Genomic and plant breeding studies on host-plant resistance to Pythium root and stalk rot of maize (Zea mays L.)

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GENERAL INTRODUCTION

Maize and its genetical importance of breeding

Maize (*Zea mays* L.) is one of the major crops and its production is about 1.2 billion tones in the world (FAO 2023). While maize is grown as a staple food and feed in the world, Japan has almost entirely relied on imports. The current policies regarding self-sufficiency in the food supply have resulted in a gradual increase in the area under cultivation with maize for grain usage in Japan. The development of new maize F_1 varieties better suited to Japanese climates will further help increase maize production (MAFF 2021).

Recently, maize varieties are often developed from a cross between two different parental inbred lines to utilize their hybrid vigor called heterosis (Stupar et al. 2008). The combination of inbred lines which express greater heterosis than others is called a 'heterotic pattern', and each group is called a 'heterotic group' (Melchinger and Gumber 1998). As in Europe, common heterotic groups in Japan are dent inbred lines and flint inbred lines (Enoki 2007). The heterosis between dent and flint inbred lines is expressed in the root lodging resistance and dry matter weight in the Japanese public sector and has contributed to the effective breeding of superior maize F_1 varieties (Koinuma 2001). Thus,

a proper understanding of genetic diversity and population structure of inbred lines is essential to develop superior maize F_1 varieties. This will enable the classification of heterotic groups of inbred lines, selection of efficient mates or testers in F_1 development, and the introgression of superior genes from diverse genetic resources. The degree of genetic diversity among the inbred lines has usually been assessed based on morphological data such as the endosperm type, the pedigree record, and the amount of heterosis expressed in the crosses (Enoki 2007). However, these methods present several limitations. The morphological characteristics often do not rely on their genetic relationships. The degree of genetic diversity in inbred lines are affected by selection and random genetic drift during inbreeding (Messmer et al. 1993). Testcross designs with several testers are extremely expensive and overworked. On the other hand, genotyping is one of the most reliable approaches to documenting polymorphisms in selecting inbred lines (Adu et al. 2019). Hence, the development of genotyping tools is essential to achieve this object.

Maize is extremely diverse both phenotypically and genotypically, and numerous tools have been developed to exploit its diversity in hybrid breeding (Riedelsheimer et al. 2012). Moreover, the genetic studies on maize have made remarkable progress in recent years; genome-wide association studies (GWAS) and marker-assisted selection (MAS)

have contributed to the improvement of its important traits including disease resistance (Kump et al. 2011), flowering time (Chardon et al. 2004), and leaf architecture (Tian et al. 2011). As the cost of molecular genotyping has decreased, genome-wide marker polymorphisms have become useful not only for detecting causal genes for agronomic traits through association mapping (e.g., GWAS) but also for predicting the agronomic performance of genotyped individuals without observed phenotypic value in major crops.

'Genomic selection (GS)' or 'Genomic Prediction (GP)' are breeding techniques where a part of phenotypic selection can be replaced by selection of individuals based on their breeding values predicted from genotypic information. The proposal by Meuwissen et al. (2001) has created a remarkable achievement in dairy cattle breeding by genomic breeding values predicted only by genotypic marker information (Hayes et al. 2009b). It has also attracted the attention of crop breeding; Bernardo and Yu (2007) attempted applying GP and GS to maize breeding. A previous computer simulation study, in which the author was also involved, indicated that GP and GS could be powerful tool for maize breeding teams in the Japanese public sector (Tamaki et al. 2012). However, application of GP and GS to actual maize breeding in Japan have not been achieved yet.

Pythium Root and stalk rot (RSR) by Pythium spp. on maize

Root and stalk rot (RSR) of gramineous plants, caused by soil-borne disease pathogens of the *Pythium* spp., restricts crop production in plants across many countries. Pythium RSR of maize caused by *P. graminicola* Subramanian, has been known to cause wilting of the plants in the maturing stages since the 1950s (Dickson 1956, Erwin and Cameron 1957). This disease became common in Japan during the 1980s, spreading in wide areas, especially in hot and wet summers. It has found that *P. arrhenomanes* Drechsler (Drechsler 1928), also affects forage maize in Japan, and has been isolated in Tochigi Prefecture as described below.

Symptoms of the disease include wilting or lodging of whole plants and drooping of ears; consequently, it becomes difficult to process the crop for forage because the plants are too soft to be cut (Tsukiboshi et al. 2016), or their quality and nutritional value reduce. It is known that the disease is triggered under hot and humid conditions, particularly by heavy rainfall during the ripening stage (Deep and Lipps 1996, Reyes-Tena et al. 2018, Yanar et al. 1997). Therefore, recent outbreaks of the disease can be attributed to the effects of global warming, which causes heavy rainfall during the maturing stages of the crop. Soil- or foliar-applied fungicides cannot be used to control the disease because of labor and other costs as well as of legal constraints on forage maize production in Japan, which means that breeding resistant varieties is the only feasible option for its control.

However, this new disease has not been well studied for breeding resistant varieties. Especially, genetic studies of Pythium RSR resistance are important, but only a few studies on quantitative trait locus (QTL) analyses for *P. inflatum* and *P. aristosporum* have been reported in China (Duan et al. 2019, Hou et al. 2023, Song et al. 2015).

First report of Pythium RSR by P. arrhenomanes in Japan and other information to date

In September 2009, typical Pythium RSR symptoms were detected on maize plants cultivated in Tochigi prefecture. The symptoms of the disease included wilting of whole plants after the yellow-ripening stage with drooping ears. Roots turned black and their number decreased. Further, the stalks became hollow, soft, and harbored white hyphae. The author obtained seven isolates of a *Pythium*-like organism by single hypha isolation from surface-sterilized pieces of diseased roots and stems on water agar. One of the isolates was grown in the dark on V8 juice agar medium for 10 d to produce oogonia. The oogonia were globose, light brown to yellow, smooth, 23.9 to 30.5 μm in size, and had one to eight antheridia. Oospores were mostly plerotic, and oogonia walls were 1.3 to 2.7 μm thick. The morphology of the isolate was similar to that of *P. arrhenomanes* Drechsler and consistent with the species description (Van der Plaats-Niterink 1981). The author's team analyzed the rDNA-ITS region sequences of the isolate as described by Kageyama

et al. (2003). The sequence showed 99.1% (783/790 bp) similarity with that of *P. arrhenomanes*. Based on morphological and rDNA sequence similarities, the author's team identified the isolate obtained from maize as *P. arrhenomanes*. The pathogenicity of the isolate was confirmed by planting seedlings of the commercial maize F_1 hybrid '36B08' immediately after germination in five replicate pots containing soil mixed with 5% boiled barley grain by weight, incubated with or without the isolate for 7 d. After 10 d of incubation in a greenhouse at 20 to 25°C, only the inoculated plants exhibited symptoms of Pythium RSR. Since the inoculated organism was readily re-isolated from the diseased stems and roots, the pathogenicity of the isolate was confirmed.

P. arrhenomanes causes severe root rot symptoms in maize and other plants of the *Poaceae* family, but it also infects a wide range of hosts, including green onions (Yamamoto-Tamura and Yoneyama 2014). To date, the genome size of *P. arrhenomanes* has been reported to be approximately 44.7 Mb (NCBI 2024). However, this information is based on short-read assembly, resulting in a fragmented state with 10,972 contigs. Additionally, around 13,800 genes have been annotated (FungiDB 2024).

Objectives of this study

The frequency of isolation of *P. arrhenomanes* is high in Hokkaido, Tochigi and Nagano

pref., and its virulence is high, so it is considered to be a recent major pathogenic species in Japan. The optimum temperature for growth of *P. arrhenomanes* is around 30°C (Tsukiboshi et al. 2016), which is lower than that of *P. graminicola* mainly isolated in the Kyushu area, and therefore, it is expected to increase in northern Japan and cooler regions due to recent warming. Thus, breeding research against this disease will become even more important in the future.

The present study is carried out to develop genome wide molecular breeding method to accelerate the breeding of excellent maize varieties with Pythium RSR resistance by *P. arrhenomanes*. The objectives are to 1) analyze genetic diversity of maize inbred lines for further breeding, 2) confirm the applicability of a bottom stalk toothpick inoculation method and to evaluate the resistance of maize hybrids to Pythium RSR, 3) evaluate simple parental-progeny-based Best linear unbiased prediction (BLUP) in predicting single-cross performance of the resistance to Pythium RSR, 4) develop GP model and to estimate the Pythium RSR resistance of maize inbred lines that have been never evaluated for this disease, and 5) propose genomic approach for improving Pythium RSR resistance in maize F_1 plants. In addition to pursuing these research objectives, the author demonstrates and validates a breeding process of two inbred lines and one novel F_1 variety in the present study.

CHAPTER I. Genetic diversity among maize inbred lines adapted to Japanese climates

Introduction

As described in the previous section, understanding the genetic diversity and population structure of inbred lines is essential to development of superior F_1 varieties (Akaogu et al. 2013, Badu-Apraku et al. 2021, Benchimol et al. 2008,) and will enable the classification of inbred lines into heterotic groups, the selection of efficient mates or testers in F_1 development, and the introgression of superior genes from diverse genetic resources (Betrán et al. 2013, Prasanna 2012, Solomon et al. 2012). Japan spans a long distance from north to south, with the northernmost region, Hokkaido, opting to grow maize varieties and inbred lines with earlier ripening adapted to cold climates. The genetic basis of these inbred lines was previously reported (Enoki et al. 2002) and can be potentially exploited as an essential tool for breeding early maturing varieties in warmer regions. Additionally, reports on the genetic diversity of inbred lines grown south of the Kanto region are available (Tamaki et al. 2014, 2016). However, these inbred lines have not been comprehensively analyzed yet. The objective of this chapter is to extensively deal with Japanese maize inbred lines and to analyze their genetic diversity.

Materials and methods

Plant materials

Table 1 lists details of the 127 inbred lines used in this chapter. All of these were developed in the public sector research stations, namely, Hokkaido, Miyakonojo, and Nasushiobara stations of NARO (The National Agriculture and Food Research Organization, Japan), or Prefectural public breeding sections in Nagano, Japan. The names of these inbred lines start with the first letter of the related locations; 'Ho (Hokkaido)', 'Mi (Miyakonojo)', 'Na (Nasushiobara)', and 'CHU (Chushin area)', respectively. 'Ki' is used as the name of an inbred line in Nagano Pref. and the first letter for the Kikyogahara area in Shiojiri, Nagano Pref., but it is also the name of an inbred line at Kasetsart University in Thailand (Jenweerawat et al. 2009). To avoid confusion, the author used conservation names starting with 'J' and 'JC' instead of 'Ki'. All the inbred lines had clear derivation based on breeding history documented by the developing sectors. The inbred lines were classified into some heterotic groups, where heterotic patterns were expected based on their genetic backgrounds and derivations as described by Enoki et al. (2002), and partially modified following Tamaki et al. (2016). These included that flint mainly developed or derived from the European region (EF), Japanese tropical inbred lines mainly developed from hybrids for summer seeding (RD), flint

mainly derived from Japanese landraces (JF), dent mainly derived from US corn-belt dent (D), and miscellaneous origin (MIS).

DNA preparation

DNA was extracted as previously described by the author's team (Tamaki et al. 2016). Briefly, a fresh leaf section weighing about 1 g from each seedling was cut using scissors, frozen with liquid nitrogen, and milled using 'Multi-beads shocker®' (Yasui Kikai Corporation, Osaka, Japan) thrice for 10 seconds at 1,500 rpm under frozen conditions. DNA was extracted using the 'DNeasy Plant Mini Kit™' (Qiagen, Venlo, Netherlands) per the manufacturer's instructions using 100 µl of the supernatant collected post-milling. DNA concentration was measured using a 'Qubit™ 2.0 Fluorometer' and 'dsDNA HS assay kit' (Thermo Fisher Scientific, Massachusetts, USA). DNA concentrations were adjusted to 10 ng/µl for genotyping reactions.

Genotyping

All inbred lines were genotyped using 'Maize LD Bead chip' (Illumina Inc, San Diego, USA) containing 3,047 single-nucleotide polymorphisms (SNPs) and analyzed using the software 'GenomeStudio 2.0'. While the software allows operators to adjust settings to

judge genotypes on each SNP locus manually, the author opted to follow the automatic judgment made by the software. A custom report in PLINK format using Report Wizard was generated after analysis.

Markers with more than 5% missing data and less than 3% minor allele frequency were removed by 'PLINK' (Purcell et al. 2007) version 1.90. Highly correlated SNPs were eliminated through linkage disequilibrium pruning, utilizing a Variance Inflation Factor of 2.00. Also, the PLINK format data were converted to VCF format using PLINK. The VCF file was then converted to FASTA format using the 'vcf2phylip.py' script (Ortiz 2019). These approaches ensured that the genetic information, including both homozygous and heterozygous genotypes, was preserved for subsequent analyses.

Statistics and population structure analyses

Statistical analyses were adopted to investigate the genetic distinction of the inbred lines based on the SNP marker profiles. Population structure was estimated using principal component analysis (PCA) and phylogenetic tree. PCA was performed using PLINK. A phylogenetic tree was constructed using 'MEGA X' ver. 10.18 (Kumar et al. 2018). The tree was drawn using the unweighted pair group method with the arithmetic mean

(UPGMA) algorithm. The evolutionary distances were computed using the Tamura-Nei (1993) method and were in the units of the number of base substitutions per site. Also, the mean pairwise genetic distance of proportion (p) of nucleotide sites was calculated by 'MEGA X' as genetic distance (GD). The FASTA format sequence was aligned using the MUSCLE algorithm integrated within MEGA X. The aligned sequences were visually inspected using the alignment viewer in MEGA X.

Table 1. Parental inbred lines used in Chapter 1

Inbred name	Derivation	Group †	Year	Developed in ‡
Ho49	N85/Ho4	EF	1995	HARC, NARO
Ho87	Astrid	EF	2001	HARC, NARO
Ho90	Raïssa/To38	EF	2002	HARC, NARO
Ho96	RAA45/TH8913	EF	2004	HARC, NARO
Ho99	EF99-7	EF	2005	HARC, NARO
Ho100	EF95-8	EF	2006	HARC, NARO
Ho120	(302101/Ho92)/TI-024	EF	2009	HARC, NARO
Ho119	(599646-1/Ho87)/(Ho82/Ho87)	EF	2009	HARC, NARO
Ho121	Blizzak/(Tiberius/Ho96)S_1	EF	2011	HARC, NARO
Ho130	(302101/Oh43Ht)/Tiberius	EF	2011	HARC, NARO
Ho128	EF04-12	EF	2012	HARC, NARO
Ho124	NEF02-9	EF	2012	HARC, NARO
Ho126	Ho96/LG3215	EF	2013	HARC, NARO
Ho127	(To90/303132)/{(TI9804/Ho84)/(Ho73/Ho87)}S_3	EF	2013	HARC, NARO
Ho129	NEF07-3	EF	2014	HARC, NARO
Ho131	EF07-4	EF	2018	HARC, NARO
Mi71	RD92-9	RD	1998	KARC, NARO
Mi62	P3286/P3470	RD	1997	KARC, NARO
Mi91	RD96-12	RD	2002	KARC, NARO
Mi93	RD97-6	RD	2003	KARC, NARO

Table 1. Continued

Inbred name	Derivation	Group †	Year	Developed in ‡
Mi106	RD98-5	RD	2007	KARC, NARO
Na2	Hirano	JF	1985	ILGS, NARO
Na5	Eboshi2	JF	1986	ILGS, NARO
Na4	Kuma	JF	1986	ILGS, NARO
Na30	JF2C2	JF	1989	ILGS, NARO
Na50	JF1C1S_3/Tateishi1	JF	1991	ILGS, NARO
Na57	{P3747/(H84/B37Ht)}/Na4	MIS (D*JF)	1992	ILGS, NARO
Na66	Na1/JF2C2	JF	1994	ILGS, NARO
Mi47	MZ021/MZ025	JF	1995	KARC, NARO
Na76	JF4C2	JF	1997	ILGS, NARO
Na79	MZ-029/MZ-019	JF	1998	ILGS, NARO
J1608	MF91-11	JF	1999	CAES, Nagano pref.
Na80	JF5C1-46	JF	2000	ILGS, NARO
CHU44	MF91-8	JF	2002	CAES, Nagano pref.
Ho95	94GPHA	JF	2003	HARC, NARO
Na84	MF93-11	JF	2003	ILGS, NARO
Na83	MF90-12	JF	2003	ILGS, NARO
JC-009	SF97-2	JF	2004	CAES, Nagano pref.
J1785	SF95-10	JF	2004	CAES, Nagano pref.
Mi103	MF96-2	JF	2005	KARC, NARO

Table 1. Continued

Inbred name	Derivation	Group †	Year	Developed in ‡
JC-026	NF98	JF	2006	CAES, Nagano pref.
Na85	JF5C2	JF	2006	ILGS, NARO
Na89	SF97-2	JF	2006	ILGS, NARO
Na88	SF96-15	JF	2006	ILGS, NARO
Na91	SF97- 6	JF	2006	ILGS, NARO
CHU68	NF98	JF	2007	CAES, Nagano pref.
Na92	RF99	JF	2007	ILGS, NARO
JC-053	NF00-4	JF	2008	CAES, Nagano pref.
Na93	MC99- 6	JF	2008	ILGS, NARO
Na94	B73/Na28	MIS (D*JF)	2008	ILGS, NARO
Na95	JF99	JF	2008	ILGS, NARO
JC-035	(Mi47/J1690)(J1700/J1608)	JF	2009	CAES, Nagano pref.
Na97	JF2001dig	JF	2009	ILGS, NARO
Na101	JF2000dig	JF	2009	ILGS, NARO
Na96	Y02-44	JF	2009	ILGS, NARO
Mi111	MF02-14	JF	2010	KARC, NARO
Na103	SF01-3-2	JF	2010	ILGS, NARO
Na104	MF02-6	JF	2011	ILGS, NARO
Ho125	(J1785/J1711)/(Na80/Ho95)	JF	2013	HARC, NARO
Na106	JF2004-47	JF	2013	ILGS, NARO

Table 1. Continued

Inbred name	Derivation	Group †	Year	Developed in ‡
Mi115	Mi47/Na50	JF	2014	KARC, NARO
Na111	NFM05	JF	2016	ILGS, NARO
Na112	EF072-11	MIS (EF*JF)	2016	ILGS, NARO
Na113	Na50/IL#18a	JF	2016	ILGS, NARO
Na9	PX77A	D	1986	ILGS, NARO
Na7	P3424	D	1986	ILGS, NARO
Na17	G4553	D	1987	ILGS, NARO
Na13	P3747	D	1987	ILGS, NARO
Na25	(MS142/Mo17Ht)/P3358	D	1988	ILGS, NARO
Na32	H84/H95	D	1989	ILGS, NARO
Na29	H84/RB259	D	1989	ILGS, NARO
Na36	(H93/Pa91)/P3358	D	1989	ILGS, NARO
Na38	{(B37Ht/H84)/Mo17Ht}/H84	D	1989	ILGS, NARO
Na34	P3358	D	1989	ILGS, NARO
Na41	(H84/R2040)/H84	D	1990	ILGS, NARO
Na43	(Pa91/H93)/P3358	D	1990	ILGS, NARO
Na42	(Oh43ht/MS142)/P3358	D	1990	ILGS, NARO
Mi29	P3358BC/NX220	D	1991	KARC, NARO
Na49	(P3358/P3732S3)/P3358	D	1991	ILGS, NARO

Table 1. Continued

Inbred name	Derivation	Group †	Year	Developed in ‡
Na54	H84/PX77A	D	1992	ILGS, NARO
Na55	P3747/(H84/B37Ht)	D	1992	ILGS, NARO
Na56	H84/P3747	D	1992	ILGS, NARO
Na53	P3358/(Oh43Ht/H84)	D	1992	ILGS, NARO
Na58	(H84/Pa91)/R2040	D	1993	ILGS, NARO
Na62	Na7/Na23	D	1993	ILGS, NARO
Na60	P3352(H84/R2040)	D	1993	ILGS, NARO
Na61	P3358/(A509/H84)	D	1993	ILGS, NARO
J1383	P85264	D	1994	CAES, Nagano pref.
J1417	Manitoba	D	1994	CAES, Nagano pref.
Na65	P3352/(H84/R2040)	D	1994	ILGS, NARO
Ho52	P3732	D	1995	HARC, NARO
Ho57	PH4304	D	1995	HARC, NARO
J1539	P85264 OPEN	D	1996	CAES, Nagano pref.
Na74	TX8766	D	1997	ILGS, NARO
Na71	Na7/Na23	D	1997	ILGS, NARO
Na69	P3352/(H84/R2040)	D	1997	ILGS, NARO
Na72	P3358/(OkuduruwaseS_2/HiranoS_2)	MIS (D*JF)	1997	ILGS, NARO
Na70	P3358/(A509/H84)	D	1997	ILGS, NARO
Ho68	DK403	D	1998	HARC, NARO

Table 1. Continued

Inbred name	Derivation	Group †	Year	Developed in ‡
Na78	P3358/(P3737S_4/(H84/B73HtS_4))	D	1998	ILGS, NARO
Na81	Na7/Na33	D	2001	ILGS, NARO
Mi83	SD95-4	D	2001	KARC, NARO
Mi88	SD95-1	D	2002	KARC, NARO
J1706	(W642/Ho58)/H95rhm	D	2003	CAES, Nagano pref.
J1707	P3358/(OkuduruwaseS_2/HiranoS_2)	MIS (D*JF)	2003	CAES, Nagano pref.
J1698	MD93-6	D	2004	CAES, Nagano pref.
JC-002	96GPTI	D	2004	CAES, Nagano pref.
Na86	G4655	D	2006	ILGS, NARO
JC-014	(J1657/Na65)(J1563/J1539)	D	2006	CAES, Nagano pref.
Ho102	Na7/Mi29^2	D	2006	HARC, NARO
Ho104	(Ho72/Ho40)/Clarica	D	2007	HARC, NARO
JC-036	ED99-6	D	2007	CAES, Nagano pref.
JC-028	(Mi49/J1560)(J612/J1605)	D	2007	CAES, Nagano pref.
Ho106	MLD99-4	D	2008	HARC, NARO
Ho110	{(Ho72/Ho40)/Clarica}S_2/(MLD99-4)S_2	D	2009	HARC, NARO
JC-037	ND99	D	2008	CAES, Nagano pref.
JC-050	ND00	D	2008	CAES, Nagano pref.
JC-046	(J1703/J1605)(Na65/J1539)	D	2009	CAES, Nagano pref.
JC-038	ND99-21	D	2009	CAES, Nagano pref.

