Euphytica **135:** 145–155, 2004.

Genetic diversity of maize inbred lines in relation to downy mildew

Maria Luz George[1,*], Ellen Regalado[1], Marilyn Warburton[2], Surinder Vasal[2,3] & David Hoisington[2]

[1]*CIMMYT-Asian Maize Biotechnology Network, c/o IRRI, DAPO 7777, MetroManila, Philippines;* [2]*CIMMYT (International Maize and Wheat Improvement Center), Apdo. Postal 6-641, 06600 Mexico D.F., Mexico;* [3]*CIMMYT-Asian Regional Maize Program, P.O. Box 9-188, Bangkok, Thailand; (*author for correspondence; e-mail: m.george@cgiar.org)*

Received 26 February 2003; accepted 21 October 2003

Key words: Asia, downy mildew, genetic diversity, maize, simple sequence repeats (SSR)

Summary

A major emphasis in maize breeding in Asian countries has been the improvement for resistance to downy mildew, a serious disease that causes significant yield losses. A total of 102 inbred lines, including lines from Asian breeding programs, Mexico, USA and Germany, were analyzed with 76 SSR markers to measure diversity and investigate the effect of selection for downy mildew resistance. A mean polymorphism information content of 0.59, with a range of 0.14 to 0.83, was observed. Diversity at the gene level showed an average of 5.4 alleles per locus and a range of two to 16 alleles per locus, with a total of 409 alleles. About half of the alleles in the Asian lines had frequencies of 0.10 or less, and only 2% had frequencies > 0.80, indicating the presence of many alleles, and thus a high level of diversity. Some of the high-frequency alleles were in chromosomal regions associated with disease resistance. However, the frequencies of alleles in three SSR loci that are linked to a QTL for resistance to downy mildews in Asia were not significantly different in the subtropical/tropical Asian lines as compared to all the lines in the study. Lines from the US, Germany, and China, comprised three clusters of temperate maize (GS = 0.31), while those from India, Indonesia, Philippines, Thailand, Vietnam and CIMMYT comprised seven indistinct clusters of subtropical and subtropical maize (GS = 0.29). We conclude that maize breeding activity in Asia has not caused a decline in the overall amount of diversity in the region.

Introduction

Knowledge of genetic diversity in maize germplasm helps to ensure that a broad genetic base of breeding materials is maintained, not just for sustaining genetic improvement but also for reducing genetic vulnerability to pests and diseases. This knowledge, which may be obtained from pedigree and test cross data, morphological and biochemical traits or molecular markers, is important for maximizing heterosis. Because molecular markers can characterize lines directly and precisely at the DNA level, they can help maize breeders efficiently assign lines to heterotic groups and guide them in the choice of parents for the development of new hybrids. As well, markers provide a means for distinctly identifying individual plants with their unique allelic profiles, an application that is becoming important in varietal protection.

Among the molecular markers, SSRs (Simple Sequence Repeats) or microsatellites have become the marker of choice for many genetic analyses because of their high level of polymorphism, repeatability, low cost, and amenability to automation. SSRs are also abundant and their chromosomal locations are mapped, so the genome can be uniformly sampled and analyzed. The codominant nature of SSR markers, which allows the allelic contribution of each parent to be detected, is particularly important in maize hybrid testing. Several studies have successfully used SSRs in germplasm analysis across a range of crops (Plaschke et al., 1995; Rongwen et al., 1995; Charters et al., 1996; Senior et al., 1998; Kubik et al., 2001).

Over the past three decades, national programs in Asia and the Asian Regional Maize Program (ARMP, headquartered in Thailand) of the International Maize and Wheat Improvement Center (CIMMYT) have developed locally adapted open pollinated varieties and more recently, inbred lines for use in hybrid maize production. A major emphasis in the breeding programs has been the improvement for resistance to downy mildew, one of the most destructive diseases of maize in the humid subtropical and tropical areas in South and Southeast Asia. For inbred lines specifically developed for these environments, resistance to the important downy mildew pathogens – *Peronosclerospora sorghi* (sorghum downy mildew) and *P. heteropogoni* (Rajasthan downy mildew) in India, *P. maydis* (Java downy mildew) in Indonesia, *P. zeae* in Thailand and *P. philippinensis* in the Philippines, is an essential trait. In these countries, improved materials are routinely converted to downy mildew resistance before release. For example, in Indonesia where downy mildew is the most serious of maize diseases, all varieties released by the government since 1987 have resistance (Baco et al., 2000).

Heterotic groupings of maize lines in use in Asia are not yet clearly defined. In a study of a set of CIMMYT Maize Lines (CMLs) developed for Asian tropical environments, clustering by SSR marker data did not correspond to the expected heterotic groupings as assigned by field evaluations using testers (Warburton et al., 2002). However, lines that have similar heterotic partners did cluster together. The authors concluded that CIMMYT materials, being developed from very diverse germplasm sources, contain a lot of variation that may not sufficiently be represented by the low number of heterotic groups in use, and the low number of testers used to define these groups. Genetic diversity studies to characterize inbred lines and assign them to heterotic groups have been conducted in several countries in Asia, including China (Yuan et al., 2001), India (Pushpavalli et al., 2002), Indonesia, Philippines, Thailand and Vietnam (unpublished data) as part of a collaborative activity under the Asian Maize Biotechnology Network (AMBIONET, www.cimmyt.org/ambionet). This work is part of the AMBIONET effort and provides a region-wide perspective on the relationships of maize lines that are important to the breeding objectives of the national programs. This study aimed to (1) fingerprint representative maize inbred lines from public sector breeding programs in Asia; (2) investigate the amount of diversity in these materials; and (3) study the effect of selection for downy mildew resistance on this diversity.

