified into three groups in terms of their genetic background (they are hereafter called genetic groups); dent mainly derived from U.S. corn-belt dent (MD, 12 lines), semi-dent mainly developed from hybrids for summer seeding (RD, 5 lines) and flint mainly derived from Japanese landraces (JF, 14 lines). The numbers from 33 to 38 in Table 1 are the second individuals of the six inbred lines, 'Mi29', 'Mi88' and 'Na71' in MD, 'Mi62' in RD and 'Na50' and 'Na101' in JF, to examine the extent of intra-inbred-line polymorphisms. Their seeds are provided from the same seed lots of the first individuals.

DNA preparation

A part of a fresh leaf (about 1g) of each individual (seedling) growing in a greenhouse of NARO-ILGS was cut by scissors, frozen with liquid nitrogen, and milled with 'Multi-beads shocker®', manufactured by Yasui Kikai Corporation (Osaka, Japan), for 6 times (each time consists of an operation for 5 seconds at 1000 rpm) in the frozen condition. After 300 µl of 'PrepMan® Ultra Reagent', manufactured by Life Technologies Corporation (Carlsbad, CA, U.S.), was added to the milled leaf, the mixture was moved to a 1.5-mL Eppendorf® tube, and the tube was kept in boiling water for 10 minutes. After the stock was cooled down to room temperature, it was processed with protease (incubated in 37 degree centigrade for 60 minutes), and then the DNA was

purified with the procedures consisting of extraction with Tris-buffered 50% phenol, 48% chloroform, 2% isoamyl alcohol solution (in the second time and afterward, it was substituted with chloroform), ethanol precipitation, and dissolution in TE buffer (10 mM Tris-Cl, pH 7.5 and 1 mM EDTA). The procedures repeated for up to four times until each DNA solution exceeded 1.75 in the ratio of absorbance at 260 to 280 nm. After the purification, the density of DNA was adjusted to 50 ng μl^{-1} with the TE buffer.

SNP analysis

The SNP analysis was made with the combination of products manufactured by Illumina Inc. (San Diego, CA, U.S.), 'MaizeSNP50 BeadChip' and an analyzing system including the software 'GenomeStudio', which genotypes up to 56110 SNP loci of maize over its whole genome at once. The datasheet and the manifest file (including the list of the SNP loci) of the BeadChip are in the website of Illumina, http://res.illumina.com/documents/products/datasheets/datasheet_maize_snp50.pdf and http://supportres.illumina.com/documents/downloads/productfiles/maizesnp50/maizesnp50_a.csv, respectively (both sites were cited on January 24th, 2014). Although 'GenomeStudio' allows operators to adjust the setting for judging genotypes on each SNP locus manually, the authors fully followed the automatic judgment made by the software in this study.

Dendrogram drawing

A dendrogram was drawn based on the results to investigate in whether they would accord with the past similar studies and/or our breeding records, i.e. to evaluate their reliability, because the authors thought that it was necessary to verify them prior to the discussion on the primary purpose of this study, the SNPs obtained with the products. The methods adopted for the drawing were mostly equivalent to Enoki *et al.* [8] where the genetic similarity (GS) of maize inbred lines developed in Japanese public sectors had been investigated with simple sequence repeat (SSR) markers; i.e. adopted here were the unweighted pair group method with arithmetic mean (UPGMA) and the computation presented by Dice [5] and Nei *et al.* [15]. On the other hand, the following two slight modifications were made in this study; (1) that genetic distance (GD) was adopted instead of GS, both of which can be converted to each other with the equation that GD=1−GS, and (2) that a combination of a heterozygous genotype "AB" with homozygous "AA" or "BB" was counted as 0.5, which had not been considered in the past study with SSR markers.