Table 1. Continued

Inbred name	Derivation	Group †	Year	Developed in ‡
Na98	AD99syn1	D	2009	ILGS, NARO
Na99	AD99syn1	D	2009	ILGS, NARO
Na100	AD99syn1	D	2009	ILGS, NARO
JC-054	(Na65/Na42)/(Ho59/Ho72)	D	2010	CAES, Nagano pref.
JC-064	DK567/(J1704/Mi58)	D	2010	CAES, Nagano pref.
Na102	AD99syn1	D	2010	ILGS, NARO
Na109	AD2002	D	2014	ILGS, NARO
Mi29SRR	P3358BC/NX220	D	2014	KARC, NARO

† Classification by heterotic groups, defined based on the origin of each inbred lines, where heterotic patterns were expected.

EF, RD, JF, D, and MIS indicate flint mainly developed or derived from the European region, Japanese tropical inbred lines mainly developed from hybrids for summer seeding, Japanese flint landraces, Japanese dent mainly derived from US corn-belt dent, and miscellaneous origin, respectively. * is derived from crossing between each group.

‡ HARC, KARC, ILGS and CAES are abbreviations for Hokkaido Agricultural Research Center, NARO, Kyushu Okinawa Agricultural Research Center, NARO, Institute of Livestock and Grassland Science, NARO, and Chushin Agricultural Experiment Station, Nagano pref., respectively.

Results

The genotyping procedures in this study yielded 1,007 SNPs. The frequency distribution of pairwise GDs for 127 maize inbred lines genotyped at 1,007 SNPs is shown in Figure 1. Table 2 lists the maximum, minimum, and mean GD within and among groups. Supplementary Table 1 also lists all the GD matrices of 127 inbred lines. The GDs between pairwise comparisons of the inbred lines varied from 0.004 to 0.421, and the overall mean distance was 0.332. Most (48.9%) of the GDs fell between 0.350 and 0.400, with the lowest GD (0.004) being observed between 'Ho120' and 'Ho128', both of which were in the EF group but of different derivations. The highest GD of 0.421 was observed between 'Na94' and 'Ho124', which were classified with MIS (D*JF) and EF groups and were derived from different derivations. The minimum GDs within the same groups were relatively low, except for the RD inbred lines with lower N numbers. However, certain inbred lines had high GDs even within the same groups.

The PCA results classified EF, RD, JF, and D well (Figure 2). Inbred lines belonging to MIS, including 'Na57', 'J1707', and 'Na72' were classified near the midpoint between the JF and D groups. Notably, 'Na94', of MIS origin of D and JF, and 'Na112', also of EF and JF, were classified in the JF group.

The results of the population structure analysis were confirmed using a phylogenetic tree, in which the 127 genotyped inbred lines formed four heterotic groups,

with each group further divided into subgroups (Figure 3). The heterotic groups agreed with the previously ascertained classification by each derivation. For example, 'Ho95', derived from the JF mass population, was classified to the edge of the JF group as described in Tamaki et al. (2016).

Discussion

The genetic diversity of Japanese maize inbred lines was analyzed using multiple methods. PCA and phylogenetic tree reflected the heterotic groups borders according to the derivation of each inbred line, with marginal variations due to differences in each method.

The lowest GD value was 0.004 between 'Ho120' and 'Ho128' (EF group), derived from completely different material of three-way cross ([302101/Ho92]/TI-024) and mass selection (EF04-12). The breeding years of the two EF inbred lines were close and the same breeders selected them. On the other hand, the GD value of 'Na89' and 'JC-009' (JF) was 0.074, derived from the same population (SF97-2) but selected in different regions (Table 1). As a result, the EF inbred lines of different origins had more significant genetic similarities than JF inbred lines of the same origin. Warburton et al. (2002) have reported that DNA markers may be better indicators of inbred lines in cases where those derived from the same population are more distinct than those derived from different

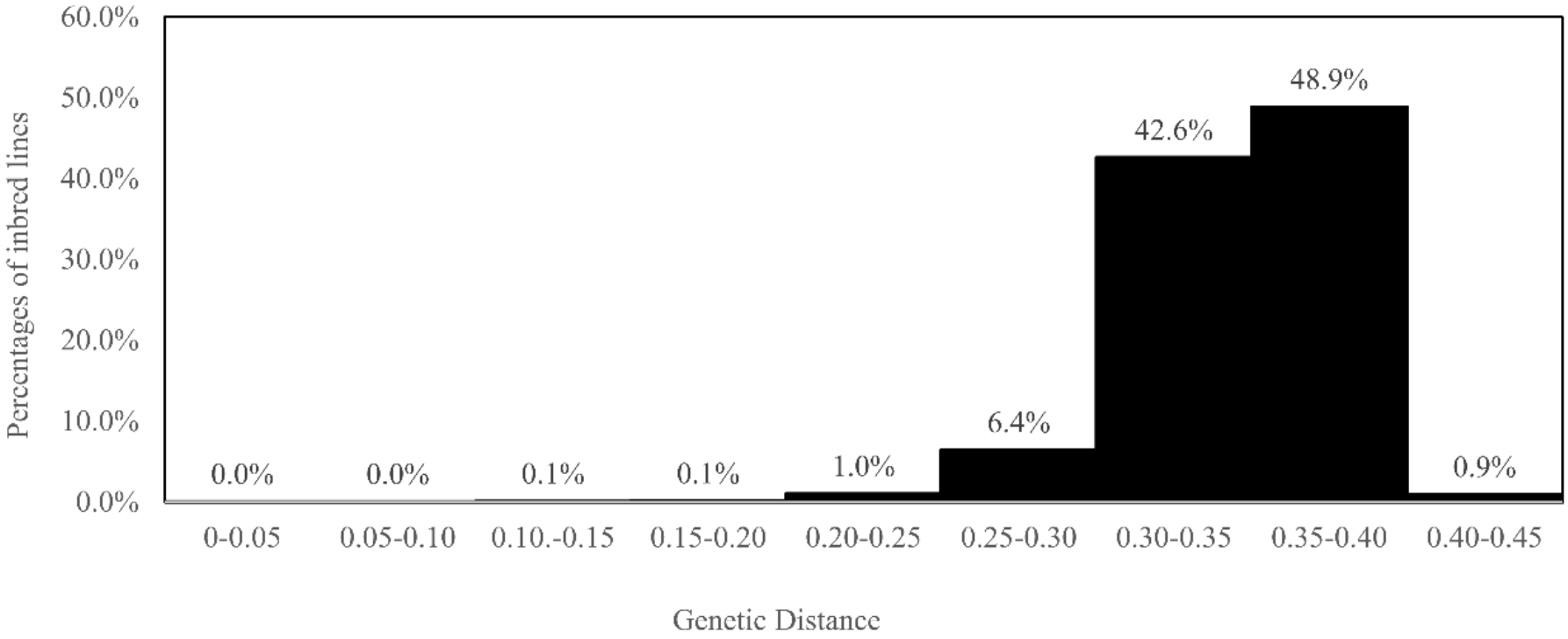

Fig. 1. Frequency distribution of pairwise genetic distances (GDs: p-distance) for 127 maize inbred lines genotyped at 1,007 SNPs.

Table 2. Genetic distances (GDs) within and between heterotic groups of 127 maize inbred lines

	EF (*N* = 16)			RD (*N* = 5)			JF (*N* = 43)			D (*N* = 63)		
	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean
EF (*N* = 16)	0.389	0.004	0.319	0.415	0.341	0.379	0.421	0.333	0.372	0.404	0.261	0.348
RD (*N* = 5)				0.320	0.278	0.301	0.396	0.313	0.359	0.398	0.272	0.352
JF (*N* = 43)							0.400	0.074	0.309	0.406	0.192	0.342
D (*N* = 63)										0.392	0.012	0.298

The estimated mean GD between 127 inbred lines (GD_M) was 0.332.

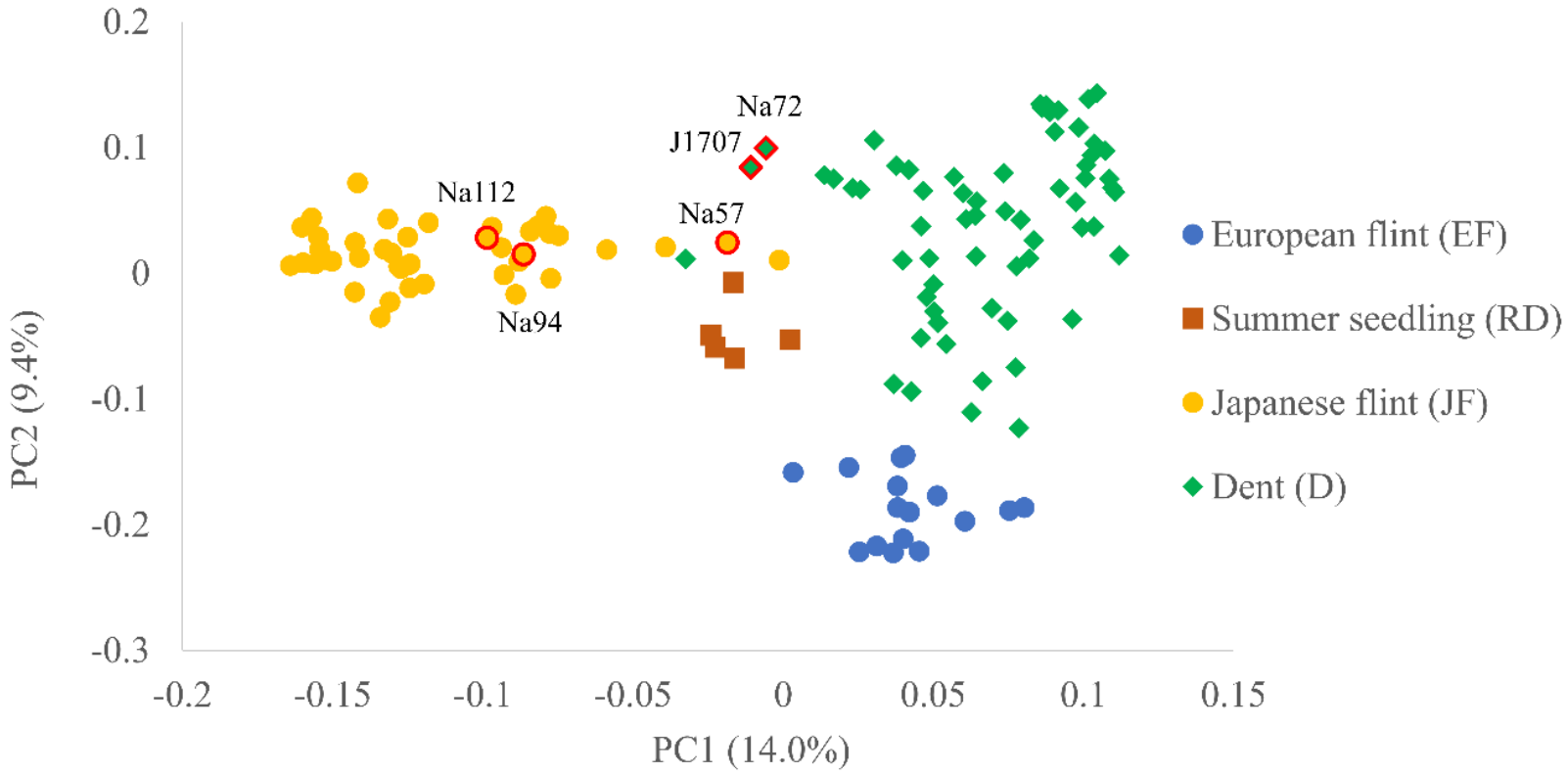

Fig. 2. A plot of principal component (PC) 1 and PC 2 scores based on 1,007 SNP markers of the 127 inbred lines. Red line shape: Miscellaneous origin.

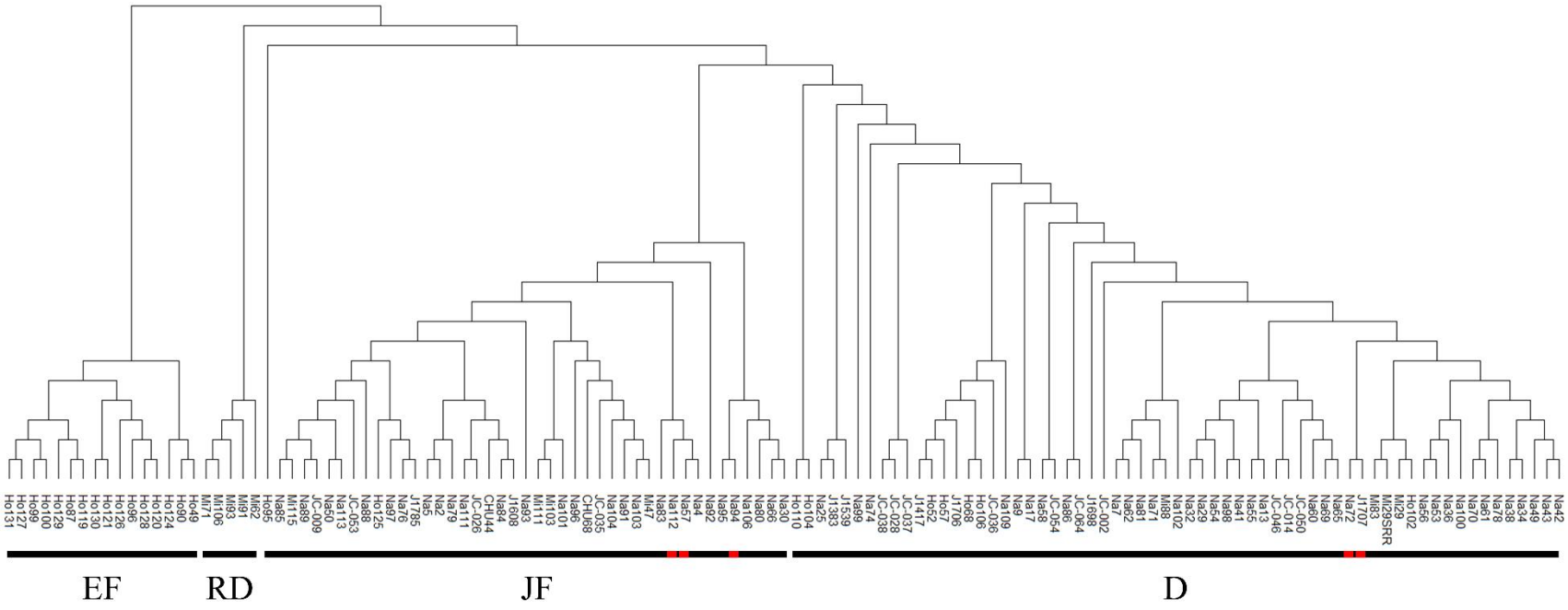

Fig. 3. The unweighted pair group method with the arithmetic mean (UPGMA) dendrogram of genetic relationships among 127 inbred lines was calculated based on genetic distances according to the Tamura-Nei model (1993). Black lines: Classification by groups in Table 1. Red shapes: Miscellaneous origin.

populations. The findings of the author's results concur with this insight.

Certain inbred lines had high GD even within the same group. For instance, the D group's 'J1383' and 'J1706' had the highest GD at 0.392. Based on the author's empirical findings, F_1 hybrids between D inbred lines tend to be superior in terms of grain yield than those between D and JF combinations. Dent testers are significantly higher for grain yield and lower for grain moisture than flint testers (Brun and Dudley 1989), may indicate that dent hybrids are superior for grain usage. However, F_1 hybrids between dent and flint inbred lines have higher effective organic matter degradability in whole plants, stems, and leaves than dent hybrids, making them suitable for silage use (Mlynekova et al. 2016). Starch degradability in the rumen is lower in flint hybrids than in dent hybrids (Philippeau and Michalet-Doreau 1998). Therefore, high GD within a same group does not necessarily mean that these will immediately lead to the production of superior F_1 hybrids in crosses between inbred lines within the group, such as D or JF hybrids. When developing new F_1 hybrids, it is necessary to consider their intended use in advance. However, information on GD may improve the values of target traits by producing F_1 hybrids that are expected to have high heterosis within the group. In the future, it will be necessary to evaluate the F_1 hybrids produced based on GD.

PCA results indicated a clear genetic distinction between the EF, RD, JF, and

D, except in cases where certain JF and D inbred lines were close. While ‘J1539’, classified with D groups, was located close to the border with JF, ‘J1383’, with the same origin, was classified with D groups. Notably, the former is derived from an open-pollinated population from a classic F_1 variety, and the latter, from self-pollination of itself (‘P85264’). Their GD value was 0.303, which was greater than the average GD within the D group (Table 2). Thus, the findings suggest that open pollination, a classic and effective tool for inducing genetic modification, could be essential in modern breeding.

‘Ho95’ belongs to the JF group, which is based on the Caribbean-type flint breeding population (94GPHA) and has a relatively later ripening period than other inbred lines bred in Hokkaido. A previous study (Tamaki et al. 2016) classified this inbred line as separate from other JF groups, which the phylogenetic tree in this study confirmed. ‘Na112’ is a MIS inbred line derived from both EF and JF according to its derivation (EF072-11) but had a minimum GD of 0.358 from EF group, which is greater than the overall mean (0.332) and was classified to JF group by PCA and phylogenetic tree. Relying on these classifications will only be possible after examining them based on the origin of the underlying population and DNA markers (Makumbi et al. 2011). Since the origin of these inbred lines especially before the 1990s, is based on old

handwritten records, further details, including the possibility of human error, should be examined in the future.

The low cost of SNP arrays allows the analysis of numerous samples. However, given that they are developed using reference genomes, they can be confounding in diversity studies. This has been exemplified by the observation of significant confirmation bias by Ganal et al. (2011) using ‘maize SNP50’ by Illumina Inc. The present findings do not contradict previous study findings on genetic diversity by RAD-seq (Tamaki et al. 2016), and their derivations. This suggests that the SNP array analysis used in this chapter helps in understanding genetic diversity. The development of accurate, inexpensive, and reproducible genotyping platforms has been a primary driver of genotypic studies, including those on GP (Washburn et al. 2020). The SNPs array ‘Maize LD Bead chip’ has already been discontinued and alternative methods will be needed in the future. Other tools for genome-wide SNP analysis should also be considered for association studies. In the future, these techniques may be used to obtain more SNPs and related phenotypic data, which could provide further insight (Abu et al. 2021, Akaogu et al. 2013, Benchimol et al. 2008, Betrán et al. 2013, Sant’Ana et al. 2020).