Materials and methods

Plant material

A total of 102 inbred lines (Table 1) were analyzed in this study. The Asian lines were from the national programs of China, India, Indonesia, Philippines, Thailand, Vietnam and the Asian Regional Maize Program of CIMMYT. The ARMP lines, most of which have resistance to the different downy mildews (Table 1), were collected or developed by the program for use in resistance breeding in the region. To maximize the genetic variability across the data set, tropical and subtropical inbred lines from CIMMYT, Mexico and temperate lines from the USA and Germany were also included in the study. Two important dent lines in the US Corn Belt, B73 from the Iowa Stiff Stalk Synthetic (BSSS) and Mo17 from the Lancaster Sure Crop (LSC) heterotic groups, were included for comparison.

Samples of leaf tissue or DNA were provided by S. Zhang and X. Li (China), B.M. Prasanna and N.N. Singh (India), M. Dahlan (Indonesia), A. Salazar and P. Guzman (Philippines) and P. Grudloyma (Thailand), and the Asian Regional Maize Program and Applied Biotechnology Center of CIMMYT. Pedigree information, when known, is provided in Table 1. Leaves from 6–8 plants (3–4 leaf stage) were bulked and ground to a fine powder with liquid nitrogen using a mortar and pestle. DNA was extracted using a modified CTAB procedure (CIMMYT, 2001). Lines with greater than 20% heterozygosity or greater than 15% missing data were excluded in the study.

SSR markers

Eighty six SSR loci, mostly possessing a repeat unit greater than two nucleotides and representing 6–10 bins per chromosome, were selected based on bin location for uniform genomic coverage (MaizeDB, www.agron.missouri.edu). The primers were synthesized through Research Genetics, Inc. (Huntsville, AL, USA).

Ten SSR markers (*phi006, phi008, phi041, phi049, phi452693, umc1122, umc1277, umc1555, phi420701, phi100175*) with more than 15% missing data were excluded in the final analysis. Of the 76 SSRs used, four (5%) were di-repeats, 30 (39%) were tri-repeats, 31 (41%) were tetra-repeats, six (8%) were

Table 1. Germplasm sources and pedigrees of maize lines used in the study

Maize Line	Origin/ Pedigree[a]	Type[b]
Huangzao4	China: TangSiPingTou	Temperate Y F
Dan340	China: Baigu lu × Pod corn	Temperate Y SD
Ye478	China: U8112 × 5003	Temperate Y D
Zi330	China: Keli 67 × Oh43	Temperate Y SD
BIO4	India: B96-1-b-#-1-2 × CM119	Tropical Y F
BIO5	India: LM5	Subtropical Y F
CM133	India: A632	Tropical Y F
LM5	India: Tuxpeno Pool C2-I-C2	Subtropical Y F
GM12	Indonesia: CIMMYT Pop 28	Tropical Y F
GM15	Indonesia: Malang Composite 9	Tropical Y SD
GM15-1-3-1#	Indonesia: GM15	Tropical Y SF
GM19-6#	Indonesia: CIMMYT Pop 28	Tropical Y F
GM27	Indonesia: Bogor Pool 1, local germplasm × Suwan-2	Tropical Y SF
J1-19	Indonesia: Malang Synthetic J1, Harapan, Kalingga, Wiyasa, Malang Composite 9, 11, Muneng Synthetic, CIMMYT Pop 27, 28	Tropical Y F
J1-46-2-2-3#	Indonesia: Malang Synthetic J1	Tropical Y F
J2-102	Indonesia: Malang Synthetic J2, Tuxpeno, GM 4, 12, 15, 11, Suwan-1 & 3; CIMMYT Pop 28	Tropical Y SD
K1	Indonesia: Malang Synthetic K1, Arjuna, Arjunax, Bogor Pool 2, Malang Comp A & F CIMMYT Pop 31/ K1-388-2-2-1-###-b	Tropical Y F
K2	Indonesia: Malang Synthetic K2, Suwan-2 / K2-229-1-3-1#	Tropical Y F
Pool26	Indonesia: CIMMYT Pool 26 / Pool26-41-1-3-1-2-###	Tropical Y F
SW2	Indonesia: Suwan-2 / SW2-43-1-1-1#-1-2-2#	Tropical Y SF
SW3-3	Indonesia: Suwan-3 / SW3-3-1-1-2-4#	Tropical Y F
SW3-109	Indonesia: Suwan-3 / SW3-109-3-2-2-2-##	Tropical Y F
YCPG	Philippines: Pop YCPG	Tropical Y F
P2S2	Philippines: Pop cg5401	Tropical Y F
P1	Philippines: Pop E2	Tropical W F
P8	Philippines: Pop E4	Tropical W F
P12	Philippines: Pop IITA 1822-2	Tropical W F
P22	Philippines: Pop IITA AIL 75B	Tropical W F
Pi 21	Philippines: Pop P3228	Tropical Y F
Pi17	Philippines: Pop SMC E9	Tropical Y F
Pi23	Philippines: Pop P3228	Tropical Y F
Pi31	Philippines: Pop SMC E9	Tropical Y F
Nei9008	Thailand (Dept of Agriculture): SW1(s)C9-germplasm / (DA9-1(s)-7-3-1 × SW C9)-S9-177-1	Tropical Y F
Nei9202	Thailand (Dept of Agriculture): Pop28(HS)C6 / Pop28(HS)C6-S9-129	Tropical Y F
Nei9203	Thailand (Dept of Agriculture): Pop28(HS)C6 / Pop28(HS)C6-S9-410	Tropical Y F-SD
Nei9204	Thailand (Dept of Agriculture): Pop28(HS)C6 × SW(s)C8 / NS1(s)C1-S9-251	Tropical Y F
Nei402004	Thailand (Dept of Agriculture): PIO.3228 × SW1(s)C9/ (M3228 × Nei9007)-S9-132-b-2	Tropical Y F
Nei402011	Thailand (Dept of Agriculture): Pop24 × TF-Comp.DMR#1	Tropical Y F
Nei402025	Thailand (Dept of Agriculture): PIO.1352A / P1325 A-S9	Tropical Y F
Nei412004	Thailand (Dept of Agriculture): SS23(S)C2-S7-190-1-2-1-BBBB3	Tropical Y F-SD
Ki3	Thailand (Kasetsart Univ): SW1(s)C4/ SW1(s)C4-S8-19-5	Tropical Y F DMR
Ki14	Thailand (Kasetsart Univ): Suwan-1 (S)C4-S8-19-5 (2028)	Tropical Y F
Ki44	Thailand (Kasetsart Univ): KS6(s)C2 / KS6(s)C2-S7-366	Tropical Y F
Ki45	Thailand (Kasetsart Univ): Ki21-improved / Ki21 × Tzi 15	Tropical Y F
ARMP1	Asia: CA00304(AMATLCOHS170-2-3-2-1-1-1-B-B-B)-B	Tropical DMR
ARMP2	Asia: P345 / CA34502(P345C5S1B-15-4-2-1-2-1-2-B-B)-B	Tropical DMR