Highly distinguishable SNP loci within a genetic group

SNP loci were examined on the ability to distinguish inbred lines within a genetic group i.e. on the potential usefulness in GwS. Let us assume here that N inbred lines ($N \geq 2$), all of which belong to a genetic group G, were genotyped, and that numbers of inbred lines genotyped as "AA" and "BB" are a and b ($0<(a+b)\leq N$), respectively, in an SNP locus L. In this case, D is calculated from the following equation

$$D(\mathrm{L}, G) = \frac{ab}{\mathrm{C}_2^N} \quad \ldots (1)$$

where C_2^N is the number of all possible pairs made in G. If $D>0.45$, L is judged as a 'highly distinguishable locus' in G. The threshold 0.45 has been set based on that D exceeds it if N is between 5 (in RD) and 14 (in JF), if all inbred lines are successfully genotyped as "AA" or "BB", and if the ratio of a to b is between 1:2 and 2:1. In other words, D is lower than the threshold and then the SNP locus is not judged highly distinguishable if genotyping has been failed in one (in RD) or two (in MD and JF) inbred line(s) of the relevant group, or if the genotypes are extremely one-sided to "AA" or "BB".

Intra-inbred-line polymorphic loci

In examining the intra-inbred-line polymorphisms, an SNP locus was regarded polymorphic in the relevant inbred line if at least one of the two individuals had been genotyped heterozygous or if they had been genotyped differently (i.e. combinations of "AA" and "BB"). Combinations of homozygous and unidentified genotypes were not regarded polymorphic.

After identification of intra-inbred-line

polymorphic loci, their ratio was computed in each genome region. A genome region was defined to consist of 500 (a somewhat different number in the end of each chromosome) 'polymorphic and reliable loci' that, according to the SNP list in the website of Illumina, could be regarded consecutive on a single chromosome. See Results and Discussion for the detailed definition of 'polymorphic and reliable loci'.

Results and Discussion

The SNP information was thought reliable

Two indices are often used to evaluate the results of SNP genotyping with the products of Illumina; one is 'Call Rate', the ratio of SNP loci successfully genotyped for each sample, and the other is 'GenTrain Score' to evaluate the confidence of the genotyping for one SNP on all samples [2,4]. The Call Rates exceeded 0.9 in all samples (Table 1), from which it was concluded that no materials need to be excluded, considering that many studies in the past have abandoned samples only if they are below 0.9 [10,18].

GenTrain Scores of the 56110 SNP loci ranged in this study from zero to 0.993. The scores of 12231 loci were below 0.7, which authors screened out, considering that studies in the past including Han *et al.* [9] and Namjou *et al.* [14] on human (*Homo sapiens* L.) as well as Pasam *et al.* [16] on barley (*Hordeum vulgare* L.) adopt this value as the threshold. In addition, another 4561 loci were excluded because the software judged that more than 10% of the materials successfully genotyped on the loci were heterozygous (such high frequency of heterozygosity was thought unrealistic because all of the materials were inbred lines and because almost all of their neighboring loci seemed fixed), or because no polymorphisms could be detected among the 32 inbred

Table 1. The parental inbred lines whose single nucleotide polymorphisms (SNPs) were examined in this study and their 'Call Rate'

Developed in †	No.	Name	Group‡	CR§	Developed in †	No.	Name	Group‡	CR§
(U.S.)	1	B73	-	.986	NARO-ILGS	21	Na50	JF	.950
NARO/HARC	2	Ho95	JF	.954		22	Na65	MD	.961
	3	Ho102	MD	.957		23	Na71	MD	.957
	4	Ho104	MD	.959		24	Na91	MD	.934
	5	Ho110	MD	.955		25	Na96	JF	.942
NAIES	6	Ki66	MD	.962		26	Na99	MD	.942
	7	Ki68	JF	.944		27	Na101	JF	.947
	8	Ki70	MD	.962		28	Na102	MD	.963
	9	Ki74	MD	.960		29	Na103	JF	.945
	10	Ki75	JF	.948		30	Na104	JF	.948
NARO/KARC	11	Mi29	MD	.956		31	N09-07	JF	.932
	12	Mi47	JF	.949		32	N10-03	JF	.945
	13	Mi62	RD	.952	NARO/KARC	33	Mi29¶	MD	.945
	14	Mi71	RD	.957		34	Mi62¶	RD	.940
	15	Mi88	MD	.949		35	Mi88¶	MD	.956
	16	Mi91	RD	.945	NARO-ILGS	36	Na50¶	JF	.931
	17	Mi93	RD	.947		37	Na71¶	MD	.949
	18	Mi103	JF	.930		38	Na101¶	JF	.943
	19	Mi106	RD	.960					
	20	Mi111	JF	.951					

