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Application of Microsatellite Markers to Fingerprint and Determine the Representational Diversity within a Recently Established Elite Maize Inbred Line Breeding Program

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Abstract

This study focused on the implementation of microsatellite markers to determine the diversity among elite inbred lines representing the Vietnamese maize breeding program. Genetic relationships were assessed using twenty loci to gain an understanding of the width of the program compared to other Asia programs, as well as develop a DNA fingerprint database. The PIC value of the 20 loci ranged from 0.10 to 0.83 with those containing a tetra-repeat the most informative (mean PIC = 0.60). The amount of genetic diversity among the lines assessed was similar to the levels identified in other Asian breeding programs. Future monitoring will be important to maintain genetic breadth of the Vietnamese program and minimize risks from narrowing in future targeted selective breeding strategies. This will be facilitated through implementation of the developed robust SSR-based DNA fingerprint database, which will also be used for true-to-type identification, hybrid confirmation and potential trait selection.

Keywords: Genetic diversity, High throughput genotyping, Inbred lines, Maize, Microsatellite markers

1. Introduction

Maize is an important crop in Vietnam with 1.09 million ha sown in 2009 producing 4.38 million tons, used mainly for animal feed (FAOSTAT Database 2009). By 2020, the Vietnamese population is projected to increase to 100 million, and the annual demand for maize for animal feeds is projected to increase to over 8 million tons (Le et al., 2010). Therefore, there is urgency to increase productivity in order to reduce the need for massive future imports, currently at 0.5-0.7 million tons per annum. This is an ambitious target in light of the finite resource of arable land suitable for growing maize, together with the diverse ecological, social and economic zones in Vietnam. To meet this demand, current strategies within the Vietnam maize breeding program are to develop hybrids with; 1) high and stable yields for intensive and combined conventional and modern maize farming practices; 2) tolerance to herbicides, and abiotic and biotic stresses and 3) shorter life cycles, for flexible crop rotation (Le et al., 2010).

In order to maximize heterosis effects from optimal hybrid combinations as well as maintain breadth in the breeding programs, knowledge of diversity and relationships among current genetic resources is required (Warburton et al., 2002). A narrow and/or non-representative genetic base will ultimately reduce the ability to develop genetically elite lines due to the lack of novel and/or alternate alleles potentially leading to vulnerability to biotic and abiotic stresses (Ali et al, 2006).

Short sequence repeat (SSR) microsatellite markers are widely used to assess genetic relationships within maize breeding programs (Prasanna et al., 2010) due to their co-dominant inheritance, chromosome specific location and amenability to automation (Kalia et al., 2011). In particular, they have been used to assess programs in China (Li et al., 2002), India (Pushpavalli et al., 2002; Ranatunga et al., 2009), Indonesia (Pabendon et al., 2007), The Philippines (Sales et al., 2004), Thailand (Phumichai et al., 2008), Japan (Enoki et al., 2002) and Iran (Choukan et al., 2006). SSR markers have also been applied to assess genetic diversity within the Vietnamese program (Bui et al., 2007a). However, these studies were limited by conventional PCR and PAGE technologies, resulting in limited accuracy and the germplasm assessed was not truly representative of the sources of the genetic material within the entire program.

Fluorescence-based SSR detection and allele sizing on an automated DNA fragment analyzer was developed to provide fast and accurate genotyping (Ziegle et al., 1992). This is based on the separation of fluorescently labeled SSR amplicons by capillary or gel electrophoresis, and requires one of the PCR primers labeled with a fluorescent dye (Oetting et al., 1995). Most recently, the multiplex-ready PCR technique was developed to increase throughput and further reduce assay costs (Hayden et al., 2008). Therefore, the aims of this study were to genotype a representative set of the elite inbred Vietnamese maize lines using the high throughput fluorescent SSR method to: 1) Determine the current genetic relationships and the width of the Vietnamese breeding program and to compare this to the width of other Asian maize inbred programs, and 2) Develop a robust DNA fingerprint database tool for future diversity assessment, true-to-type identification and hybrid confirmation.