Table 1. Continued

Maize Line	Origin/ Pedigree[a]	Type[b]
ARMP3	Asia: Pioneer derivative / CA00314(PIO3011F2-3-5-3-1-B-B)-B	Tropical
ARMP4	Thailand: KU / CA00320(KSX3601F2-5-3-2-7-B-B)-B	Tropical
ARMP5	Asia: P145 / CA14505(SW92145-2P9S2-#-#-B)-B	Tropical DMR
ARMP6	Asia: CIMMYT P31 / CA03113(P31C4S5B-6-#-#-B)-B	Tropical DMR
ARMP7	Asia: CIMMYT P31 / CA03116(P31C4S5B-39-#-#-1-B-B-B)-B	Tropical DMR
ARMP8	Asia: CIMMYT P145 / CA14502(SW92145-2P9S2-#-#-4-B-B-B)	Tropical DMR
ARMP9	Vietnam: NMRI / DF7-162(E)	Tropical
ARMP10	Vietnam: NMRI / DM9	Tropical
ARMP11	Asia: AMATLCOHS115-1-2-3-3-1-2-B-B	Tropical DMR
ARMP12	Asia: AMATLCOHS233-1-1-1-1-2-2-B-B-B	Tropical DMR
ARMP13	Asia: P345 / P345C3S3B-40-8-1-1-2-2-B	Tropical DMR
ARMP14	Asia: P345 / P345C3S3B-46-1-1-1-1-2-B	Tropical DMR
ARMP15	Asia: P345 / P345C5S1B-15-4-2-1-2-1-2-B	Tropical DMR
ARMP16	Asia: AMATLCOHS9-1-1-1-1-1-2-B	Tropical DMR
ARMP17	Asia: P345 / P345C4S2B-46-2-2-1-2-B-B-B	Tropical DMR
ARMP18	Thailand: NS1, FCRC / NS1C1S5-261-7-3-1-2-1-1-B-B	Tropical
ARMP19	Philippines: IPB / IPB9204-1-3-1-2-4-B	Tropical DMR
ARMP20	Asia: CIMMYT P24 / (24STE-5*24STE-17)-BBBB-###-B-1-B-2-B- B-B	Tropical
ARMP21	Asia: CIMMYT P24 / (24STE-5*24STE-17)-BBBB-###-B-5-B-4-B- B-B	Tropical
ARMP22	Asia: CIMMYT P24 / P24STEC1HC16-1-3-3-1-2-BBB-1-###-9-BBBBBBB	Tropical
ARMP23	Asia: CIMMYT P24 / P24STEC2-29-BBBB-#-3-BBBBBBB	Tropical
ARMP25	Asia: AMATLCOHS245-1-1-1-2-1-1-B-B	Tropical DMR
CML20	Mexico: Pop 24 / Pob24HC34-2-3-B-###	Tropical Y D
CML51	Mexico: Pop 79 / STA.ROSA8079-1-2-3-###	Tropical Y F
CML202	Mexico: ZSR / ZSR923S4BULK-5-1-b-b	Tropical W SD
CML206	Mexico: EV7992 / [EV7992#/EVPO44-SRBC3]#bF37sr-2-3-sr-2-4-3- b-b	Tropical W SD
CML236	Mexico: P32 / [LB(1)8232-SR(BC3)]-140-1-1-1-b	Tropical W F
CML270	Mexico: Pop 29 / Pob29STEC1HC17-4-1-1-2-1-BB-f	Tropical W SD
CML272	Mexico: Pop 29 / Pob29STEC1HC1-3-1-1-4-2-BB-f	Tropical W SF
CML281	Mexico: Pop 43 / (43*PORILLO8043)-5-1-2-2-BB-F	Tropical W D
CML289	Mexico: Pop 24 / Pob24STEC1HC23-5-2-1-2-3-BB-f	Tropical Y F
CML292	Mexico: Pop 28 / (Pob28xTSR)-33-2-7-1-2-BB-f	Tropical Y F
CML385	Mexico: Pop502 / P502c1#-771-1-1-1-B-B	Subtropical W F
CML387	Mexico: ZM609 / [EV7992#/EV8449-SR]C1F2-334-1(OSU8i)-1-1-B- B-3-B*4	Tropical W F
CML396	Mexico: Pop 21 / P21C5HC109-3-1-5-4-B-4-3-##-2-B* 6	Tropical W D
CML452	Mexico: Pop 28 / Ac8328BNC6-166-1-1-1-BBBBBBB	Tropical Y
CML453	Mexico: Pop 24 / P24STEC1HC21-3-1-1-#-BBB-f-##-BBB	Tropical Y
Mo17	US: Lancaster Sure Crop (LSC)	Temperate Y D
B73	US: Iowa Stiff Stalk Synthetic C5 (BSSS)	Temperate Y D
B90	US: BSCB1(R)C7	Temperate
B91	US: BSCB1(R)C7	Temperate
B94	US BSSS (R) C8	Temperate
B97	US: BSCB1(R)C9-2	Temperate
B99	US: BSCB1(R)C10-7233]	Temperate
B100	US: (B85 × H99)H99-361	Temperate
B102	US: (B85 × H99)H99-336	Temperate
B104	US: BS13 BS13(S)C5	Temperate
B105	US: BSSS(R)C9	Temperate
B114	US: NT Pool 41-C15	Temperate

Table 1. Continued

Maize Line	Origin/ Pedigree[a]	Type[b]
R228	US: [900 × Mo17ˆ 2]S6	Temperate
R229	US: [479 × B73ˆ 2]S6	Temperate
R230	US: [509 × B73ˆ 2]S6	Temperate
026	Germany	Temperate
049	Germany	Temperate
304	Germany	Temperate
305	Germany	Temperate

[a] Pedigree: Pop or P = population; C = Cycle; HC = Full Sibs; B = Selfed and Bulked; –1,–2,–3 = ear to row; # = sibbing; SR = Streak resistance; EV = Experimental Variety; STE = inbreeding tolerant population; AMATL=Asia Mildew Acid Tolerant.
[b] Type: Y = Yellow grain, W = White grain; D = Dent, SD = Semindent, F = Flint, SF = Semiflint, DR = Downy Mildew Resistant.

penta-repeats, three (4%) were hexa-repeats, and two (3%) were compound repeats (Table 2).