† NARO/HARC, NAIES, NARO/KARC and NARO-ILGS are the abbreviations of NARO Hokkaido Agricultural Research Center, Nagano Animal Industry Experiment Station, NARO Kyushu Okinawa Agricultural Research Center and NARO Institute of Livestock and Grassland Science, respectively.

‡ JF, MD and RD indicate flint mainly derived from Japanese landraces, dent mainly derived from U.S. corn-belt dent, and semi-dent mainly derived from hybrids for summer seeding, respectively.

§ Means 'Call Rate', the ratio of SNP loci successfully genotyped for each sample.

¶ The second individuals for examining intra-inbred-line polymorphisms.

lines. Therefore further analyses were made with the remaining 39318 loci that were judged polymorphic and reliable. On the SNP list of Illumina are the chromosomes of 35508 loci out of the 39318, and the coordinates of 35424 loci out of the 35508. Based on these 35424 loci will be discussed the intra-inbred-line polymorphisms in each genome region (Table 2).

The dendrogram based on the polymorphisms observed in the 39318 SNP loci (Fig. 1) mostly accords with the breeding records and/or the past studies, including that the genetic distances among the three genetic groups, MD, RD and JF, are roughly equivalent [13], that 'Ho102' has been developed from a crossing including 'Mi29', and that 'N09-07' and 'Mi111' have been developed from the same random-mating population. Therefore the authors concluded that the results were reliable on the whole (though it seems to have some errors), and that they would help the maize breeders in Japanese public sectors promote molecular breeding in the future.

The only discrepancy between the results in this study and those in the past is that the American public line 'B73' is very distantly located from MD group, to which it was assumed to belong. This seems to reflect the policy of the manufacturer in designing the BeadChip; the datasheet in the website of Illumina mentions that it has approximately 4000 SNPs that will less perform in other samples than in

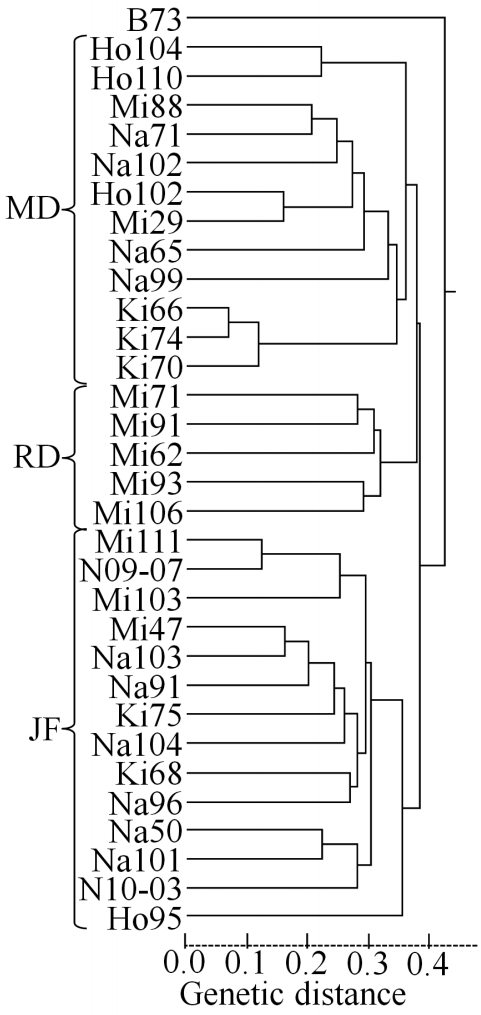


Fig. 1. The dendrogram of the 32 inbred lines drawn based on the genetic distance (GD) having been calculated from the single nucleotide polymorphisms (SNPs) detected in this study