CHAPTER II. Evaluation of resistance to Pythium RSR by *P. arrhenomanes* in maize by using a bottom stalk toothpick inoculation method

Introduction

As described earlier, considering labor and other costs and legal constraints on maize feed production in Japan, breeding resistant varieties is the only feasible option for Pythium RSR control. A 'toothpick method' developed by Young (1943) is one of the reliable methods to evaluate the resistance to ear and stalk rot of maize caused by *Fusarium* spp., and a lot of research have confirmed its effectiveness (Mesterhazy et al. 2012, Todd and Kommedahl 1994). However, the method has not yet been examined for Pythium RSR resistance. The objective of this chapter is to confirm the applicability of the bottom stalk toothpick inoculation method and evaluating resistance of maize hybrids to Pythium RSR.

Materials and methods

Field test

Field experiments were made between 2011 and 2013 at the Institute of Livestock and Grassland Science (ILGS), NARO, Nasushiobara, Tochigi, Japan (N36°55'04,

E139°56'29, 320 m above mean sea level). Maize–Sudan grass (*Sorghum Sudanense Piper.*) rotation was made for the fields, meaning that the experiments were made on the same field in 2011 and 2013, but not in 2012 (on the neighboring one). In the early spring of each year, 50 metric tons/ha of manure, 600 or 700 kg/ha of fertilizer (containing 17 or 14% each of N, P_2O_5 and K_2O) and 60 kg/ha insecticide (Diazinon granule) were applied before sowing. Some herbicides (6.0 L/ha of Alachlor emulsion and 2.0 L/ha of Atrazine wettable powder) were also applied after sowing.

Table 3 shows the seven maize hybrids tested in this chapter, three Pioneer brand hybrids, three hybrids bred in the author's institute, and a hybrid bred in the prefectural public breeding sections in Nagano prefecture. The experimental design was a randomized complete block with a split plot with two replications, where the main plots were inoculation and natural infection, and the subplots were the tested hybrids. All the hybrids were grown in single row plots (3.6 m in length, 0.75m apart) each of which consisted of 19 plants. They were sown on May 10th, 12th, and 18th in 2011, 2012, and 2013, respectively.

Table 3. The maize hybrids used in Chapter 2

Hybrid	Origin †
32F27	Pioneer Hi-bred
36B08	Pioneer Hi-bred
Cecilia	Pioneer Hi-bred
Na62×Na50	ILGS, NARO
Na65×Na50	ILGS, NARO
Na65×CHU44 (‘Takanestar’)	CAES, Nagano pref.
Mi29×Na50 (‘Yumesodachi’)	KARC, NARO

† ILGS, KARC, and CAES are abbreviations for Institute of Livestock and Grassland Science, NARO, Kyushu Okinawa Agricultural Research Center, NARO, and Chushin Agricultural Experiment Station, Nagano pref., respectively.

Field inoculation with natural infection

The inoculums were prepared from the isolate called CP-1 of *P. arrhenomanes* having been collected from Nagano Pref. in 2010. The isolate was deposited in the NARO Genebank, Japan as MAFF 511548.

The isolate was preliminarily multiplied in petri dishes filled with V8 juice agar (180 ml of Cambell's V8 juice, 3 g of $CaCO_3$, and 15 g of agar per liter) and incubated at 25°C for 2 d. Wooden toothpick pieces (60 mm in length and 2 mm in diameter) having been autoclaved at 120°C for 20 min with potato dextrose (PD) broth, were placed on the petri dishes filled with multiplied isolate. After the toothpicks had been placed, the dishes were again incubated at 25°C for another 10 d in the dark, until the toothpicks were completely covered with the hyphae. The inoculation was made at the silking stage of each hybrids (between July 19th and August 11th), an infested toothpick was inserted into a hole (2.5 mm in diameter) on the bottom stalk of each plant (about 5 cm height from the ground surface) soon after the hole had been punctured with an awl (in 2011 and 2012) or electric drill 'LXD10-2' (Stanley Black and Decker, Inc., Connecticut, US; in 2013). No holes were punctured in natural infection plots.

Given that the experimental fields were estimated to be highly contaminated with the Pythium RSR pathogen, it was inferred that the inoculation plots would also

be affected under natural infection (inoculation with natural infection).

Evaluation of the disease severity and infection frequency

When each hybrid reached to the yellow-ripe stage (about 50 d after silking). The plants were cut at about 5 cm above the ground, and the rotting degree on the cut surface of the stalks were observed. The surface was just above the hole for the inoculated plants. The author modified the method reported by Nemoto et al. (1987) and scored the disease severities as 0 = no symptoms, 1 = light browning, 2 = browning, 3 = below 50% of the section was rotten and hollowed, 4 = over 50% was rotten and hollowed (Figure 4).

The infection frequency was calculated as the percentage of plants whose scores were two or more and was adopted as the scale for the disease resistance, since plants with scores of two or more were clearly distinguishable from those having scores less than two. Differences in infection frequencies between varieties in natural infection plots and inoculation with natural infection plots, as well as the annual variations of each method, were tested for significance using the Mann-Whitney U test. Additionally, analysis of variance (ANOVA) with aligned rank transform (ART) was conducted for each year and method, with variety as a factor (Wobbrock et al. 2011).

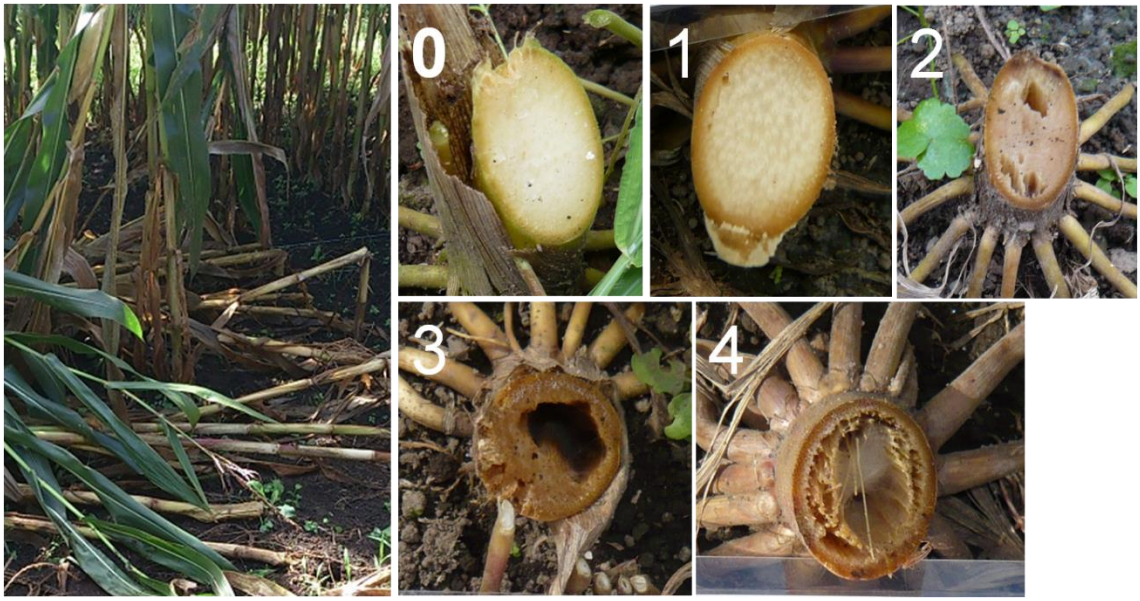

Fig. 4. Symptoms of Pythium RSR in maize at the maturing stage.

Left end: Plants infected by *Pythium* spp. show a withered body and dangling ears due to Pythium RSR or water shortage.

0–4: Severity scores used in this study; 0 = no symptoms, 1 = a little light browning, 2 = browning, 3 = below 50% of the section was rotten and hollow, 4 = over 50% was rotten and hollow in the cut stalk section.

Results

Tables 4 and 5 depict the results of the field tests, natural infection, and inoculation with natural infection plots. Throughout the experimental period from 2011 to 2013, Pythium RSR occurred under natural infection in the field. But the average infection frequency remarkably varied in each test plot; higher in 2011 and 2013 than in 2012. The hybrid used in this chapter, '32F27' consistently showed high resistance, with infection frequencies of 0.00–2.63% under inoculation with natural infection. Meanwhile, 'Mi29×Na50', which is sold as a F_1 hybrid 'Yumesodachi' in Japan, was always susceptible, showing high infection frequencies of 21.05–45.98% under inoculation with natural infection. 'Yumesodachi' is known as one of the most susceptible hybrids in Japan, and the results of this chapter confirm this practical knowledge. Comparing the differences between natural infection and inoculation with natural infection plots for each variety, only the variety 'Cecilia' showed a significantly higher infection frequencies under inoculation with natural infection.

Table 6 indicates the results of significance tests of the annual variation for Pythium RSR, and the ANOVA with ART on effect of different varieties. The inoculation with natural infection plots showed no significant annual variation, and the differences between varieties were significant in all experimental years.

Table 4. Infection frequencies (IF) of Pythium RSR under natural infection in the field in 2011, 2012, and 2013

Hybrid	IF ± Standard Error (%) †			
	2011	2012	2013	AVG
32F27	0.00 ± 0.00	2.63 ± 2.63	2.63 ± 2.63	1.75 ± 1.11
36B08	2.63 ± 2.63	7.89 ± 7.89	5.26 ± 5.26	5.26 ± 2.72
Cecilia	11.46 ± 5.21	5.26 ± 0.00	10.53 ± 10.53	9.08 ± 3.27
Na62×Na50	13.45 ± 2.34	5.26 ± 0.00	18.42 ± 2.63	12.38 ± 2.59
Na65×Na50	47.52 ± 5.42	5.26 ± 0.00	50.00 ± 7.89	34.26 ± 9.51
Na65×CHU44	5.56 ± 5.56	2.63 ± 2.63	13.16 ± 2.63	7.12 ± 2.63
Mi29×Na50	36.84 ± 15.79	11.51 ± 0.99	47.37 ± 0.00	31.91 ± 7.87

† The average percentage of plants with a score of two or higher, 19 individuals per section with two replicates.

Table 5. Infection frequencies (IF) of Pythium RSR under artificial inoculation with natural infection by using a bottom stalk toothpick inoculation method in the field in 2011, 2012, and 2013

Hybrid	IF ± Standard Error (%) †				
	2011	2012	2013	AVG	Significance test ††
32F27	2.63 ± 2.63	0.00 ± 0.00	2.63 ± 2.63	1.75 ± 1.11	N.S.
36B08	5.41 ± 0.15	7.89 ± 7.89	2.63 ± 2.63	5.31 ± 2.35	N.S.
Cecilia	20.49 ± 1.74	24.27 ± 2.05	13.16 ± 2.63	19.30 ± 2.28	*
Na62×Na50	13.89 ± 2.78	2.63 ± 2.63	13.16 ± 2.63	9.89 ± 2.59	N.S.
Na65×Na50	55.26 ± 7.89	15.79 ± 5.26	44.74 ± 7.89	38.60 ± 8.12	N.S.
Na65×CHU44	8.51 ± 3.25	0.00 ± 0.00	5.26 ± 0.00	4.59 ± 1.78	N.S.
Mi29×Na50	45.98 ± 22.45	21.05 ± 0.00	42.11 ± 21.05	36.38 ± 9.33	N.S.

† The average percentage of plants with a score of two or higher, 19 individuals per section with two replicates.

†† Mann-Whitney U-test for IF of each hybrid in comparison to Table 4. *: $p < 0.05$, N.S.: Not significant.

Table 6. Results of significance tests of the annual variation in infection frequency (IF) of Pythium RSR, and the Analysis of variance (ANOVA) with Aligned rank transform (ART) on effect of variety

Test plot	Annual variation in IF †			Effect of variety ††		
	2011–2012	2011–2013	2012–2013	2011	2012	2013
Natural infection	**	N.S.	*	**	N.S.	*
Inoculation with natural infection	N.S.	N.S.	N.S.	**	**	**

† Mann-Whitney U-test for each year in comparison.

†† ANOVA with ART on effect of variety. *: $p < 0.05$, **: $p < 0.01$, N.S.: Not significant.

Discussion

In 2012, the overall infection frequencies were lower than in the other two years due to suboptimal climate conditions (Tables 4 and 5). Nevertheless, at the inoculation with natural infection plots throughout the experimental years, the infection frequencies of highly susceptible and resistant hybrids were stable regardless of climate conditions (Table 5). Therefore, this method is useful for screening, especially for discarding highly susceptible hybrids to the disease based on the data obtained from a single year, even if it is optimal or suboptimal for infection.

High temperature, humidity and poor drainage are known to deteriorate symptoms of Pythium RSR (Deep and Lipps 1996, Nemoto et al. 1987, Reyes-Tena et al. 2018, Yanar et al. 1997). The precipitation around the milk- to glue-ripening stage was almost the same each year. On the other hand, ground temperature and relative humidity were lower in 2012 than in other years. In the author's preliminary study, a large outbreak may occur when several conditions favorable to Pythium RSR are present, rather than just one condition, at the time when the disease is most likely to occur (data not shown). Thus, these conditions are considered to have influenced the differences in infection frequencies between 2011, 2013, and 2012.

In 2012, the infection frequency was low, and there were no significant

differences between varieties in natural infection (Table 6). In such an unfavorable condition for this disease, it would be difficult to efficiently select many varieties under natural infection. Inoculation with natural infection was less effective in significantly increasing from the infection frequency of each variety compared to natural infection (Table 5). However, in the inoculation with natural infection plots, the results of ANOVA with ART showed that the effect of variety was significant in all experimental years, and there was no annual variation (Table 6).

Therefore, the bottom stalk toothpick inoculation method proposed in this chapter is deemed useful for stably evaluating Pythium RSR and screening resistant or susceptible varieties, regardless of weather conditions, as it can complement natural infection.

CHAPTER III. Parental-progeny-based best linear unbiased prediction (BLUP) for determining maize single-cross performance and resistance to Pythium RSR

Introduction

The author has tried the bottom stalk toothpick inoculation method developed by Young (1943) and has confirmed its effectiveness and reliability for evaluating Pythium RSR resistance in the previous chapter. However, problems persist in constructing effective breeding systems against Pythium RSR, including that the resistance of F_1 hybrids often does not align with that of their parental inbred lines. For example, 'Yumesodachi (Mi29×Na50)' and 'Na65×Na50' showed high infection frequencies in Chapter 2 but their common parental inbred line 'Na50' was resistant in field inoculation with natural infection plots (0.00% between 2011 and 2013, data not shown). This makes it difficult to develop resistant hybrids effectively.

BLUP is a standard method for estimating the random effects of a mixed model equation among large set of genotypes. This method was originally developed in the field of animal breeding for the estimation of breeding values, and currently, it has been applied for analyzing many plant breeding systems (Huang et al. 1995, Kerr et al. 2002,

Roudbari et al. 2017). Bernardo (1994) suggested that BLUP was highly suitable for predicting single-cross hybrids grain yield in maize. Also, Bernardo (1996) evaluated the effectiveness of BLUP for predicting grain yield, grain moisture, and stalk or root lodging rate.

The objective of this chapter is to evaluate simple parental-progeny-based BLUP in predicting single-cross performance and to determine the importance of general combining ability (GCA) of the resistance to Pythium RSR to build effective breeding programs. In conventional genetic analyses for unknown traits to evaluate the effects of GCA and special combining ability (SCA), maize breeders have often adopted diallel crosses which requires highly balanced materials. However, they substantially restrict the number of parents, and it often causes inefficiency of the breeding which needs large numbers of plants to estimate. Oliveira et al. (2016) compared the values of GCA and SCA in classical population genetics with those obtained using BLUP and found moderate to high correlations for multiple traits in parental inbred lines. Thus, in this chapter, BLUP is adopted for predicting GCA of the parental inbred lines instead of such breeding programs which need large number of inbred lines. Another important aspect of GCA is, as mentioned above, the low parent-progeny correlations in their phenotypes. Generally, maize disease resistance is known to have high parental-progeny

correlations or narrow-sense heritability, meaning that the effect of GCA is greater than that of SCA (Dorrance et al. 1998, Legesse et al. 2009). The low correlations in the phenotypic scores of Pythium RSR indicate the possibility that this resistance might be different from such diseases. In this chapter, the potential of the BLUP for Pythium RSR resistance is discussed in terms of the correlation between the predicted GCA of parental inbred lines and the phenotypic values of the F_1 hybrids.

Materials and methods

Plant materials

A total 449 F_1 hybrids were tested in this chapter, derived from crosses among 100 parental inbred lines developed at the Nasushiobara, Hokkaido, and Miyakonojo research stations of NARO and Prefectural public breeding sections in Nagano, Japan. All hybrids were evaluated for their resistance to Pythium RSR in the field tests, as described in Chapter 2.

Field test 1

Field experiments were made for four years from 2014 to 2017, at the ILGS, NARO, Nasushiobara, Tochigi, Japan. A maize–Sudan grass (*Sorghum Sudanese* [Piper] Stapf.)

annual rotation, manure, fertilizer, insecticide, and herbicides were applied as described in Chapter 2. All field tests were made in a randomized complete block with two or three replicates. All entries were grown in single row plots (2.4 or 3.6 m in length, and 0.75 m apart), each of which consisted of 13 or 19 plants.

When each F_1 hybrid reached the yellow-ripe stage (about 50 d after silking), the plants were cut at about 5 cm above the ground, and the degree of rotting on the cut surface of the stalks was recorded. Pythium RSR resistance was evaluated by the infection frequency as the percentage of plants whose scores were two or more. F_1 hybrids were evaluated under natural infection. The details of the evaluation and scoring were the same as those in Chapter 2. Table 7 shows the scale and outline of field test 1.

Table 7. The scale and outline of field test 1, which tested 449 F_1 hybrids derived from 100 inbred lines through natural infection from 2014 to 2017 in Chapter 3

Field experiment number	Number of				Broad-sense heritability (H^2)
	Hybrids	Inbred lines crossed for hybrids	Plants in a single row	Replications	
2014-1	39	29	19	2	0.908
2014-2	19	30	19	3	0.924
2015-1	34	29	19	2	0.789
2015-2	16	22	19	3	0.815
2016-1	7	12	19	3	0.050 †
2016-2	14	14	19	2	0.970
2016-3	23	22	19	2	0.651
2016-4	77	41	13	2	0.517
2016-5	61	44	13	2	0.562
2017-1	11	13	19	2	0.824
2017-2	20	25	19	2	0.840
2017-3	136	42	13	2	0.082 †
2017-4	58	35	13	2	0.204
2017-5	13	19	19	3	0.867

Sowing and observation dates were different for each year or environment at the ILGS, NARO, Nasushiobara, Tochigi, Japan. A total of 449 F_1 hybrids were tested, obtaining 528 data. These F_1 hybrids were derived from crosses among 100 inbred lines.

† These were excluded from the calculation of the mean as exaggerated values (Smirnov-Grubbs test $p < 0.05$). The average value of H^2 was 0.739.

BLUP to predict the GCAs of each inbred line

To predict the breeding values (i.e., GCAs) of individual inbred lines, the author partly modified the BLUP mixed model matrix equation in maize by Bernardo (1996) and Tamaki et al. (2012):

$$y = X\beta + Z_g a_1 + Z_s a_2 + \varepsilon \quad (1)$$

y is the phenotypic values via field test 1 (The infection frequencies of Pythium RSR, i.e., percentages of plants scoring more than two), X is a design matrix to express which F_1 combination is tested in which field experiments, β is an unknown vector for environmental values of each experiment, a_1 and a_2 are vectors for the GCA and SCA of each inbred line used in this chapter. Z_g is a design matrix to express which inbred lines are the parents of each F_1 combination, Z_s is a design matrix that indicates the combination of F_1 for each data, and ε is a residual effect. In each component of the design matrix Z_g, the directionality of the crosses is not considered. The inbred lines used for F_1 combination are defined as 1, while inbred lines not used are defined as 0. This model did not consider the effects of epistasis.