Amplification and detection conditions

Approximately 10 ng of DNA was used as template for PCR in a 10-μl reaction in a 96-well microtiter plate containing 1× PCR buffer, 2.0 mM $MgCl_2$, 1.0 mM dNTPs, 0.25 μM each of the forward and reverse primers, and 0.5 units of BIOTAQ^{TM} DNA Polymerase (Bioline USA Inc., NJ, USA) or *Taq* DNA Polymerase in Storage Buffer B (Promega Corp., Madison, USA). Amplifications were carried out using a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA, USA), with the following amplification conditions: initial denaturation at 94 °C (2 min); 30 cycles of 94 °C (30 sec) denaturation, 56 °C (1 min) annealing, and 72 °C (1 min) extension; and then a final extension at 72 °C (5 min). The φX174/*Hin*f I markers (Promega, Madison, WI, USA) served as molecular weight standards. Six CMLs (CML51, 292, 202, 206, 236, 396), serving as reference lines, were run with the samples in all gels.

Denaturing polyacrylamide gel electrophoresis was used with 4.5% acrylamide using the 38 × 30 cm SequiGen-GT System and protocol (Bio-Rad Laboratories, Inc, Hercules, CA, USA). The bands were detected by staining with silver based on the Promega Silver Sequence^{TM} DNA Sequencing System protocol.

Gel scoring

Alleles were named based on the positions of the bands relative to the φX174/*Hin*f I fragments. Data were scored as present ('1') or absent ('0') for a particular allele/maize line combination. Bands that were diffused or too difficult to score were considered as missing data ('9'). In cases when a line has multiple bands of varying intensity, the most intense band is scored as '1', and the others as '9'.

Data analysis

The discriminatory power of each SSR locus was measured using the polymorphism information content (PIC) and calculated according to Smith et al. (1997). To examine the relationship of the SSR marker repeat type (excluding the compound repeat type) with the PIC value, and the repeat type with the number of alleles, we calculated the simple correlation coefficients using the Correlation Analysis Tool in MS Excel.

A matrix of binary data, with columns equal to genotypes (inbred lines), and rows equal to the alleles of each primer, was compiled in MS Excel and analyzed with NTSYS-pc version 2.02 (Rohlf, 1999). We used three coefficients (Jaccard, Dice and Simple Matching similarity coefficients) to calculate the matrices of genetic similarities among pair-wise comparisons of genotypes, and compared them using the matrix comparison function (MXCOMP program) of NTSYS. The matrices were similar ($r_{\text{jaccard vs. dice}} = 0.99$; $r_{\text{jaccard vs. simple matching}} = 0.94$; $r_{\text{dice vs. simple matching}} = 0.95$) and thus, we used only the Jaccard coefficient for further calculations. Cluster analysis was done with the unweighted pair group method using arithmetic averages (UPGMA) and the relationships between inbred lines were visualized in a dendrogram. To determine if the dendrogram obtained was a good fit to the similarity matrix, the cophenetic coefficient was computed and tested using the Mantel matrix correspondence test (MXCOMP program). A principal components analysis (PCA) was also done to better visualize differences in the lines and discern groups.

The robustness of the clusters was assessed using bootstrap analysis for obtaining an estimate of the confidence limits for the groupings produced by the dendrogram. The WINBOOT program (Yap & Nelson, 1996) was used with 400 repeated samplings with replacement for > 95% accuracy of the bootstrap (Hedges, 1992). The bootstrap values, reflecting

Table 2. Bin location, PIC and number of alleles at each locus of SSRs used in the maize study

SSR locus	Bin[a]	PIC[b]	No. of alleles	SSR locus	Bin[a]	PIC[b]	No. of alleles
phi056	1.00-1.01	0.60	4	umc1153	5.09	0.78	6
phi109275	1.03	0.78	6	umc1143	6.00	0.81	8
phi339017	1.03	0.39	5	bnlg391	6.01	0.78	8
umc1124	1.05	0.51	3	phi423796	6.01	0.26	6
phi002	1.07	0.55	3	bnlg1702	6.05	0.79	16
phi011	1.09	0.46	3	mmc0241	6.05	0.76	8
phi308707	1.10	0.66	6	phi078	6.05	0.69	8
phi064	1.11	0.83	12	phi123	6.07	0.55	3
phi227562	1.11	0.72	7	phi299852	6.07	0.77	9
phi96100	2.00	0.72	6	phi089	6.08	0.41	2
phi109642	2.03-.04	0.46	3	umc1545	7.00	0.65	6
phi083	2.04	0.71	5	phi112	7.01	0.48	7
nc133	2.05	0.43	3	phi034	7.02	0.71	7
phi127	2.08	0.59	5	phi114	7.03	0.60	4
phi101049	2.10	0.80	11	phi328175	7.04	0.63	5
phi104127	3.01	0.52	3	phi116	7.06	0.77	7
phi374118	3.02	0.74	6	umc1304	8.02	0.36	2
phi029	3.04	0.68	6	phi121	8.03	0.14	2
phi053	3.05	0.77	6	phi014	8.04	0.57	5
phi073	3.05	0.69	4	umc1161	8.06	0.61	8
phi102228	3.06	0.43	3	phi015	8.08	0.70	7
umc1399	3.07	0.70	5	phi080	8.08	0.72	7
phi046	3.08	0.49	2	phi233376	8.09	0.69	5
phi047	3.09	0.67	5	umc1279	9.00	0.37	4
umc1136	3.10	0.71	9	phi033	9.01	0.44	6
phi072	4.00-.01	0.65	6	phi065	9.03	0.60	4
phi213984	4.01	0.18	2	phi032	9.04	0.49	4
phi079	4.05	0.58	6	phi108411	9.05	0.31	4
phi093	4.08	0.64	4	phi448880	9.06–07	0.44	4
umc1109	4.10	0.53	4	phi059	10.02	0.51	4
phi076	4.11	0.67	5	phi063	10.02	0.65	7
nc130	5.00	0.45	4	phi96342	10.02	0.39	5
phi024	5.01	0.72	5	umc1152	10.02	0.74	7
phi109188	5.03	0.62	8	phi050	10.03	0.44	3
phi113	5.03–5.04	0.68	6	phi062	10.04	0.37	2
phi331888	5.04	0.56	4	phi084	10.04	0.47	4
phi087	5.06	0.68	4	umc1061	10.06	0.55	4
bnlg118	5.08	0.76	7	umc1196	10.07	0.70	5