Table 2. The SNP loci adopted for the analyses in this study

Description	Numbers
Total† (a)	56110
GenTrain Score‡ < 0.7 (b)	12231
GenTrain Score‡ ≥ 0.7 but frequency of heterozygosity§ > 0.1 (c)	457
GenTrain Score‡ ≥ 0.7 but no polymorphisms found among all inbred lines (d)	4104
SNP loci with which further analyses were made in this study (e)=(a)-(b)-(c)-(d)	39318
SNP loci in (e) whose chromosomes are identified on the SNP list of Illumina (f)	35508
SNP loci in (f) whose coordinates are identified on the SNP list of Illumina	35424

† Total SNPs that can be detected with a product of Illumina, 'MaizeSNP50 BeadChip'.
‡ See the text for its definitions.
§ See the text for the detailed reason why these SNP loci were excluded.

this inbred line regarded as the most standard and having been used as the materials of various genetic analyses in the past [3,7]. In this study, the number of SNP loci unique to 'B73' was 481, three and 12 times as large as the second largest 'Ho95' (158) and the average of another 31 materials (39.4), respectively.

Sufficient SNPs over the whole genome

As mentioned above, the previous simulation study by the authors [20] has shown that an important key to the success of GwS is to arrange molecular markers at intervals of 20 cM or shorter. Considering the whole genome size is about 1800 cM [1], it means that more than 90 molecular markers should be arranged over the genome. Therefore the primary purpose of this study was to discover many "highly distinguishable SNP loci" i.e. SNPs which can efficiently distinguish inbred lines within a genetic group. It has been found that the numbers of such SNP loci are 7787 (in JF) or more, and that those per cM are 1.94 or more (Table 3. The information on the length of each chromosome in cM is based on the web page http://www.maizegdb.org/cgi-bin/displaycompletemaprecord.cgi?id=1203637, to which the previous simulation study by the authors has also referred). The authors have concluded from these results that sufficient SNP markers can be arranged over the whole genome for Japanese public sectors to conduct GwS.

The datasheet in the website of Illumina suggests that little attention has been paid to Japanese germplasms in choosing the 56110 SNP loci. But the products provided in this study not only more than 39000 polymorphic loci in total but also more than 7700 highly distinguishable loci within the genetic group mainly originated from Japanese landraces. These results suggest that the products have the potential to bring sufficient information on the polymorphisms within another genetic group of maize to which molecular approaches have never been made.

Many and/or large unfixed genome regions in some inbred lines

The averaged heterozygosity rates of the six inbred lines whose intra-inbred-line polymorphisms were examined in this study ranged from 0.05 (Na71) to 2.76% (Mi62) (Table 4), which seem similar to those of 'B73' and 'Mo17' (another standard inbred line in U.S.) indicated on the datasheet in the website of Illumina, 0.32 and 2.50%, respectively.

The ratio of polymorphic loci between two individuals belonging to the same inbred line ranged from 0.09 (Na71) to 4.28% (Mi62) (Table 4). The more important for the authors interested in introducing GwS is that the polymorphic loci seem to concentrate in some genome regions (Fig. 2). Inbred lines have been developed in Japanese public sectors

Table 3. Numbers of 'highly distinguishable SNP loci' within each genetic group and those per cM in each chromosome