The solution of the matrix equation Eqn. (1) follows the previous studies by Bernardo (1996) and Tamaki et al. (2012):

$$\begin{pmatrix} X'R^{-1}y \\ {Z_g}'R^{-1}y \\ {Z_s}'R^{-1}y \end{pmatrix} = \begin{pmatrix} X'R^{-1}X & X'R^{-1}Z_g & X'R^{-1}Z_s \\ {Z_g}'R^{-1}X & {Z_g}'R^{-1}Z_g + G_1^{-1} & {Z_g}'R^{-1}Z_s \\ {Z_s}'R^{-1}X & {Z_s}'R^{-1}Z_g & {Z_s}'R^{-1}Z_s + G_2^{-1} \end{pmatrix} \begin{pmatrix} \hat{\beta} \\ \widehat{a_1} \\ \widehat{a_2} \end{pmatrix} \quad (2)$$

where X', ${Z_g}'$, and ${Z_s}'$ are the transposed matrices, R is a variance-covariance matrix for residual effects, and G_1 and G_2 are the variance-covariance matrix for the effect of traits. If G_1 and G_2 can be approximated with $A\sigma_a^2$ and $D\sigma_d^2$ where A and D are matrices of coefficient of coancestry among the inbred lines and combination of F_1 ($G_1 = A\sigma_a^2,\ G_2 = D\sigma_d^2$), R can be described as the product of the identity matrix I and a value for the residual effect variance σ_e^2 (Sasaki 2007), Eqn. (2) can be transformed as follows:

$$\begin{pmatrix} \hat{\beta} \\ \hat{a}_1 \\ \hat{a}_2 \end{pmatrix} = \begin{pmatrix} X'X & X'Z_g & X'Z_s \\ {Z_g}'X & {Z_g}'Z_g + A^{-1}(\sigma_e^2 / \sigma_a^2) & {Z_g}'Z_s \\ {Z_s}'X & {Z_s}'Z_g & {Z_s}'Z_s + D^{-1}(\sigma_e^2 / \sigma_d^2) \end{pmatrix}^{-1} \begin{pmatrix} X'y \\ {Z_g}'y \\ {Z_s}'y \end{pmatrix} \quad (3).$$

It is now quite common to adopt the coefficient of coancestry in the BLUP equations for both animal and plant breeding, which is expected to lead to higher prediction accuracy, based on strong assumptions related to the underlying quantitative genetic theory (Piepho et al. 2008). However, the author adopted in this chapter only the pedigree structure that was simple back to a common set of parents and formulated simple mixed models without the coefficient of coancestry. This was because the breeding processes of many parental inbred lines were unknown, and subsequently, the coefficient of coancestry could not be calculated in many cases. Therefore, the author assumed $A = I$, and $D = I$. In addition, the additive genetic variances σ_a^2 and σ_e^2 are assumed to be $h^2\sigma_y^2$ and $(1 - h^2)\ \sigma_y^2$ using the narrow-

sense heritability (h^2), respectively. Therefore, σ_e^2 / σ_a^2 can be described as follows:

$$\sigma_e^2 / \sigma_a^2 = \left(\frac{1-h^2}{h^2}\right) \quad (4).$$

Also, σ_e^2 / σ_d^2 can also be calculated using the broad-sense heritability (H^2), in the same way.

$$\sigma_e^2 / \sigma_d^2 = \left(\frac{1-H^2}{H^2}\right) \quad (5)$$

Therefore, the author reduces the normal equation to estimate BLUE and BLUP as follows:

$$\begin{pmatrix} \hat{\beta} \\ \hat{a}_1 \\ \hat{a}_2 \end{pmatrix} = \begin{pmatrix} X'X & X'Z_g & X'Z_s \\ Z_g{}'X & Z_g{}'Z_g + \left(\frac{1-h^2}{h^2}\right)I & Z_g{}'Z_s \\ Z_s{}'X & Z_s{}'Z_g & Z_s{}'Z_s + \left(\frac{1-H^2}{H^2}\right)I \end{pmatrix}^{-1} \begin{pmatrix} X'y \\ Z_g{}'y \\ Z_s{}'y \end{pmatrix} \quad (6).$$

The results that were tested only once in the experiments were excluded from the equation, to maximize the accuracy of the comparison. Broad-sense heritability (H^2) was defined as the mean of the values obtained from the ANOVA for each test plot (Table 7). In this chapter, the narrow-sense heritability (h^2) of Pythium RSR was unknown. Therefore, based on the results of previous research for root rot disease in maize, the author calculated the ratio of narrow-sense heritability (0.51) to broad-sense heritability (0.81) for root rot discoloration (Lazaro 2001), regarded it as a hypothetical ratio, and multiplied it by average H^2 (0.739) to obtain the value 0.465. The program for the solution was written in R 4.0 by the R Core Team (2020).

Field test 2

To evaluate the potential of simple parental-progeny-based BLUP, the sum of the predicted GCAs of parental inbred lines was compared with Pythium RSR infection frequency (percentage of individuals with symptoms whose scores were two or more) in F_1 hybrids derived from combinations of these inbred lines, under field inoculation with natural infection tests conducted for two continuous years from 2018. This is because, as noted in the Introduction, the purpose of this chapter is to determine the importance of GCA in resistance to Pythium RSR. In addition, field inoculation with natural infection tests were conducted for three continuous years from 2012 to compare the predicted GCAs and infection frequencies of parental inbred lines. Details of these field inoculation were the same as those used in field test 1 and Chapter 2.

Results

Comparing the breeding values (GCAs) by BLUP, which were predicted from the data of F_1 hybrids, and infection frequencies of parental inbred lines from 2012 to 2014 under inoculation with natural infection, the correlation coefficients were not significant in each year (Table 8). For example, F_1 hybrids from a parental inbred line 'Na50' are known to be very susceptible to Pythium RSR, but the parent itself is notably resistant, and its field inoculation with natural infection data and GCA by BLUP were 0.00% and 4.46%, respectively. In contrast, field inoculation with natural infection data and GCA by BLUP of another inbred line, 'Na71', were 1.28% and -7.07%, respectively. 'Mi29' is known to be susceptible in both inbred line itself and F_1 progenies, and its field inoculation with natural infection data and GCA by BLUP were 16.99% and 5.97%, respectively. Supplementary Table 2 lists all the results of 100 inbred lines.

Tables 9–11 and Figure 5 present the comparisons and correlation coefficients between sum of the GCAs by BLUP and infection frequencies of F_1 hybrids under inoculation with natural infection from 2018 to 2019. Relatively high correlations were observed (R = 0.776 and 0.793) and both were significant ($p < 0.01$). 'Mi29×Na50' ('Yumesodachi'), which is the most susceptible variety and used in Chapter 2, had the highest combinations of GCAs by BLUP (Table 8) and one of the highest infection

frequencies under inoculation with natural infection data (Tables 10 and 11). The result of ‘Yumesodachi’ was concordant with conventional findings in Japan. Focusing only on unknown F_1 combinations, which were not used to predict the GCAs, high correlations were also observed (R = 0.952 and 0.905, $p < 0.01$).

Table 8. Comparison of infection frequencies of F_1 hybrids under inoculation with natural infection and general combining abilities (GCAs) by best linear unbiased prediction (BLUP) of Pythium RSR in eight inbred lines, from 2012 to 2014

Name (inbred lines)	Infection frequency (IF) ± Standard Error (%) †				Predicted GCAs by BLUP (%)
	2012	2013	2014	AVG	
Na50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	**4.46**
Na65	0.00 ± 0.00	3.85 ± 3.85	0.00 ± 0.00	1.28 ± 1.28	0.22
Na71	0.00 ± 0.00	3.85 ± 3.85	0.00 ± 0.00	1.28 ± 1.28	-7.07
Mi29	0.00 ± 0.00	7.69 ± 7.69	43.27 ± 18.27	16.99 ± 9.86	**5.97**
Mi47	-	3.85 ± 3.85	-	3.85 ± 3.85	-0.32
Mi91	-	0.00 ± 0.00	-	0.00 ± 0.00	0.08
CHU44	-	15.38 ± 7.69	-	15.38 ± 7.69	1.19
CHU68	-	15.38 ± 0.00	-	15.38 ± 0.00	-0.57
	N.S. ‡	N.S.	N.S.	N.S.	-

Sowing and observation dates were different for each year or environment at the ILGS, NARO, Nasushiobara, Tochigi, Japan. Bold letters are the two highest values in this result.

† The field inoculation with natural infection data in Chapter 2, i.e. percentages of plants scoring more than two.

‡ Results of the significance test comparing IF and predicted GCAs by BLUP (Pearson's correlation coefficient). N.S.: Not significant.

Table 9. Correlation coefficients between sum of the predicted GCAs by BLUP (2014–2017) and infection frequencies of F_1 hybrids under inoculation with natural infection on Pythium RSR resistance (2018–2019)

Year	2018	2019
Number of tested hybrids (N)	20	22
Unknown F_1 combinations, which were not used to predict the GCAs, derived from the inbred lines used in field test 1	5	9
Number of inbred lines for predicting infection frequencies of F_1 hybrids	21	18
Correlation coefficient	0.776^{**}	0.793^{**}
Only unknown F_1 combinations which were not used to predict the GCAs	0.952^{**}	0.905^{**}

Pearson's correlation coefficients were significant (**, $p < 0.01$).

Table 10. Correlations between sum of the predicted GCAs by BLUP of inbred lines and infection frequency of F_1 hybrids under inoculation with natural infection for Pythium RSR resistance in 2018

F_1 hybrids name	Predicted GCAs by BLUP			Infection frequency ± Standard Error (%) ‡
	Seed parent	Pollen parent	F_1 hybrids	
Na71×N13-07	-7.07	-7.21	-14.29	0.00 ± 0.00
Na71×N09-07	-7.07	-4.86	-11.94	0.00 ± 0.00
Na98×N10-08	-7.84	-3.68	-11.52	5.88 ± 0.00
Na71×CHU68	-7.07	-0.57	-7.64	15.07 ± 2.57
Na102×Mi103	-4.08	-3.34	-7.42	18.04 ± 11.37
Mi103×Na109 †	-3.34	-2.58	-5.92	9.01 ± 2.76
Na65×N09-07 †	0.22	-4.86	-4.64	2.94 ± 2.94
Na65×Mi111 †	0.22	-4.37	-4.15	2.94 ± 2.94
Na65×N10-08	0.22	-3.68	-3.46	0.00 ± 0.00
JC-028×Mi111	0.99	-4.37	-3.38	5.88 ± 0.00
JC-037×Na113	-4.78	2.08	-2.71	15.26 ± 3.49
Na102×Na113	-4.08	2.08	-2.00	14.71 ± 2.94
Na109×CHU44	-2.58	1.19	-1.39	28.13 ± 9.38
Na100×N11-02	3.21	-4.17	-0.96	14.71 ± 2.94
Na98×N13-06 †	-7.84	8.44	0.60	27.39 ± 3.86
Na65×CHU44	0.22	1.19	1.41	5.88 ± 5.88
Mi109×N13-07	11.13	-7.21	3.92	10.00 ± 10.00
Na102×N13-06 †	-4.08	8.44	4.36	38.24 ± 8.82
Mi29×Na50	5.97	4.46	10.42	58.82 ± 0.00
Mi109×CHU68	11.13	-0.57	10.57	45.75 ± 1.61

Sowing and observation dates were different for each year or environment at the ILGS, NARO, Nasushiobara, Tochigi, Japan.

† Unknown combination of F_1 hybrids, which were not used to predict the GCAs by BLUP, derived from the inbred lines used in field test 1.

‡ The field inoculation with natural infection data in Chapter 2, i.e. percentages of plants scoring more than two.

Table 11. Correlations between sum of the predicted GCAs by BLUP of inbred lines and infection frequency of F_1 hybrids under inoculation with natural infection for Pythium RSR resistance in 2019

F_1 hybrids name	Predicted GCAs by BLUP			Infection frequency ± Standard Error (%) ‡
	Seed parent	Pollen parent	F_1 hybrids	
Na71×Na112	-7.07	-1.23	-8.30	3.13 ± 3.13
Na71×CHU68	-7.07	-0.57	-7.64	2.94 ± 2.94
Na102×Mi103	-4.08	-3.34	-7.42	17.65 ± 5.88
Na102×N17-D03 †	-4.08	-3.09	-7.17	0.00 ± 0.00
Na102×Na109 †	-4.08	-2.58	-6.66	9.01 ± 2.76
JC-037×Na112	-4.78	-1.23	-6.01	0.00 ± 0.00
N17-D03×Na105	-3.09	-2.62	-5.71	0.00 ± 0.00
Na109×Na105 †	-2.58	-2.62	-5.20	6.25 ± 6.25
Na65×Mi111 †	0.22	-4.37	-4.15	0.00 ± 0.00
JC-028×Mi111	0.99	-4.37	-3.38	0.00 ± 0.00
Na102×CHU44	-4.08	1.19	-2.89	2.94 ± 2.94
JC-037×Na113	-4.78	2.08	-2.71	0.00 ± 0.00
JC-028×Mi103	0.99	-3.34	-2.35	18.20 ± 0.55
Na102×Na113	-4.08	2.08	-2.00	5.88 ± 5.88
Na109×CHU44	-2.58	1.19	-1.39	9.09 ± 9.09
Na102×Na50 †	-4.08	4.46	0.38	8.82 ± 2.94
Na65×CHU44	0.22	1.19	1.41	8.82 ± 2.94
JC-028×Na111 †	0.99	1.58	2.57	26.67 ± 13.33
Mi29×CHU68 †	5.97	-0.57	5.40	35.29 ± 11.76
Mi109×Na102 †	11.13	-4.08	7.05	26.47 ± 2.94
Mi109×N17-D03 †	11.13	-3.09	8.04	47.06 ± 11.76
Mi29×Na50	5.97	4.46	10.42	26.47 ± 2.94

Sowing and observation dates were different for each year or environment at the ILGS, NARO, Nasushiobara, Tochigi, Japan.

† Unknown combination of F_1 hybrids, which were not used to predict the GCAs by BLUP, derived from the inbred lines used in field test 1.

‡ The field inoculation with natural infection data in Chapter 2, i.e. percentages of plants scoring more than two.

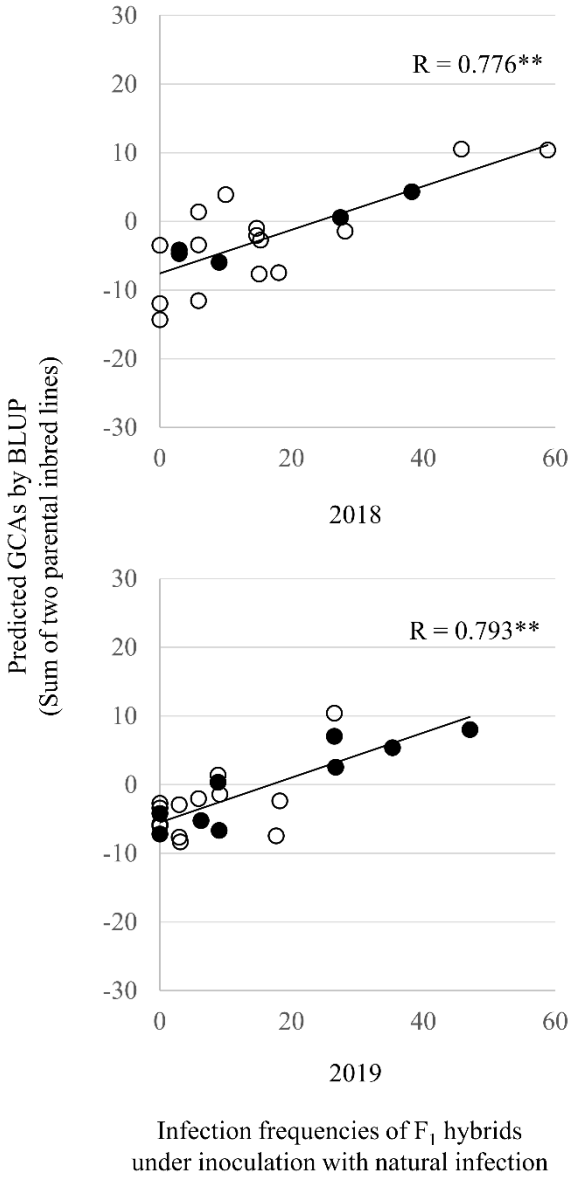

Fig. 5. Correlations between sum of the predicted GCAs by BLUP of inbred lines and infection frequencies of F_1 hybrids under inoculation with natural infection for Pythium RSR resistance (2018–2019); The open and closed circle represent known and unknown combinations derived from the inbred lines used in field test 1. Pearson's correlation coefficients were significant (**, $p < 0.01$).

Discussion

The correlation coefficient was low and not significant between the GCAs by BLUP and infection frequencies of inbred lines under inoculation with natural infection (Table 8). 'Mi29×Na50' ('Yumesodachi'), which is the most susceptible variety, had the highest combinations of GCAs by BLUP. But the combinations of infection frequencies under inoculation with natural infection data of inbred lines were different, especially in 'Na50' at 0.00 % in each year (Table 8). Thus, it is difficult to predict infection frequencies of F_1 data directly from those of inbred lines.

Bernardo (1996) reported that the correlations evaluated with cross-validation, between breeding values by BLUP and observed single-cross performance, ranged from 0.43 to 0.76 for grain yield, from 0.75 to 0.93 for grain moisture, from 0.30 to 0.74 for stalk lodging rate, and from 0.16 to 0.53 for root lodging rate in maize. This was done where all available test cross data and the coefficient of coancestry were incorporated into the predictions. The current data is consistent with these results, in the middle to high prediction accuracy for resistance of Pythium RSR (Table 9 and Figure 5).

In this chapter, the resistance to Pythium RSR was found to have a high correlation coefficient between predicted breeding values (sum of the GCAs of inbred lines) and the infection frequencies of F_1 hybrids under inoculation with natural

infection (Tables 9–11, Figure 5). This suggest that the effect of GCA is notably large in this resistance, as is known for other maize diseases (Beyene et al. 2017, Sibiya et al. 2013, Vivek et al. 2010). As shown in Table 8, it is currently unknown why a low correlation was between the infection frequencies under inoculation with natural infection and the GCAs by BLUP of parental inbred lines. However, these results strongly suggest that the resistance of parental inbred lines should be predicted not from their own phenotypes but from the predicted GCAs by BLUP. Considering the notable effect of GCA, it is possible to make effective selection for the resistance from the field test results, wherein a small number of F_1 progenies are observed.

Coefficient of coancestry is essential for improving prediction accuracy (Bromley et al. 2000, Piepho et al. 2008). However, as stated in the Materials and methods, it could not be adopted in this chapter, as the breeding processes of many parental inbred lines were unknown. Piepho et al. (2008) further presented several cases in which the coefficient of coancestry could not be used in plant breeding. For example, Tancred et al. (1995) used BLUP to estimate GCA and SCA effects for the date of ripening in apples (*Malus domestica*), despite not adopting the coefficient of coancestry. In this chapter, the author constructed a model that considered the relationships between F_1 hybrids and inbred lines using the Z_g design matrix. This process was a simple

pedigree model that showed how the GCAs of inbred lines were transmitted to the pedigrees, in which case the coefficient of coancestry might be considered unnecessary. Disadvantage of this method is that it is limited to handling simple pedigrees (Gallais 1980). However, in the author's actual breeding programs, the coefficient of coancestry is often not available, so the prediction of GCAs by BLUP becomes a practical advantage.

In conclusion, the results of this chapter indicate the potential of parental-progeny-based BLUP for maize single-cross performance to predict resistance to Pythium RSR even in untested hybrids. To obtain more accurate predictions, the author has collected genome-wide molecular data on parental inbred lines developed in the Japanese public sectors, described in Chapter 1. With these data, it will be possible to test how a full model such as the BLUP animal model, which usually includes the coefficient of coancestry, improves the prediction accuracy compared to the simpler model in the future.

CHAPTER IV. Genomic prediction of maize inbred lines using a small combined training population, and evaluation of untested inbred lines for resistance to Pythium RSR

Introduction

In the previous Chapters 1 and 3, the author has obtained SNPs array data and GCAs by BLUP of inbred lines. These values could be integrated to produce a GP model.

The preparation of large-scale training dataset is the major difficulty in GP. To overcome it, some studies have evaluated the effectiveness of GP for small populations of combined, multibreed training dataset in dairy cattle breeding (Erbe et al. 2012, Hayes et al. 2009a). In the case of the resistance breeding of maize, Technow et al. (2013) indicated that combined training dataset (the dent and flint inbred lines) provided adequate prediction accuracy by cross-validation even if the training population sizes were very small ($N \geq 25$). The objectives of this chapter are to (1) develop GP model for maize Pythium RSR resistance using data obtained in the breeding process and (2) employ GP to estimate the Pythium RSR resistance of maize inbred lines that have been never evaluated for this disease, using a combined training dataset derived from different heterotic groups.