[a] From Maize Genetics and Genome Database (http://www.maizegdb.org/ssr.php).
[b] PIC, Polymorphism Information Content.

the frequency with which each group is formed in repeated cycles of dendrogram construction, were used as a measure of the relative stability of the clusters of lines.

Differences between allele frequencies were tested for significance with the Fischer's exact test using PROC-FREQ of SAS-STAT (SAS Institute Inc., Raleigh, NC).

Results

Allelic diversity

All lines could be distinguished based on the SSR loci used in the study. Measures of allelic diversity at each SSR locus are presented in Table 2. The polymorphism information content at 76 SSR loci in 102 inbred

lines had a mean of 0.59, and ranged from 0.14 (tri-repeat SSR *phi121*) to 0.83 (tetra-repeat SSR *phi064*). Diversity at the gene level had an average of 5.4 alleles per locus and a range of two (*phi046, phi213984, phi089, umc1304, phi121, phi062*) to 16 (*bnlg1702*) alleles per locus. The highest average number of alleles per locus was found in the di-repeat class of SSR loci, but we found no correlation between the number of repeats in the SSR and the level of polymorphism detected, either estimated by the PIC values or by the number of alleles.

A total of 409 alleles was found in the 102 lines. Among the 68 Asian lines, including the temperate lines from China, and the subtropical and tropical lines from India, Indonesia, Philippines, Thailand, Vietnam and the CIMMYT Asian Regional Maize Program, there were 383 alleles (average of 5.0 alleles per locus). Without the temperate lines from China, there were 374 alleles (average of 4.9 alleles per locus) among the 64 subtropical and tropical Asian lines. The temperate maize from the US and Germany had 247 alleles (average of 3.25 alleles per locus).

Seventy seven alleles (19%) were unique to the Asian lines, not being found in the CMLs, the US or the European lines. Some of these unique alleles were distinctive of lines that originated from a specific country or breeding program, reflecting the diverse origins of these lines. Alleles unique to groups of lines, including those from China (3 alleles), India (3 alleles), Indonesia (11 alleles), Philippines (10 alleles), Thailand (6 alleles), Vietnam (1 allele) and ARMP (11 alleles) were also found. Forty-one alleles unique to the Asian lines, each found in only one line, are potentially important as diagnostic markers for particular inbred lines.

About half of the total number of alleles in the Asian lines had frequencies of 0.10 or less, and only 2% had frequencies > 0.80. Two of these alleles, located in loci *phi213984* and *phi121,* were near fixation (frequency > 0.95).

Allelic diversity associated with downy mildew resistance

Given the selection pressure for downy mildew resistance in maize for the subtropical and tropical environments of Asia, the allelic profiles of the subset of lines from India, Indonesia, Philippines, Thailand, Vietnam and the CIMMYT Asian Regional Maize Program were examined for the presence of alleles associated with resistance to downy mildew. Previous work by George et al. (2003) identified six QTLs involved in resistance to the downy mildews, including a strong QTL on chromosome 6 that influenced resistance to five different downy mildews in Asia (*P. sorghi* and *P. heteropogoni* in India, *P. maydis* in Indonesia, *P. philippinensis* in the Philippines, and *P. zeae* in Thailand). Alleles in this QTL that reduced susceptibility to the downy mildews were from the resistant line 'Ki3', a tropical yellow flint line which was derived from Suwan-1, a popular cultivar developed in Thailand for resistance to downy mildew (Sriwatanapongse et al., 1993).

The frequencies of alleles in three SSR loci (*mmc0241*, *phi078,* and *bnlg1702,* George et al., 2003) that are linked to this QTL were calculated in the subtropical/tropical group of Asian lines and compared to those of all lines in the study. The allele *mmc0241-f1*, one of eight alleles detected at the SSR *mmc0241* locus, occurred at a frequency of 0.30 in the Asian tropical/subtropical lines compared to 0.26 among all the lines, while that of the allele *bnlg1702-g9* was 0.51 compared to 0.40 among all the lines. Since *bnlg1702-g9* is one of 16 alleles at the SSR *bnlg1702* locus, this frequency is strikingly high. This high frequency was also seen in the subtropical/tropical Asian lines, as well as in all the lines in the study. The allele *phi078-i2* occurred at a frequency of 0.16 among the Asian subtropical/tropical lines compared to 0.11 among all the lines. However, the frequencies of these alleles in the subtropical/tropical group of Asian lines were not significantly different ($p > 0.5$, Fischer's exact test) from those of all the lines in the study. The frequencies of these alleles in a smaller subset of 13 lines that are known to have Suwan-1 in their background were also not significantly different from those of all lines in the study.