Chromosome number (top) and length (bottom, in cM) ‡		1	2	3	4	5	6	7	8	9	10	Total†
		286	183	211	189	173	145	158	160	164	136	
MD§	Numbers¶	1584	494	1324	1137	1478	881	1023	1281	780	804	11804
	Numbers¶ per cM	5.54	2.70	6.27	6.02	8.54	6.08	6.47	8.01	4.76	5.91	
RD§	Numbers¶	1227	449	1143	1177	1168	901	707	962	515	795	9859
	Numbers¶ per cM	4.29	2.45	5.42	6.23	6.75	6.21	4.47	6.01	3.14	5.85	
JF§	Numbers¶	1080	355	1187	1081	693	557	459	654	542	452	7787
	Numbers¶ per cM	3.78	1.94	5.63	5.72	4.01	3.84	2.91	4.09	3.30	3.32	

† The numbers of total SNP loci do not accord with those summed up on all 10 chromosomes because the carrying chromosomes have not been identified in some SNP loci.

‡ The length of each chromosome in cM has been quoted from the website http://www.maizegdb.org/cgi-bin/displaycompletemaprecord.cgi?id=1203637

§ JF, MD and RD indicate flint mainly derived from Japanese landraces, dent mainly derived from U.S. corn-belt dent, and semi-dent mainly derived from hybrids for summer seeding, respectively.

¶ Numbers of 'highly distinguishable SNP loci'. See the text for their definition.

through repeated selfing for five or six times [19,21]. The results shown in this study suggest that some genome regions have remained unfixed during the procedures of repeated selfing. Some inbred lines have been found to have large and/or many unfixed genome regions, suggesting the necessity to revise the assumption having been made in the previous simulation study by the authors [20] that the intra-inbred-line polymorphisms can be negligible. In addition, the supposition of unfixed genome regions in inbred lines will make the choice of molecular markers for GwS more complicated. In the case mentioned in Introduction where the training population has been developed from a three-way cross $(D_1 \times D_2) \times F_T$, heterozygous loci on F_T must be avoided to distinguish genotypes of individuals in the training population accurately, i.e. careful consideration will be required in choosing marker loci in the genome regions that have remained unfixed in F_T. On the other hand, such unfixed genome regions may raise the efficiency of yield improvement. Again in the case mentioned in Introduction, let us assume a genome region where the inbred lines D_1 and D_2 have been unfixed and fixed, respectively. As shown in Fig. 3, two and one candidate genome regions from D_1 and D_2, respectively, i.e. three candidates in total, can accurately be distinguished by two marker loci in a haplotype. It means that breeders can select the best candidate from the three, which may be better than the selection from two in the case both D_1 and D_2 have been fixed.

Table 4. Heterozygosity rate and ratio of intra-inbred-line polymorphic loci of the six inbred lines

Name (genetic group) †	Heterozygosity rate‡	Ratio of 'intra-inbred-line polymorphic loci§'
Mi29(MD)	.0007	.0011
Mi62(RD)	.0276	.0428
Mi88(MD)	.0093	.0191
Na50(JF)	.0016	.0020
Na71(MD)	.0005	.0009
Na101(JF)	.0154	.0172

† JF, MD and RD indicate flint mainly derived from Japanese landraces, dent mainly derived from U.S. corn-belt dent, and semi-dent mainly derived from hybrids for summer seeding, respectively.

‡ The ratio of heterozygous loci to the 'polymorphic and reliable loci (see the text for their definition)' successfully genotyped in the relevant individual. Shown is the average of two individuals each.

§ See the text for their definition.

