Nepal Journal of Science and Technology 11 (2010) 1-8

Analysis of Genetic Diversity within Nepalese Maize Populations Using SSR Markers

Dil Bahadur Gurung,[1] Maria Luz C. George,[2] and Quirino D. Dela Cruz[3]
[1]*Nepal Agricultural Research Council, National Maize Research Program, Rampur, Chitwan*
[2]*International Coconut Genetic Resources Network (COGENT), Biodiversity International Regional Office for Asia, the Pacific and Oceania, Malaysia*
[3]*Central Luzon State University, Munoz, Nueva Ecija, Philippines*
e-mail:gurung_dilbahadur@yahoo.com

Abstract

Information on genetic diversity and relationships among breeding materials is necessary for hybrid maize breeding. Four open-pollinated varieties were analyzed using SSR markers to determine the genetic diversity within the varieties. In each variety, 15 individuals were genotyped with 30 SSR markers. Average heterozygosity percentage of the varieties was 45.07%, ranging from 35.23% in Rampur Composite to 54.64% in Khumal Yellow, indicating the higher level of heterozygosity in these two varieties. An average PIC value across all the polymorphic SSR loci was 0.50; which ranged from 0.47 in Manakamana-2 to 0.52 in Khumal Yellow and Arun-4. At the genotype level, the range was from 0.07 in umc1161 to 0.84 in umc1136. The total number of alleles detected was 415 for 30 SSR markers in 60 genotypes. The unique and common alleles detected respectively were 27 and 71. The average number of alleles per locus was 3.45 among the varieties, ranging from 3.21in Manakamana-2 to 3.76 in Khumal Yellow. Average gene diversity across the varieties was 0.54 and ranged from 0.51 in Manakamana-2 to 0.56 in Khumal Yellow and Arun-4. The genetic similarity coefficient of all individuals among the varieties was seen at 0.35.The MRD values were higher between Arun-4 and Manakamana-2 (0.290) and low between Khumal Yellow and Rampur Composite (0.221). Estimate of genetic distances among the varieties showed that Rampur Composite, Khumal Yellow, and Manakamana-2 were closely related sharing the similar genetic backgrounds, whereas Arun-4 was genetically more distantly related. Efforts are being made for the development and evaluation of inbred lines from these distantly related maize varieties for developing high yielding maize hybrids.

Key Words: genetic diversity, maize hybrid, SSR markers

Introduction

An analysis of genetic diversity in germplasm collections facilitates reliable classification of accessions and identification of subsets of core accessions with enormous utility for specific breeding purposes. Genetic diversity has a significant impact on crop improvement (Hallauer 1988). This information is critical in hybrid breeding and population improvement programs in assessing the level of genetic diversity and characterizing and assigning germplasm into different heterotic groups (Reif *et al.* 2003).

Genetic diversity in crop plants can be analyzed at different levels viz. inbred lines/pure lines, clones, populations, germplasm accessions and species. The development of molecular markers has made it possible to analyze plants at the DNA level to achieve a high degree of precision (Melchinger & Gumber 1998).

Genetic diversities among inbred lines in the past were studied only on the basis of pedigree records and the amount of heterosis expressed by the hybrids. The information is sometimes incomplete, unreliable, or unavailable (George *et al*. 2004). Moreover, the expression of morphological traits can be affected by environmental factors but this will not affect molecular analysis. Molecular analysis, therefore, provides reliable and complementary information.

SSR markers have been used effectively to measure genetic diversity in many crop plants, including maize. A number of molecular marker systems are currently available for molecular analyses. These include RFLPs, RAPDs, AFLPs, and SSR markers. These markers have been extensively used for genetic diversity analysis, heterosis, and combining ability studies in maize. Among the markers, SSRs has become the marker of choice for many genetic analyses because of their high level of polymorphism, repeatability, low cost and amenability to automation. SSR markers are co-dominant in nature, which can detect genes in both heterozygous and homozygous states, an important feature in maize hybrid testing.