Materials and methods

Plant materials

In total, 41 maize inbred lines consisting of 17 dent (D), 23 Japanese flint (JF), and one Japanese tropical (RD) inbred lines were tested as the training dataset for developing the GP model, and 188 inbred lines that had been never evaluated for this disease were employed to estimate Pythium RSR resistance using this model. All inbred lines were developed at the Nasushiobara, Hokkaido, and Miyakonojo research stations of NARO, Prefectural public breeding sections in Nagano, Japan, and Governmental Tokachi Agricultural Experiment Station in Hokkaido, Japan. The details of the inbred lines are described below (See the Results section).

Field test and phenotypic data

The breeding values of the 41 inbred lines used in this chapter were predicted by BLUP approach in Chapter 3. These values were derived from the natural infection frequencies of the F_1 hybrids against Pythium RSR in field tests conducted over four years from 2016 to 2019. These breeding values were utilized as the GCAs of each inbred line and as the phenotypic data for the training dataset described below.

Field experiments were conducted at the ILGS, NARO, Nasushiobara, Tochigi, Japan, following the customary practices that had been employed in the author's

breeding processes and previous chapters. A maize–Sudan grass (*Sorghum Sudanese* [Piper] Stapf.) annual rotation, manure, fertilizer, insecticide, and herbicides were applied as described in Chapters 2 and 3. All field tests were conducted in a randomized complete block design with two or four replicates. Each entry was grown in single-row plots, measuring 2.4 or 3.6 m in length, with a spacing of 0.75 m between rows. Each plot consisted of 13 or 19 plants. As the fields were presumed to have a high level of Pythium RSR pathogen contamination, F_1 hybrids were evaluated under natural infection. However, considering disease susceptibility, inbred lines were evaluated under inoculation with natural infection.

At the yellow-ripe stage of each hybrid (approximately 50 d after silking), the plants were cut approximately 5 cm above the ground, and the extent of rotting on the cut surface of the stalks was recorded. Pythium RSR resistance was evaluated based on the infection frequency, represented as the percentage of plants with scores of two or higher. The field experiments, Pythium RSR pathogen inoculation, evaluation, and scoring procedures were consistent with those described in the previous chapters. Please refer to Table 12 for further details regarding the scale and overview of the field tests.

Table 12. The scale and outline of the field test, which tested 648 F_1 hybrids derived from 105 inbred lines through natural infection from 2016 to 2019 in Chapter 4

Experiment number	Number of				Broad-sense heritability (H^2)
	Hybrids	Inbred lines crossed for hybrids	Plants in a single row	Replications	
2016-1	5	7	19	2	0.600
2016-2	22	20	19	2	0.651
2016-3	78	41	13	2	0.517
2016-4	61	44	13	2	0.562
2017-1	11	13	19	2	0.824
2017-2	20	25	19	2	0.840
2017-3	136	42	13	2	0.082 †
2017-4	58	35	13	2	0.204
2018-1	7	12	19	4	0.749
2018-2	100	35	19	2	0.278
2018-3	44	29	13	2	0.746
2018-4	47	32	13	2	0.652
2019-1	6	7	19	4	0.668
2019-2	17	24	19	2	0.796
2019-3	100	50	19	2	0.697
2019-4	10	13	13	2	0.845
2019-5	29	28	13	2	0.935

Sowing and observation dates were different for each year or environment at the ILGS, NARO, Nasushiobara, Tochigi, Japan. A total of 648 F_1 hybrids were tested, obtaining 751 data. These F_1 hybrids were derived from crosses among 105 inbred lines.

† These were excluded from the calculation of the mean as exaggerated values (Smirnov-Grubbs test $p < 0.05$). The average value of H^2 was 0.660.

BLUP to predict the GCAs of each inbred line

To predict the breeding values (GCAs) of the 41 inbred lines, the author adopted the following BLUP mixed model matrix equation described in Chapter 3.

$$y = X\beta + Z_g a_1 + Z_s a_2 + \varepsilon \quad (1)$$

$$\begin{pmatrix} X'R^{-1}y \\ Z_g{}'R^{-1}y \\ Z_s{}'R^{-1}y \end{pmatrix} = \begin{pmatrix} X'R^{-1}X & X'R^{-1}Z_g & X'R^{-1}Z_s \\ Z_g{}'R^{-1}X & Z_g{}'R^{-1}Z_g + V_{G1}^{-1} & Z_g{}'R^{-1}Z_s \\ Z_s{}'R^{-1}X & Z_s{}'R^{-1}Z_g & Z_s{}'R^{-1}Z_s + V_{G2}^{-1} \end{pmatrix} \begin{pmatrix} \hat{\beta} \\ \widehat{a_1} \\ \widehat{a_2} \end{pmatrix} \quad (2)$$

$$\begin{pmatrix} \hat{\beta} \\ \hat{a}_1 \\ \hat{a}_2 \end{pmatrix} = \begin{pmatrix} X'X & X'Z_g & X'Z_s \\ Z_g{}'X & Z_g{}'Z_g + \left(\frac{1-h^2}{h^2}\right)I & Z_g{}'Z_s \\ Z_s{}'X & Z_s{}'Z_g & Z_s{}'Z_s + \left(\frac{1-H^2}{H^2}\right)I \end{pmatrix}^{-1} \begin{pmatrix} X'y \\ Z_g{}'y \\ Z_s{}'y \end{pmatrix} \quad (3).$$

The solutions of the matrix equations Eqn. (1) to (3) follow the previous study. Broad-sense heritability (H^2) was determined 0.660, as the average of the values obtained from the analysis of variance (ANOVA) conducted for each test plot (refer to Table 12). Narrow-sense heritability (h^2) was calculated as 0.416, based on the ratio of Lazaro (2001), described in Chapter 3. The solution program was implemented using R version 4.0, developed by the R Core Team (2020). The calculated GCAs were then compared with Pythium RSR infection frequencies of inbred lines observed in the field inoculation with natural infection test conducted in 2021, as described earlier.

Genomic data and their analysis

All inbred lines were genotyped with the 'Maize LD Bead chip' (Illumina Inc, San Diego, USA) containing 3,047 single-nucleotide polymorphisms (SNPs). This process has been described in Chapter 1. Markers with more than 5% missing data were removed. 'BEAGLE' (Browning et al. 2018), version 5.4, was used to impute all remaining missing marker genotypes, resulting in 2,581 SNPs available for further analysis. The linkage disequilibrium (LD) was plotted as measured by r^2 toward marker distance in Mb for all 2,581 markers to see its pattern. LD plots and fitting were performed in R script written by Remington et al. (2001). A principal component analysis, based on the 2,581 SNP marker profiles of the inbred lines, was adopted to investigate the genetic distinction of different heterotic groups. These two indicators were calculated by 'Tassel' version 5.2.87 (Bradbury et al. 2007).

Prediction procedures

The author conducted all validation and prediction by parental inbred lines only; phenotypic data and genotypic data were substituted to develop the following linear model:

$$y_i = \mu + \sum \beta_j X_{ij} + \varepsilon_i \qquad (4)$$

In which y_i is the predicted GCA by BLUP of inbred i, μ is the overall mean, β_j represents the genetic effect of the marker j ($j = 1, 2\ldots$), X_{ij} denotes the genotype of marker j for inbred i and is defined by 1 or -1 for contrasting homozygous genotypes and 0 for heterozygous, and ε_i is the error deviation assumed to follow $N(0, \sigma^2)$. To estimate the genetic effect coefficients β_j, the author employed the Ridge regression method. Following this regression method, the author obtained a prediction model using the R package 'glmnet' (Friedman et al. 2010). The selection of hyperparameter λ in the Ridge regression was done using the cross-validation function of default.

Cross-validation and prediction

To assess the accuracy of GP, the author employed five-fold cross-validation following the method described by Zhao et al. (2012). Different marker patterns, including 250, 500, 1,000, 2,000, and all 2,581 markers, were evaluated to determine the optimal number of markers for prediction. A fixed number of markers were randomly distributed throughout the entire genome, and the dataset derived from the 41 inbred lines was randomly divided into five subsets. Four subsets were combined to form the training dataset for estimating genetic effects, whereas the remaining subset served as the

validation dataset. The author determined the prediction accuracy (r) using the correlation between the predicted GCAs by BLUP from the validation dataset and the estimated GCA (EsGCA) from the genetic effects calculated by the training model. The process of randomly locating markers was repeated 100 times, the sampling of training and validation sets was repeated 1,000 times for each marker set, and the mean of both prediction accuracy and 95% confidence interval (CI) for each marker set was calculated. Furthermore, the training model, utilizing all 41 inbred lines and 2,581 markers, was applied to estimate the EsGCAs of an additional 188 untested inbred lines for this disease.

Results

Table 13 provides details of the 41 inbred lines. Supplementary Table 3 lists all the results of GCAs for 105 inbred lines. The predicted GCAs by BLUP were inconsistent with the results of the field inoculation with natural infection test conducted in 2021, with a correlation coefficient of 0.170 (N = 17, not significant). This inconsistency was particularly evident in the case of 'Na50', a representative parental inbred line of susceptible F_1 hybrids that exhibited resistance itself, consistent with the author's previous studies and Chapter 3. Therefore, the author confirms that the results of field

inoculation with natural infection test conducted on inbred lines cannot be considered phenotypic values for GP of Pythium RSR resistance.

The PCA result illustrates the population composition within the author's dataset, clearly depicting a distinct separation between D, RD, and JF inbred lines in the training dataset. 'Na112', derived from the EF and JF groups, had been classified within the JF group in Chapter 1, and it occupied a similar position in the current analysis. In the validation dataset, each group also showed clear separation, with the MIS inbred lines generally positioned in the middle of the groups (Figure 6).

Table 13. Resistance to Pythium RSR under inoculation with natural infection and GCAs by BLUP in elite inbred lines used in Chapter 4.

Inbred name †	Group ‡	GCAs by BLUP	Observed values in the field	EsGCAs in the model	Developed by §
CHU44	JF	1.76	10.00	1.21	CAES, Nagano pref.
JC-028	D	-1.37	4.55	-1.39	CAES, Nagano pref.
CHU68	JF	-0.16	4.55	-0.11	CAES, Nagano pref.
JC-037	D	-5.06	0.00	-4.07	CAES, Nagano pref.
JC-038	D	0.91	-	-0.18	CAES, Nagano pref.
Mi47	JF	-1.78	0.00	-2.03	KARC, NARO
Mi91	RD	0.14	-	0.05	KARC, NARO
Mi103	JF	-0.71	0.00	-1.07	KARC, NARO
Mi111	JF	-5.92	0.00	-5.29	KARC, NARO
Mi115	JF	-2.32	-	-1.45	KARC, NARO
N09-07	JF	-5.68	-	-5.36	ILGS, NARO
N10-01	D	-1.68	-	-1.77	ILGS, NARO
N10-02	D	-4.80	9.55	-4.66	ILGS, NARO
N10-08	JF	-4.19	-	-3.93	ILGS, NARO
N10-12	JF	-6.01	-	-4.96	ILGS, NARO
N11-02	JF	-1.87	-	-1.74	ILGS, NARO
N12-01	D	-4.03	-	-3.84	ILGS, NARO
N12-02	JF	0.55	-	0.69	ILGS, NARO

Table 13. Continued

Inbred name †	Group ‡	GCAs by BLUP	Observed values in the field	EsGCAs in the model	Developed by §
N12-05	JF	-1.60	34.85	-1.23	ILGS, NARO
N12-07	JF	-3.66	-	-3.10	ILGS, NARO
N13-01	D	-3.27	-	-3.07	ILGS, NARO
N13-05	JF	5.05	-	5.04	ILGS, NARO
N13-06	JF	8.26	-	7.09	ILGS, NARO
N13-08	JF	0.80	-	0.34	ILGS, NARO
N14-01	D	-1.49	-	-1.65	ILGS, NARO
N14-02	D	-4.56	-	-4.30	ILGS, NARO
N15-01	D	-4.55	-	-4.19	ILGS, NARO
N16-03	D	-1.37	-	-1.91	ILGS, NARO
N16-07	JF	0.88	-	1.06	ILGS, NARO
Na50	JF	4.89	0.00	4.72	ILGS, NARO
Na65	D	0.66	27.78	0.10	ILGS, NARO
Na71	D	-6.99	0.00	-6.49	ILGS, NARO
Na83	JF	5.74	-	4.97	ILGS, NARO
Na98	D	-5.77	0.00	-4.98	ILGS, NARO
Na100	D	2.92	-	1.84	ILGS, NARO
Na102	D	-8.17	5.00	-7.33	ILGS, NARO
Na106	JF	7.21	6.25	6.41	ILGS, NARO

Table 13. Continued

Inbred name †	Group ‡	GCAs by BLUP	Observed values in the field	EsGCAs in the model	Developed by §
Na109	D	-1.22	-	-1.31	ILGS, NARO
Na111	JF	-1.83	0.00	-1.57	ILGS, NARO
Na112	MIS (EF*JF)	-0.61	0.00	-0.24	ILGS, NARO
Na113	JF	7.93	-	6.77	ILGS, NARO

GCAs were calculated from F_1 data obtained in the field test using BLUP described in Chapter 3. Observed values in the field were Pythium RSR infection frequency of field inoculation with natural infection test in 2021. The correlation coefficient between GCAs by BLUP and observed values was 0.170 (N = 17, not significant). EsGCAs were estimated in the training model using all 2,581 SNPs.

† CHU is the registered inbred lines. JC is promising inbred lines. Mi and Na are the registered or promising inbred lines. N is the superior inbred line before Na was named.

‡ EF, RD, JF, D and MIS indicate flint mainly developed or derived from the European region, Japanese tropical inbred lines mainly developed from hybrids for summer seeding, Japanese flint landraces, Japanese dent mainly derived from US corn-belt dent, and miscellaneous origin, respectively. * is derived from crossing between each group.

§ KARC, ILGS, and CAES are abbreviations for Kyushu Okinawa Agricultural Research Center, NARO, Institute of Livestock and Grassland Science, NARO, and Chushin Agricultural Experiment Station, Nagano pref., respectively.

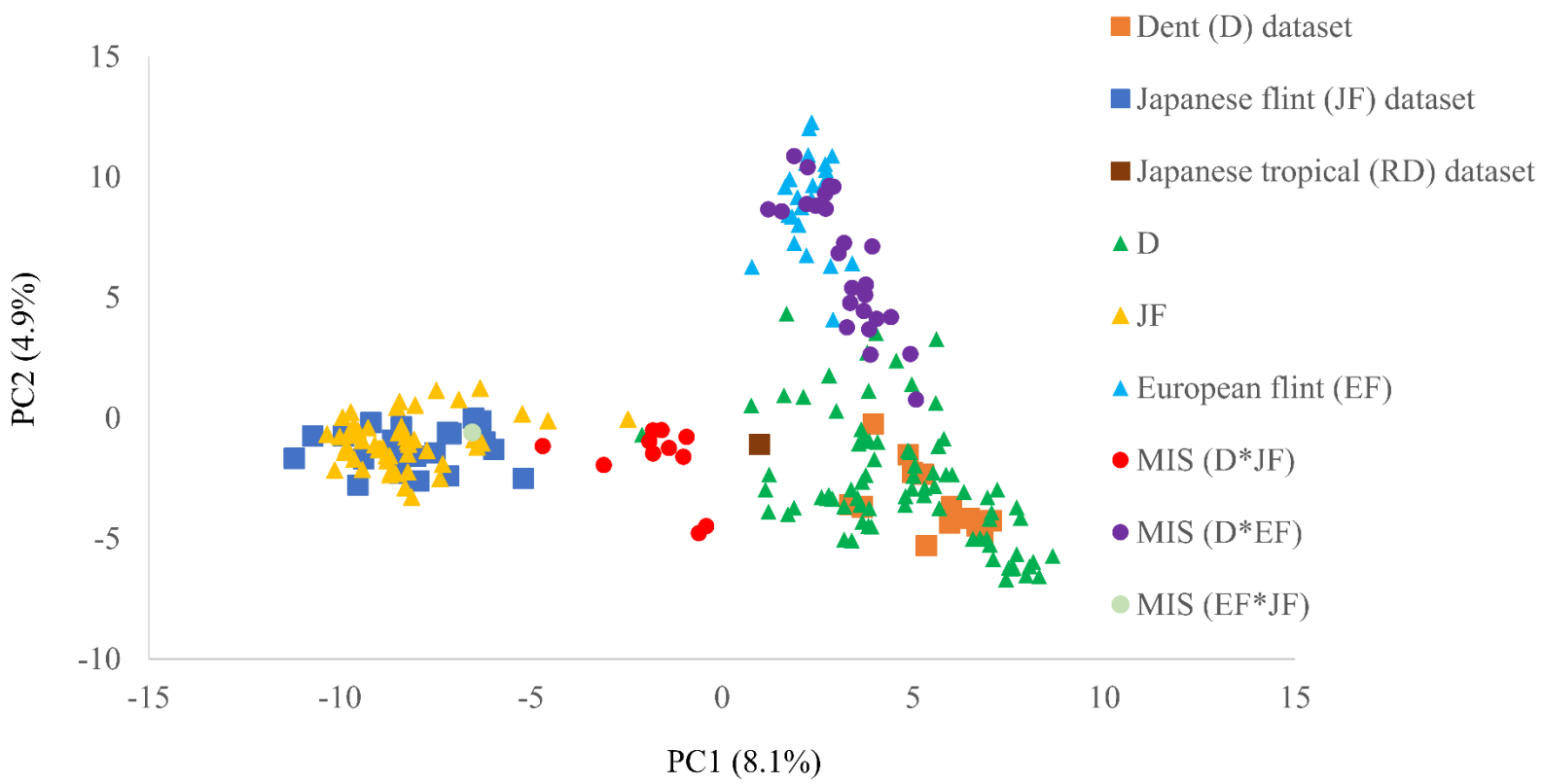

Fig. 6. The plot of principal component (PC) 1 and PC 2 scores based on 2,581 SNP markers of all the 229 inbred lines used in Chapter 4. The square sign indicates the 41 inbred lines used in the training dataset. In Chapter 1, 22 out of 41 inbred lines from the training dataset and 85 out of 188 inbred lines from the validation dataset described in Chapter 4 were used.

LD (r^2) between markers for the 41 maize inbred lines was exceeded 0.30 within a distance of less than 0.24 Mb. It gradually decreased to around 0.20 at approximately 0.60 Mb. Beyond 1.00 Mb, LD continued to decline slightly but remained above 0.10 at approximately 2.00 Mb (Figure 7).

During the repetition of randomly locating each marker set 100 times and performing sampling and validation 1,000 times for each marker set, outliers of prediction accuracy (r) were excluded using the Smirnov–Grubbs test ($p < 0.05$). The average prediction accuracy improved as the number of markers increased. However, the rate of improvement diminished when the number of markers exceeded 1,000 (r = 0.649, 95% CI: 0.644–0.653), and it nearly reached a plateau at around 1,000 to 2,000 markers (r = 0.653, 95% CI: 0.651–0.656). The prediction accuracy using all 2,581 markers was 0.656 (95% CI: 0.655–0.657; Table 14, Figure 8).

Table 15 presents the EsGCAs among the 188 untested inbred lines, in terms of resistance to Pythium RSR, based on the prediction model utilized in this chapter. The lowest EsGCAs among D, JF, and European flint inbred lines (EF) were differed (-4.21, -3.29 and -1.77). Also, the highest EsGCAs among those inbred lines are differed (0.37, 2.63 and -0.17). Inbred lines derived from crosses between each group exhibited intermediate values. This finding is consistent with the results of the PCA (Figure 6).