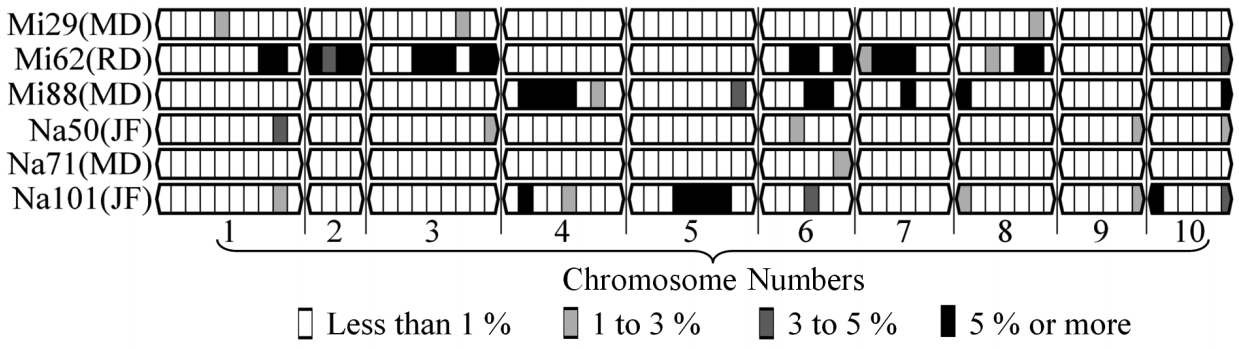
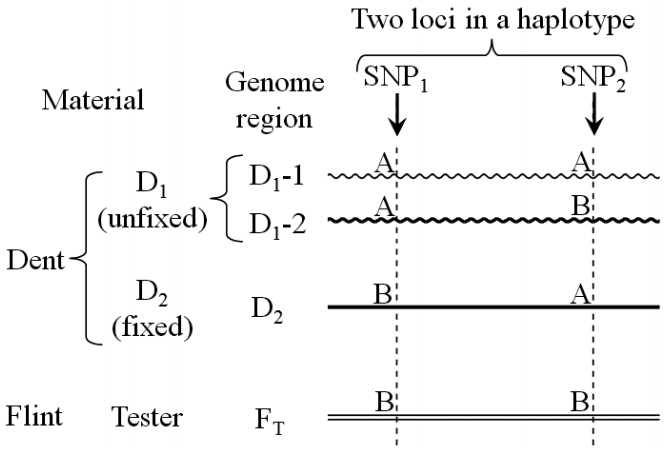


Fig. 2. The ratio of intra-inbred-line polymorphic loci in each genome region (see the text for the definitions) of the six inbred lines



SNP_1	SNP_2	Genotype*
AB	AB	D_1-1/F_T
AB	BB	D_1-2/F_T
BB	AB	D_2/F_T

*Genotype in the relevant genome region.

Fig. 3. A typical model to genotype individuals of a training population developed from a three-way cross ($D_1 \times D_2$) × F_T in a certain genome region where D_1 and D_2 have been unfixed and fixed, respectively

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日本の公的機関におけるトウモロコシ（*Zea mays* L.）育種のためのゲノムワイドセレクションに関する研究：親自殖系統群内に見られた一塩基多型

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摘　要

一塩基多型（SNP）の検知に要する費用が近年大きく減少したことにより，飼料用トウモロコシ（*Zea mays* L.）の育種を担当する日本の公的機関もゲノムワイドセレクション（GwS）の様な分子育種技術の導入が可能になりつつある。前報で著者らはコンピューター・シミュレーションを用い，分子マーカーをゲノム全体に渡って 20 センチモルガン（cM）以下の間隔で配置できれば，トウモロコシの収量性の改良を加速させる上で GwS が有効であることを示した。本研究の目的は，将来における GwS の実施を視野に，日本の公的機関で育成されたトウモロコシ自殖親系統群内における SNP の発見であった。この目的を達するため，32 自殖親系統（うち 31 が日本の公的育種機関の育成）38 個体について，最大 56110SNP 遺伝子座のジェノタイピングを試みたところ，以下の結果を得た。(1) 将来の GwS 実施に充分な数の SNP が発見された。(2) 得られた結果を基に描かれた系統樹が過去の育種記録や既往の知見とほぼ一致した。(3) その一方，2 個体をジェノタイピングした 6 自殖親系統の一部において多数または広大な未固定ゲノム領域が発見された。以上のことから，本研究により得られた結果は高い信頼性を有する一方，「自殖親系統内の多型は無視できるほど小さい」とした前報のシミュレーションにおける前提条件は，実際の GwS においては見直す必要性があると考えられた。

キーワード：ゲノムワイドセレクション，トウモロコシ育種，分子マーカー，一塩基多型

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