Molecular markers can help maize breeders to assign inbred lines efficiently to heterotic groups and to guide them in the choice of parents to develop new hybrids (George *et al.* 2004). In this context, there is a need to generate reliable information on the genetic diversity of Nepalese maize germplasm to achieve greater breeding efficiency. Therefore, this study was undertaken to analyze the genetic relationships among Nepalese maize germplasms and determine the breadth of genetic diversity.

Methodology

Four open-pollinated varieties, Rampur Composite, Khumal Yellow, Manakamana-2, and Arun-4 were used in this study. Rampur Composite and Arun-4 are the varieties recommended for both Terai and hill environments, whereas Khumal Yellow and Manakamana-2 are suitable to the hill environments. Khumal Yellow and Rampur Composite are still very popular varieties in the Terai and hills, even though they were released in 1966 and 1975, respectively. We obtained the seeds of these varieties from the National Maize Research Program (NMRP), Rampur, Chitwan, Nepal. Seeds were grown on plastic trays in the greenhouse for DNA isolation. The detail description of varieties is given in Table 1.

Table 1. Description of maize varieties used for molecular analysis

Variety/ Germplasm	Pedigree/ Parentage	Grain type	Maturity days	Average yield (t/ha)	Germplasm description
Arun-4	Formed using the introduced germplasm and local landraces	Yellow flint	113	4.0	Open pollinated variety with early maturing type, Yellow flint grain type, and a promising pipe line experimental variety suitable for Terai, Inner Terai and mid hills
Rampur Composite	DMR version of Thai composite #1 x Suwan-1	Yellow flint	110	3.0-4.5	Yellow flint, open pollinated and full season variety released in 1975 for Terai production environments
Manakamana-2	Formed using elite introduced germplasm and local landraces	Yellow bold flint	139	5.9	Open pollinated promising pipeline variety, flint grain type, and long intermediate maturity suitable for mid hills
Khumal yellow	Antigua G_2D x Guatemala	Yellow flint	130	3.0-4.5	Yellow flint, tall plant type, full season open pollinated variety released in 1965 for mid hill environments

SSR markers

Thirty SSR marker loci, possessing a repeat unit greater than two nucleotides and representing six bins per chromosome, were selected based on bin location and PIC value for uniform genome coverage (Maize DB, www.agron.missouri.edu). Markers were chosen from the 43 priority markers used by the Asian Maize Biotechnology Network (AMBIONET). Primers were synthesized through Research Genetics, Inc. (Huntsville, AL, USA). A laboratory protocol developed and used by the AMBIONET was followed for the molecular analysis of populations (varieties).

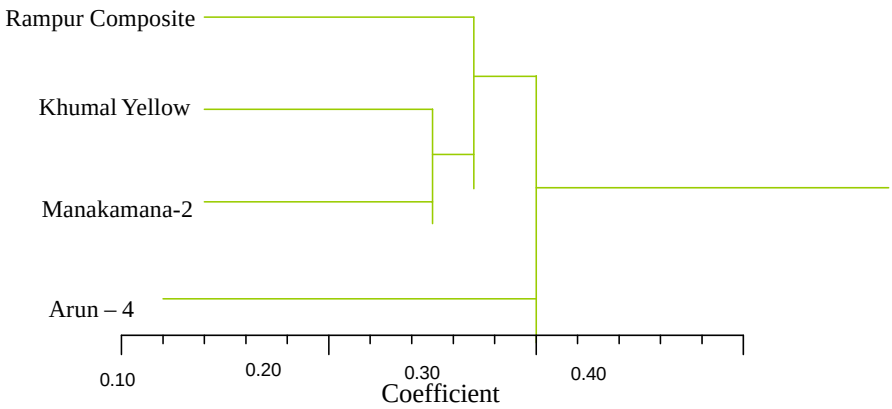

Fig. 1. Genetic distance of four Nepalese maize populations based on modified Rogers' distance

Lyophilization and DNA isolation

Leaf samples were taken from 10-day-old plants. Plants were cut at the base of the second leaf from the bottom. Fifteen individual plants from each variety were taken for sampling. These samples were kept in the silk bags and placed inside the condenser and run for 72 h at -75 ^{0}C and 25 **mottr** for lyophilization. Grinding of the samples was done in a Spex Certi-prep Inc. Geno/grinder 2000® for 30 min. The DNAs were isolated following the CTAB procedure (CIMMYT, 2001).