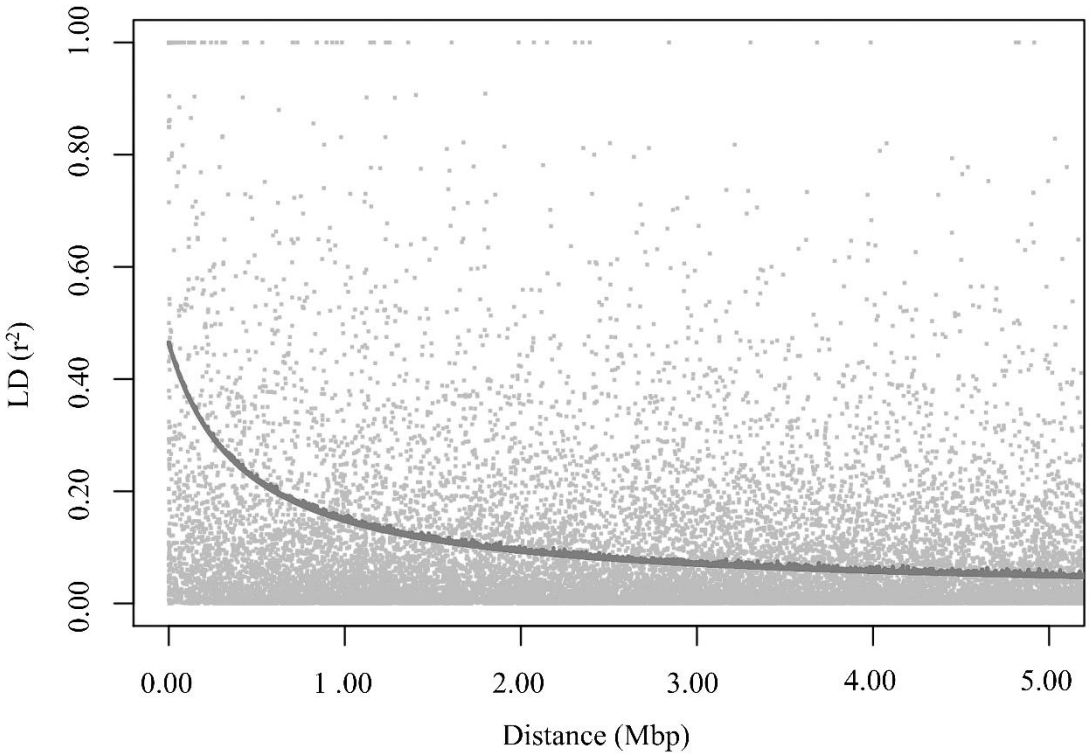

Fig. 7. Linkage disequilibrium (LD r^2) decay plot of 2,581 markers as a function of physical distance (Mb) for the 41 maize inbred lines used in Chapter 4.

Table 14. Prediction accuracy (r) of genomic predictions across populations according to the number of each marker revealed by five-fold cross-validation for Pythium RSR resistance

The number of markers	Average distance between markers (Mb) †	Prediction accuracy (r)	95% confidence interval
250	9.20	0.605	0.597–0.614
500	4.60	0.638	0.632–0.643
1000	2.30	0.649	0.644–0.653
2000	1.15	0.653	0.651–0.656
2581	0.89	0.656	0.655–0.657

The process of randomly locating markers was repeated 100 times, and the sampling of training and validation sets was repeated 1,000 times for each marker set.

† Calculated assuming a total maize genome size of 2.3 Gb.

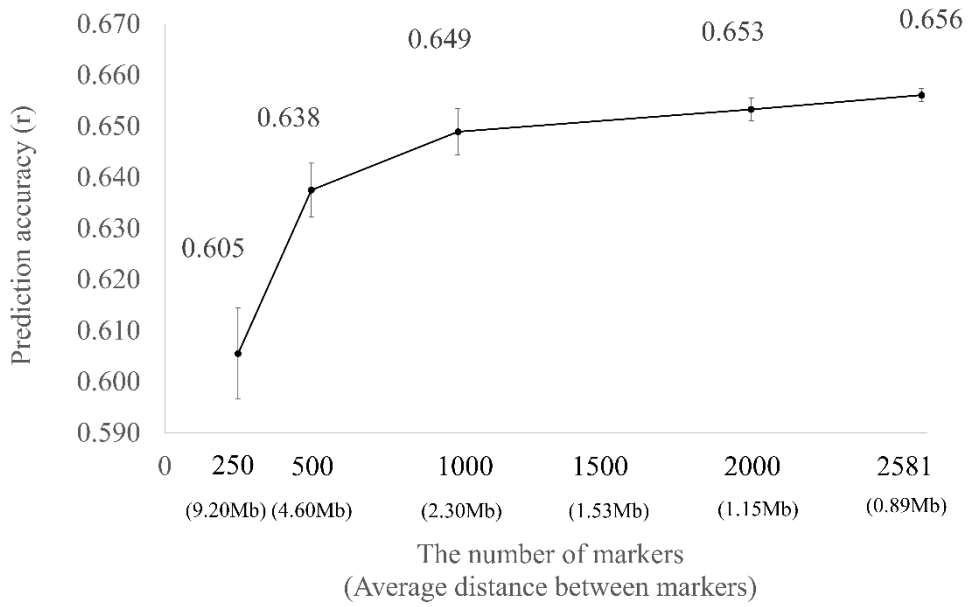

Fig. 8. Transition of the Prediction accuracy (r) of genomic predictions across populations revealed by five-fold cross-validation for Pythium RSR resistance. The process of randomly locating markers was repeated 100 times, and the sampling of training and validation sets was repeated 1,000 times for each marker set. Error bars represent 95% confidence intervals.

Table 15. Estimated general combining ability (EsGCA) for 188 inbred lines in the resistance of Pythium RSR based on the prediction model constructed in Chapter 4

Inbred name †	Group ‡	EsGCA by GP	Developed by §
Na60	D	-4.21	ILGS, NARO
Ho57	D	-4.10	HARC, NARO
Na78	D	-4.09	ILGS, NARO
Na54	D	-3.87	ILGS, NARO
J1350	D	-3.76	CAES, Nagano pref.
Na56	D	-3.69	ILGS, NARO
Na49	D	-3.58	ILGS, NARO
Na29	D	-3.49	ILGS, NARO
Na42	D	-3.42	ILGS, NARO
IM-390	D	-3.29	KARC, NARO
Na7	D	-3.27	ILGS, NARO
Na81	D	-3.25	ILGS, NARO
Na62	D	-3.21	ILGS, NARO
Ho52	D	-3.13	HARC, NARO
Na61	D	-3.10	ILGS, NARO
Na9	D	-3.10	ILGS, NARO
Na36	D	-3.03	ILGS, NARO
Na70	D	-2.97	ILGS, NARO
Na53	D	-2.91	ILGS, NARO

Table 15. Continued

Inbred name †	Group ‡	EsGCA by GP	Developed by §
Na23	D	-2.89	ILGS, NARO
Na55	D	-2.86	ILGS, NARO
J1417	D	-2.75	CAES, Nagano pref.
JC-002	D	-2.52	CAES, Nagano pref.
Na64	D	-2.52	ILGS, NARO
Na41	D	-2.50	ILGS, NARO
IM-429	D	-2.47	KARC, NARO
Na34	D	-2.42	ILGS, NARO
IM-435	D	-2.35	KARC, NARO
IM-423	D	-2.34	KARC, NARO
IM-437	D	-2.31	KARC, NARO
Na86	D	-2.30	ILGS, NARO
Na32	D	-2.28	ILGS, NARO
JC-014	D	-2.26	CAES, Nagano pref.
IM-436	D	-2.10	KARC, NARO
Na15	D	-2.09	ILGS, NARO
J1698	D	-2.08	CAES, Nagano pref.
JC-050	D	-2.07	CAES, Nagano pref.
Ho68	D	-2.06	HARC, NARO
Ho106	D	-2.05	HARC, NARO
JC-054	D	-2.04	CAES, Nagano pref.

Table 15. Continued

Inbred name	Group	EsGCA by GP	Developed by
IM-477	D	-1.98	KARC, NARO
Na77	D	-1.97	ILGS, NARO
Na58	D	-1.96	ILGS, NARO
Na8	D	-1.96	ILGS, NARO
Na17	D	-1.95	ILGS, NARO
IM-465	D	-1.94	KARC, NARO
IM-421	D	-1.93	KARC, NARO
IM-450	D	-1.91	KARC, NARO
IM-467	D	-1.90	KARC, NARO
JC-064	D	-1.84	CAES, Nagano pref.
Mi83	D	-1.82	KARC, NARO
IM-466	D	-1.79	KARC, NARO
J1706	D	-1.77	CAES, Nagano pref.
IM-422	D	-1.76	KARC, NARO
Na74	D	-1.74	ILGS, NARO
J1605	D	-1.72	CAES, Nagano pref.
Na38	D	-1.70	ILGS, NARO
Na18	D	-1.64	ILGS, NARO
J1559	D	-1.52	CAES, Nagano pref.
Na6	D	-1.46	ILGS, NARO
Na25	D	-1.44	ILGS, NARO

Table 15. Continued

Inbred name	Group	EsGCA by GP	Developed by
Na13	D	-1.38	ILGS, NARO
TI-123	D	-1.38	HARC, NARO
IM-427	D	-1.20	KARC, NARO
IM-424	D	-1.19	KARC, NARO
Na87	D	-1.11	ILGS, NARO
Ho104	D	-1.10	HARC, NARO
J1330	D	-1.08	CAES, Nagano pref.
Na69	D	-1.08	ILGS, NARO
IM-426	D	-0.91	KARC, NARO
IM-475	D	-0.78	KARC, NARO
IM-455	D	-0.65	KARC, NARO
JC-036	D	-0.32	CAES, Nagano pref.
Na43	D	-0.29	ILGS, NARO
J1407	D	-0.15	CAES, Nagano pref.
Na45	D	0.06	ILGS, NARO
J1383	D	0.22	CAES, Nagano pref.
JC-046	D	0.37	CAES, Nagano pref.
J1539	D	0.37	CAES, Nagano pref.
Na84	JF	-3.29	ILGS, NARO
IM-459	JF	-2.62	KARC, NARO

Table 15. Continued

Inbred name †	Group ‡	EsGCA by GP	Developed by §
Mi107	JF	-2.01	KARC, NARO
IM-458	JF	-1.47	KARC, NARO
J1608	JF	-1.40	CAES, Nagano pref.
JC-034	JF	-1.19	CAES, Nagano pref.
JC-053	JF	-0.89	CAES, Nagano pref.
IM-470	JF	-0.87	KARC, NARO
IM-469	JF	-0.73	KARC, NARO
IM-402	JF	-0.65	KARC, NARO
IM-468	JF	-0.62	KARC, NARO
IM-252	JF	-0.57	KARC, NARO
Na92	JF	-0.52	ILGS, NARO
IM-403	JF	-0.51	KARC, NARO
Na88	JF	-0.46	ILGS, NARO
IM-454	JF	-0.27	KARC, NARO
Na97	JF	-0.14	ILGS, NARO
Na79	JF	-0.12	ILGS, NARO
J1785	JF	-0.04	CAES, Nagano pref.
JC-026	JF	-0.01	CAES, Nagano pref.
Mi102	JF	0.01	KARC, NARO
N17-F04	JF	0.02	ILGS, NARO
IM-453	JF	0.09	KARC, NARO

Table 15. Continued

Inbred name †	Group ‡	EsGCA by GP	Developed by §
J1693	JF	0.11	CAES, Nagano pref.
IM-431	JF	0.13	KARC, NARO
IM-464	JF	0.15	KARC, NARO
IM-452	JF	0.17	KARC, NARO
IM-460	JF	0.18	KARC, NARO
N14-04	JF	0.34	ILGS, NARO
Mi105	JF	0.54	KARC, NARO
Na76	JF	0.60	ILGS, NARO
Na30	JF	0.88	ILGS, NARO
Na85	JF	1.03	ILGS, NARO
Na66	JF	1.04	ILGS, NARO
Na27	JF	1.05	ILGS, NARO
Na51	JF	1.10	ILGS, NARO
IM-461	JF	1.25	KARC, NARO
JC-009	JF	1.25	CAES, Nagano pref.
Na93	JF	1.25	ILGS, NARO
Na5	JF	1.35	ILGS, NARO
Na80	JF	1.44	ILGS, NARO
Na26	JF	1.51	ILGS, NARO
Na4	JF	1.55	ILGS, NARO
IM-472	JF	1.68	KARC, NARO

Table 15. Continued

Inbred name †	Group ‡	EsGCA by GP	Developed by §
IM-248	JF	1.88	KARC, NARO
Na89	JF	1.96	ILGS, NARO
IM-430	JF	2.37	KARC, NARO
Na28	JF	2.51	ILGS, NARO
Na2	JF	2.53	ILGS, NARO
Na95	JF	2.63	ILGS, NARO
Ho131	EF	-1.77	HARC, NARO
Ho99	EF	-1.65	HARC, NARO
TI-083	EF	-1.60	HARC, NARO
Ho120	EF	-1.24	HARC, NARO
TI-045	EF	-1.20	HARC, NARO
Ho129	EF	-1.10	HARC, NARO
Ho49	EF	-1.09	HARC, NARO
Ho90	EF	-1.08	HARC, NARO
TI-137	EF	-1.07	HARC, NARO
TI-091	EF	-1.06	HARC, NARO
Ho126	EF	-0.87	HARC, NARO
Ho96	EF	-0.82	HARC, NARO
TI-064	EF	-0.76	HARC, NARO
Ho130	EF	-0.71	HARC, NARO

Table 15. Continued

Inbred name †	Group ‡	EsGCA by GP	Developed by §
TI-092	EF	-0.63	HARC, NARO
Ho119	EF	-0.57	HARC, NARO
TI-094	EF	-0.54	HARC, NARO
Ho124	EF	-0.52	HARC, NARO
TI-106	EF	-0.50	HARC, NARO
Ho87	EF	-0.47	HARC, NARO
TI-105	EF	-0.24	HARC, NARO
Ho121	EF	-0.22	HARC, NARO
Ho127	EF	-0.17	HARC, NARO
IM-254	MIS (D*JF)	-1.92	KARC, NARO
Na72	MIS (D*JF)	-1.22	ILGS, NARO
Na57	MIS (D*JF)	-1.13	ILGS, NARO
IM-308	MIS (D*JF)	-1.06	KARC, NARO
IM-239	MIS (D*JF)	-0.75	KARC, NARO
IM-270	MIS (D*JF)	-0.56	KARC, NARO
Na82	MIS (D*JF)	-0.55	ILGS, NARO
IM-419	MIS (D*JF)	-0.44	KARC, NARO
IM-347	MIS (D*JF)	-0.36	KARC, NARO
J1707	MIS (D*JF)	-0.19	CAES, Nagano pref.
Na94	MIS (D*JF)	0.95	ILGS, NARO

Table 15. Continued

Inbred name †	Group ‡	EsGCA by GP	Developed by §
TI-098	MIS (D*EF)	-2.18	HARC, NARO
TI-133	MIS (D*EF)	-2.11	HARC, NARO
TI-114	MIS (D*EF)	-2.02	HARC, NARO
To113	MIS (D*EF)	-1.93	TAES, Hokkaido gov.
TI-145	MIS (D*EF)	-1.78	HARC, NARO
TI-107	MIS (D*EF)	-1.58	HARC, NARO
TI-044	MIS (D*EF)	-1.51	HARC, NARO
TI-086	MIS (D*EF)	-1.44	HARC, NARO
To85	MIS (D*EF)	-1.34	TAES, Hokkaido gov.
TI-126	MIS (D*EF)	-1.33	HARC, NARO
TI-132	MIS (D*EF)	-1.30	HARC, NARO
TI-097	MIS (D*EF)	-1.27	HARC, NARO
TI-096	MIS (D*EF)	-1.16	HARC, NARO
TI-118	MIS (D*EF)	-1.13	HARC, NARO
TI-111	MIS (D*EF)	-1.08	HARC, NARO
TI-061	MIS (D*EF)	-1.07	HARC, NARO
TI-130	MIS (D*EF)	-1.04	HARC, NARO
To15	MIS (D*EF)	-1.01	TAES, Hokkaido gov.
TI-131	MIS (D*EF)	-0.99	HARC, NARO
TI-136	MIS (D*EF)	-0.91	HARC, NARO
TI-095	MIS (D*EF)	-0.88	HARC, NARO

Table 15. Continued

Inbred name †	Group ‡	EsGCA by GP	Developed by §
TI-081	MIS (D*EF)	-0.83	HARC, NARO
To90	MIS (D*EF)	-0.75	TAES, Hokkaido gov.
To38	MIS (D*EF)	-0.28	TAES, Hokkaido gov.
TI-108	MIS (D*EF)	-0.02	HARC, NARO

The prediction model was derived from the training dataset of the 41 elite inbred lines and 2,581 SNPs.

† J, JC Mi, Na, Ho, and To are the registered or promising inbred lines.

IM, N, and TI are the superior inbred lines before Mi, Na, and Ho were named.

‡ Classification by heterotic groups, defined based on the origin of each inbred lines, where heterotic patterns were expected.

EF, JF, D and MIS indicate flint mainly developed or derived from the European region, Japanese flint landraces, Japanese dent mainly derived from US corn-belt dent, and miscellaneous origin, respectively. * is derived from crossing between each group.

§ HARC, KARC, ILGS, CAES and TAES are abbreviations for Hokkaido Agricultural Research Center, NARO, Kyushu Okinawa Agricultural Research Center, NARO, Institute of Livestock and Grassland Science, NARO, Chushin Agricultural Experiment Station, Nagano pref., and Tokachi Agricultural Experiment Station, Hokkaido gov., respectively.

Discussion

Labor and field capacity are limiting factors in the maize breeding system. However, it is well-known that larger population sizes lead to higher genetic gains in GP (Lorenz et al. 2011). Therefore, the author made significant efforts to design breeding schemes that enhanced efficiency while reducing labor requirements.

PCA analysis clearly indicated a distinct separation between D, JF, and RD in the training dataset (Figure 6). LD between markers was high in the close distances, and it gradually decreased but continued to decline slightly and remained above 0.10 at around 2.00 Mb (Figure 7). Technow et al. (2013) suggested that small training datasets, combining dent and flint inbred lines, achieved adequate prediction accuracy for Northern corn leaf blight (NCLB) resistance. In that study, PCA was clear distinct separation between the small dataset (D, JF, and RD), and LD continued to decrease above 1.00 Mb but remained greater than 0.10 over the entire range of distances considered. The maintenance of LD over long distances affects the accuracy of GP because, as shown by Hayes et al. (2009a), SNPs with high LD close to the target gene are better markers, but distant SNPs with low LD can also have an effect. The author's findings were similar to those of the previous study. These findings are considered appropriate for achieving high prediction accuracy even with a small population size.

Technow et al. (2013) reported that the prediction accuracies were 0.576–0.589 (N = 50) and 0.690–0.706 (N = 75) in Northern corn leaf blight. Crossa et al. (2014) showed that the prediction accuracies were 0.588–0.790 in flowering, 0.513–0.572 in anthesis-silking interval, and 0.415–0.525 in grain yield (N = 284). Considering the use of a smaller training population (N = 41), the author confirms that the GP model presented in this chapter achieves sufficient prediction accuracy compared to the previous studies (Table 14 and Figure 8).

Rashid et al. (2020) employed around 300,000 SNPs for conducting GWAS on resistance to maize NCLB, whereas Liu et al. (2021) utilized over 200,000 SNPs for resistance to Fusarium ear rot. However, to achieve cost-effectiveness, GP should be performed using lower-density markers (Heffner et al. 2010). In the author's study, the prediction accuracy improved with an increasing number of markers, but it reached closer to a plateau when the number of markers exceeded 1,000 (Table 14 and Figure 8). This observation is consistent with a previous study by Zhao et al. (2012), where prediction accuracy plateau at approximately 800 SNPs. These results indicate that GP for Pythium RSR resistance can be achieved using a small population size and lower-density markers.

In this study, some of the JF and EF inbred lines were predominantly derived

from regions with colder climates, such as Hokkaido or Northern Europe. Breeding for RSR resistance, which is more prevalent under hot and humid conditions, presents challenges in such regions. The EsGCA of JF and EF inbred lines were higher (i.e., more susceptible) than those of D inbred lines among the resistant ones (Table 15). The different selection pressures are likely reflected in these results. None of inbred lines with lowest EsGCAs have been utilized in recent breeding programs owing to their age (developed from the 1990s to early 2000s). These results suggest the potential utilization of such old germplasms as valuable materials for developing resistant hybrids.