Diversity patterns

The dendrogram in Figure 1 was calculated using the genetic similarity (GS) values for all pairs of inbred lines in the study. Besides five lines that did not cluster with any group (CML386, Huangzao4, K2, Pool26, GM19-6#), three clusters of temperate maize and seven indistinct clusters of the subtropical and tropical maize, could be discerned from the dendrogram. The cophenetic correlation coefficient did not show a good fit (r = 0.73), indicating the need for more markers for the dendrogram to more accurately represent the estimates of genetic similarity of the genotypes obtained. While bootstrap values for the branches of the

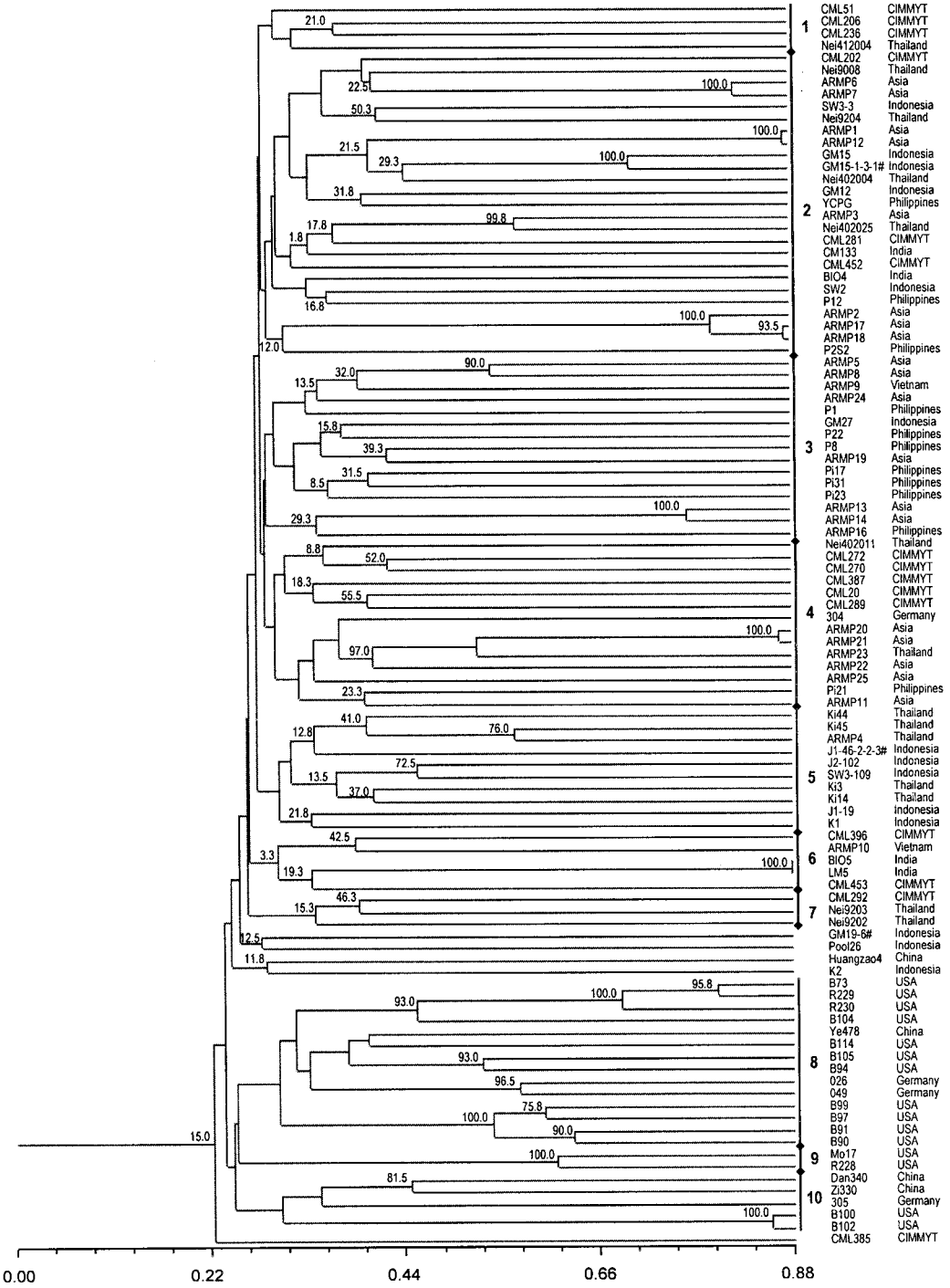

Figure 1. UPGMA dendrogram of 102 maize inbred lines based on 76 SSR markers. Genetic similarities were calculated using Jaccard's coefficient. Bootstrap values are indicated at the junction of the clusters.

temperate cluster are high, indicating that the groupings are robust, bootstrap values of the branches in the subtropical/tropical cluster were low (Figure 1).

Lines from the US, Germany, and China, comprised the temperate maize group while those from India, Indonesia, Philippines, Thailand, Vietnam and CIMMYT comprised the subtropical and tropical maize group. The exceptions were one line from China (Huangzao4) and another from Germany (304) that did not cluster with the temperate maize as expected. One subtropical line from CIMMYT (CML385) was distinct, but two subtropical lines from India (Bio5 and LM5) clustered with the tropical maize.

Within the temperate maize cluster, the groupings are quite distinct. B73 and other inbreds from the BSSS heterotic group and Mo17 of the Lancaster heterotic group clustered into separate groups (Average GS = 0.23). Of the temperate lines from China, Ye478 of the PN group clustered with the BSSS group. The lines Dan340 and Z330 of the Lu group clustered together in a separate group while the line Huangzao4 of the Tangsipingtou group was distinct and more distantly related from the rest of the temperate group.

In contrast, the large group of subtropical and tropical lines could not be delineated into clear-cut groups based on cluster or principal components analysis (range of average GS = 0.14–0.88). Although lines closely related by pedigree did cluster together as in the case of the Indian lines LM5 and Bio5 (average GS = 0.88), and the Asian lines ARMP 1 and 12, ARMP 17 and 18, and ARMP 20 and 21 (average GS = 0.87), the lines did not cluster well according to source population.