SSR analysis

The protocol for DNA extraction, amplification and detection, as described in George *et al.* (2004), was followed. PCR products were separated using 4.5% PAGE and visualized using silver staining (Promega). A laboratory protocol used by the AMBIONET Service Laboratory was followed for running the PAGE. Allele scoring was done based on standard allele markers with known molecular weights. Data were scored as present ("1") or absent ("o"), while bands that were diffused or too difficult to score were considered as missing data ("9"). In the case when a line has multiple bands of varying intensities, the most intense band is scored as ("1"). A matrix of binary data was analyzed with NTSYS-pc version 2.02 (Rohlf 1999). Cluster analysis was done with the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) and relationships between varieties were visualized in a dendrogram.

Statistical analysis

We measured the discriminatory power of each SSR locus using the polymorphism information content (PIC). The PIC values were calculated according to Smith *et al*. (1997). The number of alleles per locus, number of total unique alleles per varieties, size range, and number of missing data, percent heterozygosity, and gene frequencies were computed. Similarly, gene diversity, variance of gene diversity, and genetic distance were estimated. The genetic distance matrix was determined using the UPGMA and a dendrogram prepared based on the matrix of genetic Distances. Relationships between varieties were visualized in a dendrogram. Modified Roger's distances (MRD) between two populations were calculated according to Wright (1978) and Goodman & Stuber (1983).

Results and Discussion

Polymorphism information content

We estimated the PIC values of each of the polymorphic SSR locus detected in the study. The values ranged from 0.0 (tetra repeats SSR) in *phi089* to 0.84 (tetra repeats SSR) in *phi064* and *umc1136* (Table 3). Average PIC value at the variety level was 0.50 and the range was from 0.47 in Manakamana-2 to 0.52 in Khumal Yellow and Arun-4. SSR loci *phi064*, *phi109188*, and *phi053* showed the best average PIC values (0.76 and 0.67), respectively. SSR markers showing less than 0.50 PIC consisted of 14 loci with eight tri repeats, four with tetra, one with penta, and two with hexa repeats. The PIC value reported by Yen *et al.* (2001) in 13 Indian lines was lower (0.45) and slightly higher than the PIC value (0.69) obtained by Enoki *et al.* (2002) in 65 maize inbred lines adapted to cold regions of Japan.

Table 2. Bin location, repeat types, size range, PIC values and number of alleles at each locus of SSRs used in the study