The geographic diversity of Japan, spanning from Hokkaido in the north to Kyushu in the south, presents different challenges for breeding and selecting maize inbred lines suitable for each region. The outbreak of Pythium RSR is not frequent in Hokkaido, because *Pythium* spp. prefer to higher temperatures (Kageyama 2014). Similarly, Kyushu experiences different cropping systems compared to Kanto (Nasushiobara). However, under specific conditions such as adequate temperature and heavy rainfall during the glue-ripening stage, large outbreaks of Pythium RSR can occur even in these regions (Deep and Lipps 1996, Reyes-Tena et al. 2018, Yanar et al. 1997). Accurate prediction of Pythium RSR resistance using GP can assist in making preliminary selections based solely on genotypic data from the constructed model.

In conclusion, despite using a smaller training population (N = 41) and lower-density markers (approximately 1,000 SNPs), the GP model presented in this chapter achieves sufficient prediction accuracy. Increasing the size of the training population and using multiple populations may enhance the accuracy of GP (Hayes et al. 2009a, Lorenz et al. 2011). Based on the findings obtained in this chapter, utilizing larger populations and selecting appropriate inbred lines may lead to further improvements in prediction accuracy. The results have significant implications, particularly in regions with limited labor and field resources. However, it is crucial to validate the reliability of the EsGCAs through field tests involving infection frequencies of F_1 derived from the 188 inbred lines used in this chapter. These findings offer new possibilities for breeding maize with Pythium RSR resistance in Japan.

CHAPTER V. Genomic approaches for improving Pythium RSR resistance in maize F_1 plants and their validation

Introduction

The author has confirmed effective methods for breeding Pythium RSR resistant materials in earlier chapters. In the breeding programs, the author has utilized GD, the bottom stalk toothpick inoculation method, and BLUP, which have contributed successfully to developing new inbred lines and F_1 varieties. The objective of this chapter is to propose a genomic approach for improving Pythium RSR resistance in maize F_1 plants. In addition, the author demonstrates and validates a breeding process of two inbred lines and one novel F_1 variety in the present study.

Materials and methods

Plant materials and verification of their combinations

To verify the efficient selection of F_1 combinations with resistance to Pythium RSR, a total of 11 maize inbred lines consisting of five dent (D), five Japanese flint (JF), and one miscellaneous origin (MIS, but regarded as JF due to its origin) inbred lines were selected. Among these inbred lines, four both D and JF inbred lines were parents of commercial F_1 varieties, two JF, one MIS, and one D inbred lines were selected as

promising in recent breeding programs. The names of these inbred lines start with the first letter of the locations where they were developed; ‘Ho (Hokkaido)’, ‘Mi (Miyakonojo in Miyazaki pref.)’, ‘Na (Nasushiobara in Tochigi pref.)’, and ‘CHU (Chushin area in Nagano pref.)’, respectively.

Data collection of GD between parental inbred lines

Using the 1,007 SNPs information obtained in Chapter 1, the mean pairwise genetic distance of proportion (p) of nucleotide sites was calculated by ‘MEGA X’ as GD (Table S1). The GD between inbred lines were compared and evaluated to verify promising combinations assuming crosses within and between different heterotic groups.

Data collection of predicted GCAs of parental inbred lines based on the data from F_1 varieties

The breeding values of each inbred line used in this chapter were calculated by BLUP approach described in Chapter 3. These values were derived from the natural infection frequencies of the F_1 combinations against Pythium RSR in field tests conducted over four years from 2014 to 2017. These breeding values were utilized as the GCAs of each inbred line (Table S2). EsGCAs of each inbred line estimated by GP model are described

in Table 15 in Chapter 4, except for ‘Mi29’ and ‘Ho110’. The sums of the GCAs between inbred lines were compared and evaluated to verify promising combinations assuming crosses within and between different heterotic groups.

Development of the promising inbred lines and a novel F_1 variety

The promising inbred lines ‘Na113’ and ‘Na102’ were also used in this chapter. Detailed breeding scheme were described in the Results section following, in addition to their F_1 hybrid.

Results

GD between parental inbred lines

The GD between promising inbred lines including ‘Na113’ and ‘Na102’ is shown in Table 16. Out of fifty-five combinations, the range of GD were from 0.167 between ‘Na113’ and ‘Na50’ to 0.364 both between ‘Ho110’ and ‘Na50’, and ‘Na71’ and ‘CHU68’ (‘Takanefudo’). The GD between the heterotic groups (D and JF) is shown in Table 17, where GDs were above 0.300 between all the combinations of each inbred line except 0.293 between ‘Mi29’ and ‘Na113’. These scores were almost equivalent to the average score of 0.332 between 127 inbred lines in Chapter 1 (Table 2). The GD

within the same JF group is shown in Table 18, where GDs were nearly equivalent to 0.300 between all the combinations of each inbred line except 0.167 between 'Na113' and 'Na50'. The GD within the same D group is demonstrated in Table 19, where GDs were lower than 0.300 at ranging from 0.209 to 0.282 except the combination involving 'Ho110' with the score of ranging 0.309 to 0.349.

Sum of the predicted GCAs based on the data from parental inbred lines

The sum of the predicted GCAs by BLUP combining inbred lines including 'Na113' and 'Na102' is shown in Table 20. Out of fifty-five combinations, the range of sums of the predicted GCAs by BLUP were from -11.15 between 'Na71' and 'Na102' to 10.42 between 'Mi29' and 'Na50' ('Yumesodachi'). The sum of the predicted GCAs by BLUP between the heterotic groups (D and JF) is shown in Table 21, where sums of the predicted GCAs were below 0.00 for almost all the combinations involving 'Na71' and 'Na102'. The sum of the predicted GCAs within the same JF group is shown in Table 22, where sums of the predicted GCAs were above 0.00 between all the combinations of each inbred line except -0.04 between 'Na112' and 'CHU44', -1.70 between 'Na112' and 'CHU68'. The sum of the predicted GCAs within the same D group is demonstrated in Table 23, where sums of the predicted GCAs were lower than 0.00 for almost all the

combinations involving ‘Na71’ and ‘Na102’. These combinations also included ‘Ho110’, which had high GDs with all D inbred lines.

A promising inbred line ‘Na113’ and its breeding scheme

‘Na113’ (MAFF variety registration application No. 35623) is a Japanese flint inbred line having a flooding-resistant QTL from ‘Teosinte’ (*Z. nicaraguensis*) with back-crossing (‘Na50’ is a repeating parent). This inbred line exhibits resistance to humid conditions, such as those in converted paddy fields, and shows superior Pythium RSR resistance compared to ‘Na50’. In 2011, the prototype inbred line ‘IL#18a’, which had incorporated a flooding-resistant QTL from *Z. nicaraguensis* into a dent inbred line ‘Mi29,’ was crossed with ‘Na50’. This cross was followed by six backcrosses from ‘Na50’ and two self-crosses by 2015. In 2016, the BC_6F_3 generation seeds were propagated through sibling crosses, and tests were conducted to confirm its superiority (Figure 9). These include flooding-resistant tests using Wagner pots in a greenhouse, combining ability tests of F_1 hybrids, and field characterization tests of the inbred line. In 2018, the name ‘Na113’ was given, and thereafter, combined ability, characterization, and productivity tests have been continuously conducted.

Table 16. Genetic distances between promising inbred lines, those are dent, Japanese flint, and miscellaneous origin

Name/Group †		Na50	Na113	Na111	CHU44	CHU68	Na112	Ho110	Na71	Na102	Na65
		JF	JF	JF	JF	JF	MIS (EF*JF)	D	D	D	D
Na113	JF	0.167	-	-	-	-	-	-	-	-	-
Na111	JF	0.327	0.327	-	-	-	-	-	-	-	-
CHU44	JF	0.336	0.326	0.247	-	-	-	-	-	-	-
CHU68	JF	0.323	0.348	0.319	0.310	-	-	-	-	-	-
Na112	MIS (EF*JF)	0.319	0.307	0.322	0.277	0.336	-	-	-	-	-
Ho110	D	0.364	0.354	0.361	0.358	0.363	0.349	-	-	-	-
Na71	D	0.348	0.336	0.315	0.335	0.364 ‡	0.325	0.312	-	-	-
Na102	D	0.343	**0.322**	0.339	0.323	0.353	0.329	0.349	0.209	-	-
Na65	D	0.331	0.311	0.336	0.316 ‡	0.356	0.318	0.309	0.264	0.244	-
Mi29	D	0.334 ‡	0.293	0.319	0.319	0.338	0.301	0.325	0.282	0.258	0.236

† EF, JF, D and MIS indicate flint mainly developed or derived from the European region, Japanese flint landraces, Japanese dent mainly derived from US corn-belt dent, and miscellaneous origin, respectively. * is derived from crossing between each group.

‡ Practical combination of commercial F_1 varieties.

Bold: The combination of 'Nako No. 919'.

The estimated mean GD between 127 inbred lines (GD_M) was 0.332 in Chapter 1.

Table 17. Genetic distances between promising inbred lines, those are dent, Japanese flint, and miscellaneous origin

Name/Group †		Na50	Na113	Na111	CHU44	CHU68	Na112
		JF	JF	JF	JF	JF	MIS (EF*JF)
Ho110	D	0.364	0.354	0.361	0.358	0.363	0.349
Na71	D	0.348	0.336	0.315	0.335	0.364 ‡	0.325
Na102	D	0.343	**0.322**	0.339	0.323	0.353	0.329
Na65	D	0.331	0.311	0.336	0.316 ‡	0.356	0.318
Mi29	D	0.334 ‡	0.293	0.319	0.319	0.338	0.301

† EF, JF, D and MIS indicate flint mainly developed or derived from the European region, Japanese flint landraces, Japanese dent mainly derived from US corn-belt dent, and miscellaneous origin, respectively. * is derived from crossing between each group.

‡ Practical combination of commercial F_1 varieties.

Bold: The combination of ‘Nako No. 919’.

The estimated mean GD between 127 inbred lines (GD_M) was 0.332 in Chapter 1.

Table 18. Genetic distances between promising inbred lines, those are Japanese flint, and miscellaneous origin

Name/Group †		Na50	Na113	Na111	CHU44	CHU68
		JF	JF	JF	JF	JF
Na113	JF	0.167	-	-	-	-
Na111	JF	0.327	0.327	-	-	-
CHU44	JF	0.336	0.326	0.247	-	-
CHU68	JF	0.323	0.348	0.319	0.310	-
Na112	MIS (EF*JF)	0.319	0.307	0.322	0.277	0.336

† EF, JF and MIS indicate flint mainly developed or derived from the European region, Japanese flint landraces, and miscellaneous origin, respectively. * is derived from crossing between each group.

The estimated mean GD between 127 inbred lines (GD_M) was 0.332 in Chapter 1.

Table 19. Genetic distances between promising dent inbred lines

Name/Group †		Ho110	Na71	Na102	Na65
		D	D	D	D
Na71	D	0.312	-	-	-
Na102	D	0.349	0.209	-	-
Na65	D	0.309	0.264	0.244	-
Mi29	D	0.325	0.282	0.258	0.236

† D indicates Japanese dent mainly derived from US corn-belt dent.

The estimated mean GD between 127 inbred lines (GD_M) was 0.332 in Chapter 1.

Table 20. Sum of the predicted GCAs by BLUP combining promising inbred lines, those are dent, Japanese flint, and miscellaneous origin

Name/Group †		Na50	Na113	Na111	CHU44	CHU68	Na112	Ho110	Na71	Na102	Na65
		JF	JF	JF	JF	JF	MIS (EF*JF)	D	D	D	D
Na113	JF	6.53	-	-	-	-	-	-	-	-	-
Na111	JF	6.03	3.65	-	-	-	-	-	-	-	-
CHU44	JF	5.65	3.27	2.77	-	-	-	-	-	-	-
CHU68	JF	3.89	1.51	1.01	0.62	-	-	-	-	-	-
Na112	MIS (EF*JF)	3.23	0.85	0.35	-0.04	-1.79	-	-	-	-	-
Ho110	D	7.42	5.04	4.54	4.15	2.40	1.73	-	-	-	-
Na71	D	-2.62	-5.00	-5.50	-5.88	-7.64 ‡	-8.30	-4.11	-	-	-
Na102	D	0.38	**-2.00**	-2.50	-2.89	-4.64	-5.31	-1.12	-11.15	-	-
Na65	D	4.68	2.30	1.80	1.41 ‡	-0.34	-1.01	3.18	-6.85	-3.86	-
Mi29	D	10.42 ‡	8.04	7.54	7.16	5.40	4.74	8.93	-1.11	1.89	6.19

† EF, JF, D and MIS indicate flint mainly developed or derived from the European region, Japanese flint landraces, Japanese dent mainly derived from US corn-belt dent, and miscellaneous origin, respectively. * is derived from crossing between each group.

‡ Practical combination of commercial F_1 varieties.

Bold: The combination of ‘Nako No. 919’.

Table 21. Sum of the predicted GCAs by BLUP combining promising inbred lines, those are dent, Japanese flint, and miscellaneous origin

Name/Group †		Na50	Na113	Na111	CHU44	CHU68	Na112
		JF	JF	JF	JF	JF	MIS (EF*JF)
Ho110	D	7.42	5.04	4.54	4.15	2.40	1.73
Na71	D	-2.62	-5.00	-5.50	-5.88	-7.64 ‡	-8.30
Na102	D	0.38	**-2.00**	-2.50	-2.89	-4.64	-5.31
Na65	D	4.68	2.30	1.80	1.41 ‡	-0.34	-1.01
Mi29	D	10.42 ‡	8.04	7.54	7.16	5.40	4.74

† EF, JF, D and MIS indicate flint mainly developed or derived from the European region, Japanese flint landraces, Japanese dent mainly derived from US corn-belt dent, and miscellaneous origin, respectively. * is derived from crossing between each group.

‡ Practical combination of commercial F_1 varieties.

Bold: The combination of ‘Nako No. 919’.

Table 22. Sum of the predicted GCAs by BLUP combining promising inbred lines, those are Japanese flint, and miscellaneous origin

Name/Group †		Na50	Na113	Na111	CHU44	CHU68
		JF	JF	JF	JF	JF
Na113	JF	6.53	-	-	-	-
Na111	JF	6.03	3.65	-	-	-
CHU44	JF	5.65	3.27	2.77	-	-
CHU68	JF	3.89	1.51	1.01	0.62	-
Na112	MIS (EF*JF)	3.23	0.85	0.35	-0.04	-1.79

† EF, JF and MIS indicate flint mainly developed or derived from the European region, Japanese flint landraces, and miscellaneous origin, respectively. * is derived from crossing between each group.

Table 23. Sum of the predicted GCAs by BLUP combining promising dent inbred lines

Name/Group †		Ho110	Na71	Na102	Na65
		D	D	D	D
Na71	D	-4.11	-	-	-
Na102	D	-1.12	-11.15	-	-
Na65	D	3.18	-6.85	-3.86	-
Mi29	D	8.93	-1.11	1.89	6.19

† D indicates Japanese dent mainly derived from US corn-belt dent.

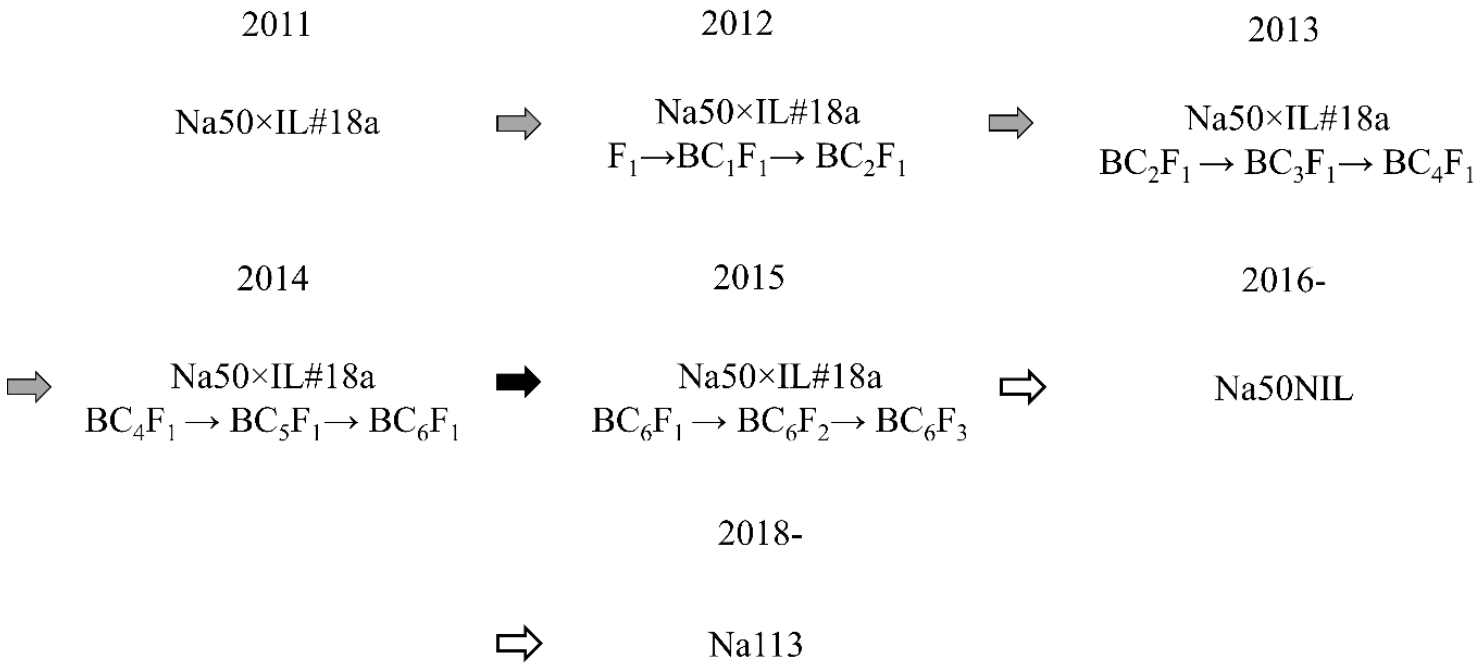

Fig. 9. Breeding scheme of ‘Na113’. The upper part indicates the year and the lower part the name of the individual and the generation, respectively. Gray arrows indicate back-cross, black arrow indicates self-cross, and white arrows indicate sibling-cross, respectively.

The other promising inbred line 'Na102' and its breeding scheme

'Na102' (No. 36933) is a dent inbred line with high resistance to NCLB, Southern corn leaf blight (SCLB), and Pythium RSR, as well as high ear yield. This inbred line is suitable for use as a parental inbred line to develop F_1 varieties in the south of the Kanto region. The material of this inbred line (S_0 generation), initially named 'IF-1-12-1' was selected in 2001 from the synthetic one generation of the dent population 'AD99,' with evaluation through self-cross and selection starting in 2002. The S_6 generation, selected in 2007, was initially named 'N08-11', and as it was found to be promising in 2008–2009, given the name 'Na102' in 2010 (Figure 10). Thereafter, combined ability, characterization, and productivity tests have been continuously conducted.

'Nako No. 919' and its characteristics

'Nako No. 919' (No. 36445) is a F_1 variety bred from the dent inbred line 'Na102' as a seed parent and the flint inbred line 'Na113' as a pollen parent. The first cross was made in 2016 and tested for flooding-resistance around the 6-leaf stage of waterlogging treatments in a simulated paddy field in 2017. In 2018, it was officially named 'Nako No. 919' and since then, continuous flooding-resistant tests and productivity tests have been conducted. Compared to 'KD731', another F_1 variety with the same maturity stage

and widely sold in the Kanto region, ‘Nako No. 919’ has slightly lower total dry weight in a normal field, but exhibits significantly high flooding-resistance, with about 19% greater dry ear weight in simulated paddy field.

Pythium RSR resistance in novel developed inbred lines and F_1 variety

Table 24 presents the Pythium RSR resistance data for ‘Na113’, ‘Na102’, ‘Na50’, ‘Nako No.919’, other commercial F_1 varieties, and their inbred lines. ‘Na102’ and ‘Na113’ were selected based on their Pythium RSR resistance confirmed by predicted GCAs by BLUP (Chapter 3), while ‘Nako No.919’ was confirmed through an inoculation with natural infection test (Chapter 2). Field inoculation with natural infection test showed that ‘Nako 919’ had stronger Pythium RSR resistance than ‘Yumesodachi’, a highly susceptible variety, and similar resistance to ‘Takanestar’ and ‘Takanefudo,’ which had been proven practical Pythium RSR resistance.