Lines originating from each country tended not to cluster together. This was expected, since the Asian lines in the study were selected to represent the diversity of breeding materials in each country. Lines from each country were clustered in at least two of the seven groups, with lines from Thailand being the most diverse, occurring in five of the seven clusters. The CIMMYT lines were dispersed in the clusters within the subtropical/tropical group of Asian lines, reflecting the common germplasm sources of these lines. The CMLs were found in six of the seven clusters, while the ARMP lines were found in five of the clusters. The average genetic similarity among the lines in the subtropical and tropical group (GS = 0.29) was not very different from that of all the lines (GS = 0.28), or to that of the lines in the temperate group (GS = 0.31). On a per country basis, there was a range of GS from to 0.29 (Thailand) to 0.45 (India).

Discussion

A high amount of genetic diversity exists in the maize germplasm in the Asian region. This is evidenced by the prevalence low-frequency alleles, as compared to high-frequency ones, in the Asian inbred lines studied. Interestingly, four (*phi213984, phi121*, *phi423796 phi062*) of the eight high-frequency alleles (> 0.80) observed were in loci in chromosomal regions associated with disease and pest resistance (summarized by McMullen & Simcox, 1995). The locus *phi213984* (bin 4.01) was located in a region associated with a gene and QTL for resistance against *Gibberella zeae* and a QTL for resistance against *Puccinia sorghi*, while the locus *phi121* (bin 8.04) was in a region associated with a QTL for resistance to the European corn borer (ECB). The locus *phi423796* was located in bin 6.01, as was a cluster of resistance genes, including the gene *mdm1* (conferring resistance to the maize dwarf mosaic virus), *wsm1* (conferring resistance to a related potyvirus, the wheat streak mosaic virus) and *rhm1* (conferring resistance to the fungal pathogen *Cochliolobus heterostrophus*), as well as a QTL for resistance to the sugarcane mosaic virus (Shihuang Zhang & Xinhai Li, personal communication). Finally, *phi062* was located in bin 10.04, as was a QTL for resistance to the European corn borer. Except for the sugar cane mosaic virus, which is an important pathogen in northern China, these diseases/pests are not of major importance in Asia. However, it is interesting to speculate that these loci may contain some favorable alleles that play a nonspecific role in the resistance to diseases/pests occurring in the region, which, in concert with other genes, may provide varying levels of adaptation.

The association of alleles with disease/pest resistance was also seen in the allelic patterns of five inbred lines (Ki3, ARMP12, ARMP14, ARMP16, and ARMP21, Table 1) that showed resistant reactions to downy mildews in several locations in the region (unpublished results). In these five lines, alleles in ten loci were the same, six of which are in loci associated with disease/pest resistance. These include three of the loci that contain high frequency alleles associated with disease/pest resistance (bins 4.01, 6.01, and 8.04), and locus *phi339017* in bin 1.03 (QTL for downy mildew resistance, Agrama et al., 1999; Agrama et al., 2002), locus *phi331888* in bin 5.04 (QTL for *G. zeae* resistance, McMullen & Simcox, 1995) and locus *phi033* in bin 9.01 (QTL for ECB resistance, McMullen & Simcox, 1995). Furthermore, alleles in 12 loci were

the same in four of the five lines, and of these, six were in loci associated with disease/pest resistance as well.

However, it appears that selection pressure applied by the breeding emphasis in the region for downy mildew resistance has not favored the selection of three alleles linked to a QTL for resistance. While this may be a consequence of a loose linkage between the SSRs in the study and the QTL, it may also be a consequence of the use of a broad base of diverse materials as sources of downy mildew resistance in the breeding programs in the region (Table 1). Since the 70's, international germplasm exchange and cooperative testing of resistance to downy mildew characterized the development of resistant varieties in Asia. Resistance sources from the Philippines (DMR-1 and DMR- 5 whose resistance came from the native cultivar Tiniguib) were crossed in Thailand into a locally developed variety (Thai composite #1- a composite with 36 varieties from Mexico, Central America, and the Caribbean), from which the improved open pollinated variety (OPV) Suwan-1 (Sriwatanapongse et al., 1993), and four additional improved Suwan varieties were developed. Because of their high level of resistance to downy mildew, the Suwan series became extremely popular as breeding material throughout Asia (Table 1, Dowswell et al., 1996). The tropical lowland maize populations 22 (Mezcla Tropical Blanca), 28 (Amarillo Dentado), and 31 (Amarillo Cristalino-2) from CIMMYT, Mexico which were improved for downy mildew resistance and four genetically broad-based populations (early maturing yellow and white and late maturing yellow and white; De Leon et al., 1993) developed at the Asian Regional Maize program were also extensively used as sources of resistance in breeding programs in the region.

The diversity patterns of the Asian inbred lines revealed a large amount of diversity that did not allow a clear cut distinction between groups of the subtropical and tropical lines. This case is similar to that of the CIMMYT populations which served as germplasm sources for many of the Asian lines (Warburton et al., 2002) where a large amount of diversity within, relative to between, source populations was observed. Due to this heterogeneous nature of CIMMYT populations and the interrelatedness of the lines resulting from the strategy of incorporating both diverse and related materials in the CIMMYT breeding germplasm pools, it is difficult to find a well defined structure of these lines. On the other hand, the heterotic groups in the US and European temperate maize were clearly differentiated in previous studies using RFLPs and SSRs (Messmer et al., 1992; Dubreuil et al., 1996; Smith et al., 1997; Senior et al., 1998). The placement of the China lines was consistent with their pedigree and grouping as described in the study of Yuan et al. (2001) which used both restriction fragment length polymorphism (RFLP) and SSR markers to group the germplasm.

This study is an initial attempt to characterize the breadth of germplasm diversity in Asia, from which we conclude that breeding activity in Asia has not caused a decline in the overall amount of diversity in the region. Furthermore, this information may be used to build a molecular marker database that will allow directed searches of linkages with important traits, such as resistance to diseases, tolerance to abiotic stresses, or improved quality traits. In conjunction with phenotypic and pedigree data, it may be possible to find putative linkages without mapping by directly selecting lines carrying pertinent alleles. This is especially useful in identifying favorable alleles of genes whose phenotypes are difficult to screen or whose effects are masked by an agronomically poor background.