No	SSR Loci	Bin No.	Repeat type	Size range (BP)	No of alleles	PIC value
1	bnlg118	5.07-0.08	di	105-121	7	0.76
2	phi029	3.04	AG/AGCG	148-162	6	0.68
3	phi034	7.02	CCT	122-146	7	0.71
4	phi053	3.05	ATAC	169-195	6	0.77
5	phi064	1.11	ATCC	73-105	12	0.83
6	phi065	9.03	CACTT	131-151	4	0.60
7	phi072	4.00-.01	AAAC	143-167	6	0.65
8	phi083	2.04	AGCT	125-137	5	0.71
9	phi084	10.04	GAA	150-156	4	0.47
10	phi089	6.08	ATGC	87-95	2	0.41
11	phi093	4.08	AGCT	274-294	4	0.64
12	phi114	7.03	GCCT	137-169	4	0.60
13	phi123	6.07	AAAG	146-160	3	0.55
14	phi127	2.08	AGAC	112-126	5	0.59
15	phi09188	5.03	AAAG	148-174	8	0.62
16	phi09642	2.03-.04	ACGG	136-144	3	0.46
17	phi227562	1.11	ACC	307-328	7	0.72
18	phi233376	8.09	CCG	142-154	5	0.69
19	phi328175	7.04	AGG	100-130	5	0.63
20	phi339017	1.03	AGG	148-163	5	0.39
21	phi423796	6.01	AGATG	121-141	6	0.26
22	phi448880	9.06-.07	AAG	173-188	4	0.44
23	umc1061	10.06	(TCG)6	101-110	4	0.55
24	umc1109	4.10	(ACG)4	104-116	4	0.53
25	umc1136	3.10	(GCA)5	132-159	9	0.71
26	umc1152	10.02	(ATAG)6	155-219	7	0.74
27	umc1153	5.09	(TCA)4	105-114	6	0.78
28	umc1161	8.06	(GCTGGG)5	134-158	8	0.61
29	umc1279	9.00	(CCT)6	92-101	4	0.37
30	umc1304	8.02	(TCGA)4	129-137	2	0.36

Number of alleles per locus

The total number of alleles detected was 415 for 30 SSR markers in 60 genotypes from the four maize varieties. The unique alleles detected were 27, whereas the total common alleles were 71 in 60 genotypes from the four maize varieties (Table 4). The alleles ranged from one in *phi089* to seven in *bnlg118, phi109188,* and *umc 1152*. Loci *bnlg118, phi064*, and *phi109188* detected seven alleles per locus, the highest number among those detected, and six alleles were detected by *phi065, phi034,* and *umc1136*. The average number of alleles among the varieties was 3.45 and the alleles ranged from 3.21 in Manakamana-2 to 3.76 in Khumal Yellow. The mean number of alleles reported by many authors in previous studies was similar as those found in this study. Warburton *et al*. (2002) found an average of 3.8 alleles in 9 Asian inbred lines and 3.04 by Yen *et al.* (2002) in 13 Indian inbred lines. Similarly mean alleles detected by George *et al*. (2003) had higher (5 alleles) in 68 Asian maize inbred lines, 4.9 alleles in 64 tropical Asian lines. They found lower mean alleles (3.25 alleles per locus) than the mean alleles of those inbred lines from the US and Germany. The more number of alleles reported by these authors might be due to more number of SSR markers used in their studies. The mean alleles obtained in this study are the same as the mean number of alleles within the varieties reported by Reif *et al*. (2003) in CIMMYT 23 maize populations. They found the mean number of 3.9 alleles in 7 tropical populations, 3.68 in 7 intermediate maturity germplasms, 3.67 in 5 subtropical early maturity populations and 4.18 in 5 temperate germplasms.

Table 3. Polymorphism information content (PIC) values for SSR loci found in four maize populations

Marker	Rampur Composite	Khumal Yellow	Manakamana-2	Arun-4
1. bnlg118	0.80	0.69	0.62	0.45
2. phi029	0.48	0.61	0.24	0.69
3. phi034	0.70	0.54	0.65	0.69
4. phi053	0.75	0.68	0.73	0.53
5. phi064	0.84	0.75	0.69	0.78
6. phi065	0.26	0.60	0.50	0.50
7. phi072	0.67	0.53	0.77	0.61
8. phi083	0.53	0.49	0.50	0.50
9. phi084	0.44	0.29	0.53	0.43
10. phi089	0.29	0.29	0.13	0.00
11. phi093	0.61	0.53	0.48	0.49
12. phi114	0.14	0.52	0.50	0.52
13. phi123	0.65	0.58	0.50	0.64
14. phi127	0.48	0.42	0.53	0.60
15. phi09188	0.79	0.71	0.76	0.80
16. phi09642	0.42	0.63	0.38	0.00
17. phi227562	0.36	0.44	0.65	0.34
18. phi233376	0.36	0.68	0.50	0.57
19. phi328175	0.62	0.63	0.43	0.66
20. phi339017	0.56	0.38	0.47	0.43
21. phi423796	0.18	0.51	0.06	0.58
22. phi448880	0.64	0.51	0.41	0.34
23. umc1061	0.46	0.51	0.14	0.53
24. umc1109	0.50	0.46	0.50	0.42
25. umc1136	0.42	0.84	0.56	0.78
26. umc1152	0.60	0.26	0.48	0.72
27. umc1153	0.55	0.55	0.36	0.58
28. umc1161	0.07	0.57	0.50	0.24
29. umc1279	0.13	0.19	0.07	0.59
30. umc1304	0.12	0.32	0.41	0.49
Average	0.48	0.52	0.47	0.52
S.D.	0.22	0.15	0.19	0.19