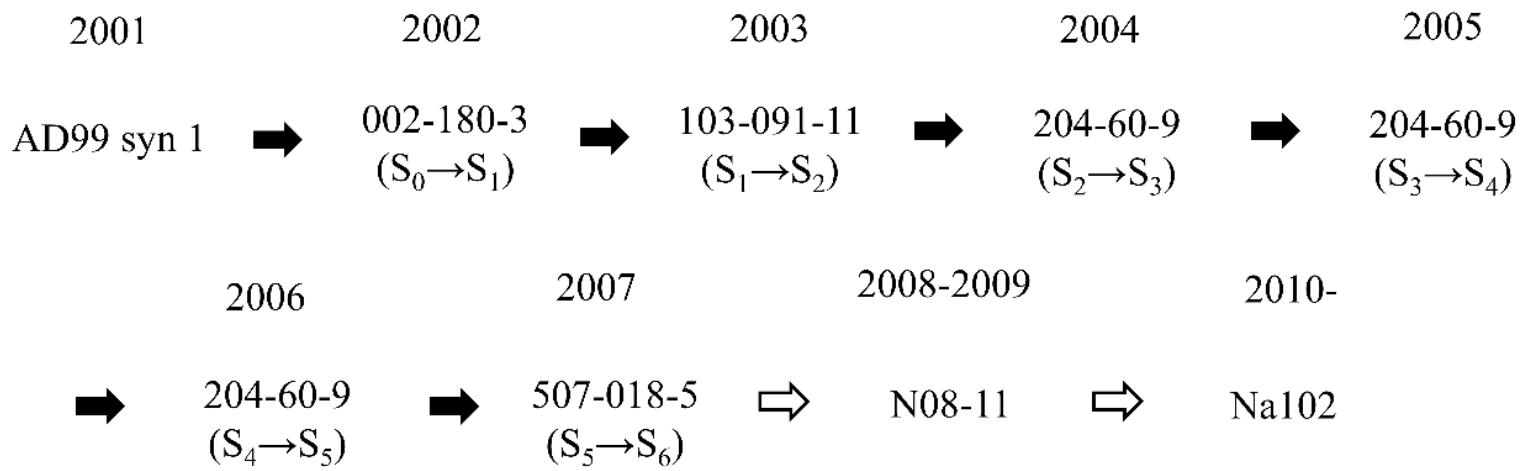

Fig. 10. Breeding scheme of 'Na102'. The upper part indicates the year and the lower part the name of the individual and the generation, respectively. Black arrows indicate self-cross, and white arrows indicate sibling-cross, respectively.

Table 24. Pythium RSR resistance of ‘Nako No.919’, ‘Na113’, ‘Na102’, other commercial F_1 varieties and inbred lines using inoculation with natural infection

Name	Pythium RSR infection frequency ± Standard Error (%) †				GCAs by BLUP ‡
	2017	2018	2019	2021	
Nako No.919 (Na102×Na113)	3.13 ± 3.13	14.71 ± 2.94	5.88 ± 5.88	2.94 ± 2.94	-2.00
Na102	-	-	0.00 §	5.00 ± 5.00	-4.08
Na113	-	-	0.00 §	-	2.08
Na50 (repeating parent of Na113)	-	-	0.00 §	0.00 ± 0.00	4.46
Yumesodachi (Mi29×Na50)	26.67 ± 6.67	58.82 ± 0.00	26.47 ± 2.94	51.65 ± 4.60	10.42
Mi29	-	-	-	13.64 ± 4.55	5.97
Na50	-	-	-	0.00 ± 0.00	4.46

Table 24. Continued

Name		Pythium RSR infection frequency ± Standard Error (%) †				GCAs by BLUP ‡
		2017	2018	2019	2021	
Takanestar (Na65×CHU44)		3.13 ± 3.13	5.88 ± 5.88	8.82 ± 2.94	-	1.41
	Na65	-	-	-	27.78 ± 5.56	0.22
	CHU44	-	-	-	10.00 ± 10.00	1.19
Takanefudo (Na71×CHU68)		3.13 ± 3.13	15.07 ± 2.57	2.94 ± 2.94	-	-7.64
	Na71	-	-	-	0.00 ± 0.00	-7.07
	CHU68	-	-	-	4.55 ± 4.55	-0.57

† The infection frequency and inoculation method were described in Chapter 2.

‡ The predicted GCAs by BLUP described in Chapter 3, those of F_1 varieties were the sum of the GCAs for each parental inbred line.

§ Infection frequencies in natural infection with no replicates.

Discussion

GDs between heterotic groups were relatively higher than within group. (Sang et al 2021). The same trend was observed for the 11 inbred lines used in this chapter. Previous studies have reported that the correlation between GD of inbred lines and hybrid performance was significant for grain yield and other traits in drought or humid conditions (Badu-Apraku et al. 2012, Suwarno et al. 2013). Therefore, the author examined the optimal combinations of inbred lines for each heterotic group using GD.

In this study, all combinations between D and JF had high GD (Tables 16 and 17). F_1 hybrids between dent and flint inbred lines have higher effective organic matter degradability in whole plants (Mlynekova et al. 2016). Thus, the conventional breeding scheme in the author's institute is considered useful for silage usage. It was observed that combinations where GD was distant and the sum of GCAs was low (negative value), expected to have strong heterosis and resistance for Pythium RSR, were frequently associated with the combination of 'Na71' and 'Na102' as a parent. 'Nako No. 919' also met these conditions. GD between 'Na102' and 'Na113' was 0.322 (Tables 16 and 17) and sum of the GCAs by BLUP was -2.00 (Tables 20 and 21). Considering its high performance for Pythium RSR resistance from inoculation with natural infection tests (Table 24) and productivity tests of 'Nako No. 919', the selection based on various

indices obtained in this study is considered effective. Pythium RSR resistance of 'Na113' was stronger than that of 'Na50'. This Pythium RSR resistance might be due to the chromosomal fragment of 'Teosinte', though further research is required at this point. The author plans to analyze this phenomenon in the future through the whole genome sequences of related F_1 varieties and parental inbred lines.

JF hybrids had high GDs within the heterotic group (Tables 16 and 18), but the sum of GCAs was predicted to be high and Pythium RSR resistance was not strong (Tables 20 and 22). Moreover, flint hybrids are lower starch degradability in the rumen than dent hybrids (Philippeau and Michalet-Doreau 1998). Thus, JF hybrids are not appropriate for forage combinations, and high GD does not always lead directly to breeding.

D hybrids had excellent materials with low GCA, such as 'Na71' and 'Na102' (Tables 20 and 23), but the GDs between promising inbred lines were low (Tables 16 and 19). On the other hand, 'Ho110' developed in Hokkaido had high GDs with any D inbred line. Dent hybrids are significantly higher for grain yield and lower for grain moisture than flint hybrids (Brun and Dudley 1989), may indicate that dent hybrids are superior for grain usage. Thus, it is considered effective to cross these inbred lines derived from distant regions to develop superior forage maize in the future. However,

as mentioned in Chapter 4, the inbred lines developed in cold regions such as Hokkaido may have low selection pressure for Pythium RSR resistance. The GCA of 'Ho110' was also not particularly superior at 2.96 (Table S2). In the future, it is expected to efficiently develop F_1 hybrids with high grain yield and Pythium RSR resistance by exploring 'Ho' inbred lines with superior GCA and combining them with promising D inbred lines.

However, positive correlation is not always observed between GD and heterosis (Fujimoto et al. 2018, Kawamura et al. 2016). Therefore, the relationship between GD and heterosis, as well as between and within heterotic groups, should be further clarified by developing F_1 combinations and testing their performances.

Based on the results of this chapter, it would be possible to select the optimal combinations with these inbred lines in advance through the findings obtained in this study, leading to more efficient breeding in the future.

CHAPTER VI. General discussion

Forage maize has become a key crop because of its high-energy content and productivity per area. Recently, there has been growing interest in using maize not only for silage but also as a grain crop in Japan. The role of maize in stabilizing and advancing livestock management in Japan, where there is heavy reliance on imported grain feed, is expected to expand (MAFF 2021). This study aims to obtain basic knowledge of breeding varieties resistant to Pythium RSR, a major pathogen in that frequently affects maize in hot and humid conditions with heavy rain in Japan (Deep and Lipps 1996, Reyes-Tena et al. 2018, Yanar et al. 1997). First, the genetic diversity of the inbred lines was analyzed. Second, the bottom stalk toothpick inoculation method was used to assess Pythium RSR infection frequency. Third, a method for predicting GCAs of inbred lines using BLUP was developed to address selection challenges during field observations. Fourth, GCAs by BLUP and SNPs information were used to attempt GP of Pythium RSR resistance and confirm sufficient prediction accuracy and estimate the EsGCAs of inbred lines that had been never evaluated for this disease. These findings led to the development of two inbred lines with superior Pythium RSR resistance and an F_1 variety by crossing these inbred lines, and to propose a genomic approach for improving

Pythium RSR resistance in maize F_1 plants. Based on the results of this study, future directions for breeding maize F_1 varieties with Pythium RSR resistance and genetic analysis of Pythium RSR are discussed below.

For the efficient breeding of superior F_1 varieties with Pythium RSR resistance, it was essential to accurately determine the resistance of the inbred lines. Despite the inoculation with natural infection test applied by the authors, it was difficult to assess Pythium RSR resistance of inbred lines in the field. Therefore, this study developed a method using the BLUP, which was able to determine of potential Pythium RSR resistance (GCAs) of inbred lines using F_1 data obtained in the author's conventional breeding scheme. Evaluation based on numerous BLUP data showed that F_1 varieties derived from two inbred lines with low GCAs demonstrated high Pythium RSR resistance (Tables 9 to 11). Additionally, the F_1 variety 'Nako No.919' which combined two parental inbred lines with low predicted GCAs by BLUP, demonstrated sufficient Pythium RSR resistance compared to commercial F_1 varieties in multi-year inoculation with natural infection tests (Table 24). The GD of each inbred line was comparable to that of the commercial F_1 varieties (Tables 16 and 17). These results facilitate the preliminary evaluation of inbred lines, overcoming a significant obstacle in breeding F_1 varieties, and open prospects for breeding Pythium RSR resistant varieties. Genetic

studies on stalk rot caused by *Fusarium* spp. have shown that GCA, SCA and epistatic effects are crucial for resistance inheritance (Donahue et al. 1989, Krishna et al. 2013, Lunsford et al. 1976, Mir et al. 2018). In Chapter 3, the incorporation of both GCA and SCA into the BLUP model suggests that the contribution of GCA is significant (Tables 9 to 11). However, clarifying the contributions of SCA and epistasis is essential knowledge for breeding F_1 varieties with Pythium RSR resistance.

The GP of Pythium RSR resistance using GCAs by BLUP and genome-wide SNPs achieved sufficient prediction accuracy, even with a small training population (Table14 and Figure 8), and it effectively predicted the capacity of numerous unknown inbred lines. These results indicate that flint inbred lines tend to have lower Pythium RSR resistance (higher EsGCAs) compared to dent inbred lines (Table 15). Whereas Pythium RSR occurs under hot and humid conditions with heavy rain in Japan, most of the flint inbred lines used in this study originate from the regions with colder climates, such as Hokkaido or Europe. The lower Pythium RSR resistance of flint inbred lines maybe due to insufficient selection pressure for Pythium RSR. Therefore, improving Pythium RSR resistance, particularly in flint inbred lines derived from cold regions, will be important in the future.

The F_1 variety 'Yumesodachi,' which is susceptible to Pythium RSR, has an

excellent ear yield. However, the author's preliminary trials revealed that Pythium RSR frequencies of this variety significantly decreased when the ears were removed soon after the silk was extracted. As competitive relationship between starch accumulation in ears and sugar accumulation in culms have been observed (Hume and Campbell 1972), removal of the ear would redirect all subsequent photosynthetic products to the culm, thus increasing culm Brix values. Currently, there are no reports on the relationships among ear yield, culm Brix values, and Pythium RSR infection frequency. Therefore, clarifying these correlations or competitive relationships is essential to develop breeding materials with both superior ear yield and Pythium RSR resistance, which must be the future breeding targets. If increased ear yield also leads to higher infection frequency of Pythium RSR, it is necessary to select the two conflicting traits simultaneously. Determining the optimal combination in advance, using predicted GCAs by BLUP or GP and GD information, helps address this issue.

MAS, including QTL analysis, has achieved notable results across various plant species, addressing traits such as insect resistance, flowering date, suitability for processing, and disease resistance (Chardon et al. 2004, Kump et al. 2011, Tian et al. 2011). In maize breeding, useful QTL have also been identified for traits like

flowering time, leaf angle and shape, and resistance to major diseases, such as NCBL, SCBL, and Fusarium ear rot (Ding et al. 2008, Wisser et al. 2006).

In general, QTL analysis of resistance to maize disease involves crossing a disease-resistant inbred line with a susceptible inbred line to develop F_1, which are then self-crossed to produce an F_2 segregating population. Furthermore, individual self-crossing from F_2 eventually produces recombinant inbred lines (RILs), which can be powerful tools for genetic mapping (Broman 2005).

The bottom stalk toothpick inoculation method used in this study provided accurate and stable evaluations year after year (Table 6). It is important to continue accumulating further results from this inoculation with natural infection test and identify experimental materials for analysis.

Few studies on QTL analysis and GWAS for Pythium RSR resistance have been reported, particularly from Chinese groups (Duan et al. 2019, Hou et al. 2023, Song et al. 2015). These studies utilized simple F_2 population or numerous inbred lines but did not mention the unique disease occurrences for the inbred lines as described by the author in Chapters 3 and 4. The author's findings regarding the discrepancy between infection frequencies of F_1 hybrids and their parental inbred lines were not covered in these studies. Therefore, it is assumed that *P. inflatum* and *P.*

aristosporum, described in these studies, are distinct from *P. arrhenomanes,* which is preferred in Japan, in terms of the pathogenicity.

In this study, the author developed a method to predict the potential Pythium RSR resistance of inbred lines using BLUP. Predicted GCAs by BLUP did not concur with the observed Pythium RSR infection frequencies of inbred lines in the field (Tables 8 and 13), highlighting it was a limiting factor for producing a segregating population for analysis. Nevertheless, BLUP results could be a powerful tool for developing a valid population for QTL analysis. In Chapter 5, the author identified inbred lines with superior GCAs using BLUP and proposed an optimal combination of F_1 hybrids by incorporating these findings and GD. The author also found several inbred lines where the predicted GCA and the direction of infection frequency were consistent. Inbred lines with both low infection frequencies and GCAs include ‘Na71’, ‘Na102’, and ‘Mi88,’ while inbred lines with both high infection frequencies and GCAs include ‘Mi29,’ ‘Ho108’, and ‘Ho112’. These inbred lines are suitable for creating segregating populations for QTL analysis. Although the genome of *P. arrhenomanes* has been partially sequenced, research focusing on the isolation of *Pythium* resistance genes has made little progress (NCBI 2024). Therefore, the author plans to identify the genetic regions involved in Pythium RSR resistance through QTL

analysis using F_2 segregating population and RILs derived from these inbred lines in the future.

SUMMARY AND CONCLUSION

Maize (*Zea mays* L.) is a major crop worldwide. Root and stalk rot (RSR) in maize, caused by *Pythium arrhenomanes* Drechsler, affects forage maize in Japan. The genome size of *P. arrhenomanes* is approximately 44.7 Mb based on short-read assembly with 10,972 contigs, and it contains around 13,800 annotated genes. Symptoms of the disease include wilting or lodging of whole plants and ear drooping. This disease occurs under hot and humid conditions, particularly during heavy rainfall during the ripening stage. Recent disease outbreaks have been attributed to global warming. Consequently, breeding research against this disease is becoming increasingly important. The objective of this study is to establish the genomic breeding method to develop maize F_1 varieties with Pythium RSR resistance, and to implement the genetic improvement of RSR resistance in F_1 varieties.

In Chapter 1, the objective is to analyze the genetic diversity of Japanese maize inbred lines. Statistical analyses of the genetic diversity of 127 inbred lines were conducted using genome-wide single nucleotide polymorphisms (SNPs). Genetic diversity cannot be accurately ascertained by traditional classifications such as dent or flint alone. By incorporating data from 1,007 SNPs, the author achieved a precise

understanding of the genetic diversity among the inbred lines. Additionally, even though they originated from the same F_1 variety, the genetic diversity between the two inbred lines was maintained by open pollination instead of self-crossing.

In Chapter 2, the objective is to develop an evaluation method for Pythium RSR using the bottom stalk toothpick inoculation method. To evaluate Pythium RSR in the field, toothpicks covered with *Pythium* hyphae of *P. arrhenomanes* were inserted into the bottom stalks of the plants. Throughout the experimental period, the average infection frequency varied remarkably, but the infection frequencies of the highly susceptible and resistant hybrids remained stable under inoculation with natural infection. This method is useful for screening regardless of weather conditions, as it can complement natural infection.

In Chapter 3, the objective is to evaluate the simple parental-progeny-based best linear unbiased prediction (BLUP) for predicting single-cross performance of resistance to Pythium RSR to resolve the low correlation between the infection frequencies of the susceptible F_1 hybrids and their parental inbred lines. The general combining abilities (GCAs) of the inbred lines were predicted from Pythium RSR infection frequencies of the 449 F_1 varieties using BLUP. Comparing the predicted GCAs by BLUP with the infection frequencies under inoculation with natural infection

data of the inbred lines revealed low correlation coefficients for three years. The correlation coefficients between GCAs by BLUP and the infection frequencies of F_1 data were high (R = 0.776 and 0.793 in 2018 and 2019, respectively). These results indicate the potential of using GCAs by BLUP to predict resistance for Pythium RSR.

In Chapter 4, the objective is to develop genomic prediction (GP) model for Pythium RSR resistance using GCAs by BLUP and genome-wide SNPs, and to use the GP model to estimate Pythium RSR resistance of inbred lines that have been never evaluated for this disease. Seventeen dent, twenty-three Japanese flint, and one Japanese tropical inbred lines were tested as training dataset for developing the GP model, and 188 inbred lines were employed to predict Pythium RSR resistance with this model. Correlation between the predicted GCAs by BLUP and the estimated GCAs (EsGCAs) by GP model from the validation dataset were calculated as prediction accuracy. During the process of randomly locating each SNPs marker set 100 times and performing sampling and validation 1,000 times for each marker set, the average prediction accuracy improved as the number of markers increased. Considering the use of a small training population (N = 41), the GP model achieves sufficient prediction accuracy.

The author has confirmed useful methods for breeding materials with Pythium RSR resistance in previous chapters. In Chapter 5, the objective is to propose a genomic

approach for improving Pythium RSR resistance in maize F_1 plants, and to demonstrate and validate a breeding process of two inbred lines and one novel F_1 variety in the present study. A total of 11 maize inbred lines were used. The genetic distance (GD, Chapter 1) and GCAs (Chapter 3) of inbred lines were compared and evaluated to verify promising combinations assuming crosses within and between different heterotic groups. All combinations of dent and Japanese flint inbred lines had high GD. It was observed that combinations where GD was distant and the sum of GCAs was low, were frequently associated with the combination of 'Na71' and 'Na102' as a parent. 'Nako No. 919', derived from a cross between two promising inbred lines 'Na102' and 'Na113', also met these conditions with high GD and low sum of the GCAs. Considering its high performance for Pythium RSR resistance from inoculation with natural infection tests and productivity tests of 'Nako No. 919', the selection based on various indices obtained in this study is considered effective.

In conclusion, using the predicted GCAs by BLUP or EsGCAs from GP, along with information on GD, may assist in selecting the optimal combination of inbred lines to develop F_1 varieties with Pythium RSR resistance. These genomic and breeding findings are anticipated to lead to the development of superior forage maize that is high quality, high yield, and with Pythium RSR resistance.

SUPPORTING INFORMATION

Supplementary Table 1 Genetic distance matrix of 127 inbred lines in Chapter 1

Supplementary Table 2 Predicted general combining abilities (GCAs) of 100 inbred lines by best linear unbiased prediction (BLUP) for Pythium RSR, which were predicted from 528 data of 449 F_1 hybrids under natural infection (2014–2017) in Chapter 3

Supplementary Table 3 Predicted GCAs of 105 inbred lines by BLUP for Pythium RSR, which were predicted from 751 data of 648 F_1 hybrids under natural infection (2016–2019) in Chapter 4

APPENDIX

Chapter 1 Genotyping data.csv

Chapter 3 Field test 1 .csv

Chapter 4 Field test.csv

Chapter 4 Genotyping data of training population.csv

Chapter 4 Genotyping data of targeting population.csv

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