Acknowledgements

We are grateful to our colleagues in the Asian Maize Biotechnology Network for providing materials and information for this study: S. Zhang & X. Li (China), B.M. Prasanna and N. N. Singh (India), M. Dahlan (Indonesia), A. Salazar & P. Guzman (Philippines), and P. Grudloyma (Thailand). We also thank M. Khairallah (formerly in CIMMYT) for her contributions in the initial part of this study and V. Bartolome (International Rice Research Institute) for help in the statistical analysis. This work was supported by the Asian Development Bank and CIMMYT.

References

Agrama, H.A., M.E. Moussa, M.E. Naser, M.A. Tarek & A.H. Ibrahim, 1999. Mapping of QTL for downy mildew resistance in maize. Theor Appl Genet 99: 519–523.

Agrama, H.A., S.F. Houssin & M.A. Terek, 2002. Cloning of AFLP markers involved in resistance to *Peronosclerospora sorghi* in maize. Mol Gen Genomics 267: 814–819.

Baco, D., J. Tandiabang & W. Wakman, 2000. Pests and diseases of maize in Indonesia. In: S.K. Vasal, F. Gonzalez Ceniceros & X.M. Fan (Eds.), Proc 7th Asian Regional Maize Workshop, pp. 267–276. PCARRD, Los Baños, Philippines.

Charters, Y.M., A. Robertson, M.J. Wilkinson & G. Ramsay, 1996. PCR analysis of oilseed rape cultivars (*Brassica napus* L. ssp. *oleifera*) using 5'-anchored simple sequence repeat (SSR) primers. Theor Appl Genet 92: 442–447.

CIMMYT, 2001. The Applied Biotechnology Center's manual of laboratory protocols. Mexico, D.F.: CIMMYT.

De Leon, C., G. Granados, R.N. Wedderburn & S. Pandey, 1993. Simultaneous improvement of downy mildew resistance and agronomic traits in tropical maize. Crop Sci 33: 100–102.

Dowswell, C.R., R.L. Paliwal & R.P. Cantrell, 1996. Maize in the Third World. Westview Press, Inc. Boulder CO, USA, 268 pp.

Dubreuil, P., P. Dufour., E. Drejci, M. Causse M., D. de Vienne, A. Gallais & A. Charcosset, 1996. Organization of RFLP diversity among inbred lines of maize representing the most significant heterotic groups. Crop Sci 36: 790–799.

George, M.L.C., B.M. Prasanna, R.S. Rathore, T.A.S. Setty, F. Kasim, M. Azrai, S. Vasal, O. Balla, D. Hautea, A. Canama, E. Regalado, M. Vargas, M. Khairallah, D. Jeffers & D. Hoisington, 2003. Identification of QTL conferring resistance to downy mildews of maize in Asia. Theor Appl Genet 107: 544–551.

Hedges, S.V., 1992. The number of replications needed for accurate estimation of the bootstrap P value in phylogenetic studies. Mol Biol Evol 9: 366–369.

Kubik, C., M. Sawkins, W.A. Meyer & B.S. Gaut, 2001. Genetic diversity in seven perennial ryegrass (*Lolium perenne* L.) cultivers based on SSR markers. Crop Sci 41: 1565–1572.

McMullen, M.D. & K. Simcox, 1995. Genomic organization of disease and insect resistance genes in maize. Mol Plant-Microbe Interact 8: 811–815.

Messmer, M.M., A.E. Melchinger, J. Boppenmaier, E. Brunklaus-Jung & R.G. Herrmann, 1992. Relationships among early European maize inbreds: I. Genetic diversity among flint and dent lines revealed by RFLPs. Crop Sci: 1301–1309.

Plaschke, J., M.W. Ganal & M.S. Roder, 1995. Detection of genetic diversity in closely related bread wheat using microsatellite markers. Theor Appl Genet 91: 1001–1007.

Pushpavalli, S.N.C.V.L., C. Sudan, S.A. Mohammadi, S.K. Nair, B.M. Prasanna, R.N. Gadag & N.N. Singh, 2002. Analysis of simple sequence repeat (SSR) polymorphism in Indian maize inbred lines. J Genet Breed 56: in press.

Rohlf, F.J., 1999. NTSYSpc: Numerical Taxonomy System, ver. 2.1. Exeter Publishing, Ltd., Setauket, NY.

Rongwen, J., M.S. Akkaya, A.A. Bhagwat, U. Lavi & P.B. Cregan, 1995. The use of microsatellite DNA markers for soybean genotype identification. Theor Appl Genet 90: 43–48.

Senior, M.L., J.P. Murphy, M.M. Goodman & C.W. Stuber, 1998. Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. Crop Sci 38: 1088–1098.

Smith, J.S.C., E.C.L. Chin, H. Shu, O.S. Smith, S.J. Wall, M.L. Senior. S.E. Mitchell, S. Kresobitch & J. Ziegle, 1997. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): Comparisons with data from RFLPs and pedigree. Theor Appl Genet 95: 163–173.

Sriwatanapongse, S., S. Jinahyon & S.K. Vasal, 1993. Suwan-1: Maize from Thailand to the world. Mexico, D.F., CIMMYT.

Warburton, M.L., X. Xianchun, J. Crossa, J. Franco, A.E. Melchinger, M. Frisch, M. Bohn & D. Hoisington, 2002. Genetic characterization of CIMMYT inbred maize lines and open pollinated populations using large scale fingerprinting methods. Crop Sci 42: 1832–1840.

Yap, I. & R.J. Nelson, 1996. WINBOOT: a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. IRRI Discussion Paper Series No. 14. International Rice Research Institute, Manila, Philippines. 22 pp.

Yuan, L.X., J.H. Fu, S.H. Zhang, X. Liu, Z. Peng, X.H. Li, M.L. Warburton & M. Khairallah, 2001. Heterotic grouping of maize inbred lines using RFLP and SSR markers. Acta Agronomica Sinica 27: 149–156.