Heterozygosity

The average heterozygosity at the variety level was 45.07%; the range was from 35.23% in Rampur Composite to 54.64% in Arun-4 (Table 6). *Phi109188* showed the maximum heterozygosity (81.03%), while *umc1109* SSR exhibited the minimum (1.69%). Average heterozygosity was 45.45% among the markers used in the study. At the variety level, Arun-4 had the highest heterozygosity (54.64%) followed by Khumal Yellow (48.21%), Manakamana-2 (42.19%), and Rampur Composite (35.23%). Manakamana-2 and Khumal Yellow had higher heterozygosity, reflecting greater gene diversity than the other two varieties (Rampur Composite and Arun-4).

Gene diversity

Khumal Yellow showed the highest gene diversity (0.62) among the varieties followed by Rampur Composite (0.59), Arun-4 (0.57) and Manakamana-2 (0.56) (Table 17). The observed average gene diversity was 0.59 among the varieties. The average variance at the genotype level ranged from 0.19 in *phi089* to 0.83 in *phi064* locus. The gene diversity in Rampur Composite ranged from 0.13 in *umc1304* to *0.93 in phi064* (Table 6).

Table 4. Number of alleles per locus as detected by 30 SSR markers in four maize populations

Marker	Rampur Composite	Khumal yellow	Manakamana-2	Arun-4	Ave	Total
1. bnlg118	3	7	3	4	4.25	
2. phi029	2	4	2	5	3.25	
3. phi034	5	6	3	5	4.75	
4. phi053	5	3	5	4	4.25	
5. phi064	7	6	6	6	6.25	
6. phi065	4	6	2	2	3.5	
7. phi072	5	4	4	4	4.25	
8. phi083	3	5	2	2	3.00	
9. phi084	4	3	3	3	3.25	
10. phi089	2	2	1	1	1.50	
11. phi093	3	3	2	2	2.50	
12. phi114	2	4	2	4	3.00	
13. phi123	3	4	3	3	3.25	
14. phi127	2	2	3	3	2.50	
15. phi09188	7	3	6	7	5.75	
16. phi09642	2	5	3	1	2.75	
17. phi227562	2	3	5	3	3.25	
18. phi233376	4	4	4	3	3.75	
19. phi328175	3	4	3	4	3.50	
20. phi339017	3	3	3	3	3.00	
21. phi423796	3	5	2	5	3.75	
22. phi448880	4	3	4	2	3.25	
23. umc1061	3	4	3	3	3.25	
24. umc1109	2	3	2	2	2.25	
25. umc1136	5	2	6	6	4.75	
26. umc1152	5	7	3	5	5.00	
27. umc1153	3	4	3	3	3.25	
28. umc1161	2	3	4	2	2.75	
29. umc1279	2	2	2	4	2.50	
30. umc1304	2	2	3	2	2.00	
Average	3.41	3.76	3.21	3.41	3.45	
S.D.	1.45	1.46	1.30	1.50	1.07	
Total	99	116	97	103		415
Total of unique/rare alleles	9	4	5	9	27	
Total common alleles						71

Table 5. Modified Rogers' Distances (genetic distance) among the four Nepalese yellow maize populations

Marker	RampurComposite	Khumal yellow	Manakamana-2	Arun-4
Rampur composite	0.00	-	-	-
Khumal Yellow	0.221	0.00	-	-
Manakamana-2	0.249	0.220	0.00	-
Arun-4	0.271	0.265	0.290	0.00

The minimum gene diversity was recorded in *phi423796* (0.07) and the maximum in *phi072* (0.83). The gene diversity at the variety level ranged from 0.51 in Manakamana-2 to 0.56 in Khumal Yellow and Arun-4. The average gene diversity was 0. 54 among the varieties. Rief *et al.* (2003) had found the similar findings in 23 CIMMYT maize populations. They found the gene diversity 0.56 in tropical maize populations and found comparable with the gene 0.62 in both subtropical early maturity and temperate populations.

The same gene diversity (0.62) was found by Reif *et al.* (2004) and Matsuoka *et al.* (2002). These results suggested that the gene diversity of the four Nepalese maize varieties was comparable with the gene diversity of the 23 CIMMYT maize populations.

Table 6. Mean PIC values, number of alleles per locus, heterozygosity percentage, gene diversity, and variance of gene diversity in four maize populations as revealed by SSR markers

Variety	PIC	Number of alleles per locus	Heterozygosity (%)	Gene diversity	Variance of gene diversity
Rampur Composite	0.48	3.41	35.23	0.52	0.06
Khumal Yellow	0.52	3.76	48.21	0.56	0.03
Manakamana-2	0.47	3.21	42.19	0.51	0.04
Arun-4	0.52	3.41	54.64	0.56	0.04
Across varieties	0.50	3.45	45.07	0.54	0.04
Range	0.00-0.84	1.00-7.00	21.00-68.97	00.00-0.93	0.00-0.31
SE ±	0.35±0.64	3.05±3.84	44.23±45.90	0.39±0.68	0.02±0.10

Cluster analysis

A dendrogram of the four varieties was constructed with Unweighted Paired Group Method Arithmetic Average (UPGMA) using NTSYS. The similarity coefficient of Manakamana-2 was observed at 0.45 coefficient value. Similarly, the similarity coefficients in Arun-4, Khumal Yellow and Rampur Composite were 0.50, 0.42, and 0.40 respectively. The overall similarity coefficient was 0.35 among the varieties, indicating that gene diversity existed in these varieties.

Genetic distance

Based on the Modified Rogers Distances (MRDs), a dendrogram was constructed according to Wright (1978) and Goodman and Stuber (1983). The highest genetic distance (GD) observed was 0.290 between Manakamana-2 and Arun-4, followed by Rampur Composite and Arun-4 (0.271), and Khumal Yellow and Arun-4 (0.265), Khumal Yellow and Manakamana-2 have shown the lowest GD (0.220) among the four varieties (Table 5).

Based on GD, Rampur Composite, Khumal Yellow, and Manakamana-2 are considered closely related, sharing similar genetic backgrounds, whereas Arun-4 is genetically away from the three varieties.

Simple-sequence repeats have proved to be accurate, reliable, and efficient tool in fine-scale genetic characterization of the Nepalese maize germplasm collections. Studies on the genetic diversity of the Nepalese germplasm need to be carried out continuously to analyze their genetic relationships and to assign them accurately into different heterotic groups so that a systematic work on hybrid maize breeding could be built on at the national maize breeding program.

Acknowledgments

The authors are very grateful to the Asian Maize Biotechnology Networks (AMBIONET), CIMMYT, Philippines, for providing the materials and technical support needed in the conduct of this study. They would also like to thank Ms. Ellen S. Regalado and Meggy Lou Katimbang of AMBIONET, CIMMYT, Philippines for their valuable help in the molecular analysis work. National Maize Research Program, Rampur, Nepal is greatly acknowledged for providing the seeds of maize varieties used in the study.

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