2. Materials and Methods

2.1 Maize Inbred Lines and gDNA Extraction

Twenty elite maize inbred lines were supplied by the National Maize Research Institute (NMRI), Vietnam, developed from commercial local and imported hybrids by a traditional selfing method. Based on their source and high yield and desirable agronomic characteristics, these lines were selected as representative of the diversity within the Vietnam maize breeding program (Table 1). The CTAB method (Saghai-Marooft et al., 1984)

was used to extract gDNA from an equal amount of fresh young leaf material from three 8-week-old plants per genotype. The gDNA was then bulked for each genotype sample.

2.2 Primer Synthesis and Amplification Optimization

Twenty SSR primer sequences were selected from the Maize GDB website (<http://www.maizegdb.org>; Table 2) based on the previously reported levels of informativeness (Phan et al., 2004; Bui et al., 2007a, b). They were also chosen based on genome location, distributed across the ten linkage groups of the maize genome. The primers were synthesized with generic non-complementary nucleotide sequences at the 5' end (Invitrogen, USA). Amplicons were obtained using the M13 genotyping method (Oetting et al., 1995). PCR reactions were optimized for annealing temperature at each locus by first observing all of the expected sized products on ethidium bromide stained agarose gel.

2.3 Fluorescent Genotyping

Genotyping was performed with a MyCycler™ PCR machine (Bio-Rad, Australia) using the multiplex-ready PCR technique (Hayden et al., 2008). Each 12 µl reaction contained 10-15 ng of gDNA template, 1x multiplex-ready PCR buffer (5x Immolase PCR buffer, 7.5 mM MgCl₂, 1 mM dNTPs and 2.5x BSA (Bioline, Australia)), 0.3 U Immolase DNA polymerase (Bioline, Australia), 75 nM of tagF primer (Applied Biosystem), 75 nM of tagR primer (Invitrogen, Australia) and an optimized amount of each of the locus specific primer ranging from 10 to 15 nM (Table 2). PCR conditions for all loci were 95°C for 10 min, followed by 2 phases: The first PCR phase consisted of five cycles of 92°C for 60 s, 54-65°C for 90 s and 72°C for 60 s, and 20 cycles of 92°C for 30 s, 54-65°C for 90 s, 72°C for 60 s; The second phase was 40 cycles of 95°C for 15 s, 54°C for 60 s, 72°C for 60 s; and a single final extension cycle of 72°C for 10 min. Automated capillary electrophoresis fragment analysis was performed on an ABI3730 DNA Analyser at the Australian Genome Research Facility, Parkville, Australia.

2.4 Data Analyses

To determine the informativeness of the SSR loci, alleles were called and analysed with GeneMapper v.4.0 software (Applied Biosystems). The observed and effective numbers of alleles were determined with POPGEN 1.32 (Francis et al., 1999). Polymorphic Information Content (PIC) calculations were determined for each primer combination by applying the formula $PIC = 1 - \sum p_i^2$, where p_i is the frequency of the i^{th} allele (Botstein et al., 1980).

For the diversity analysis, based on the presence/absence of alleles at each locus, pairwise similarity measures (S_{ij}) were determined using Jaccard's coefficient; $S_{ij} = a/(n-d)$ (Jaccard, 1908) within NTSYSpc version 2.1 (Rohlf, 2000), where a is the number of bands present in both genotypes i and j , n is the total of bands in sample (total sample size), and d is the number of bands absent in both genotypes i and j . Subsequently, the genetic relationships among the lines were visualized through an UPGMA dendrogram. Principal components analysis (PCA) was also performed using the NTSYS pc 2.1 software to determine the relationships within a multi-dimensional space.

3. Results and Discussions

3.1 SSR Genotyping and Genetic Relationships within the Vietnamese in-bred Breeding Program

The number of alleles observed per locus ranged from two to seven (mean of four) with a total of 88 alleles assessed, ranging from 100 to 350 bp. The effective allele number ranged from 1.11 to 4.08, with an average of 2.07 per locus (Table 2), which corresponded to 52% of the total number of alleles. This was somewhat lower than the allele number of 6.0 per locus observed by Rantunga et al (2009) or 6.8 observed by Warburton et al (2002). However, apart from potentially more diverse germplasm having been assessed, the allele detection and reporting methods employed may greatly influence the outcome. A more thorough checking of marker stability and reproducibility with potentially more conservative allele assignment has arrived with the era of automated genotyping.

The PIC value of each locus ranged from 0.10 (phi448880) to 0.83 (phi072). Four markers (phi423796, phi448880, umc1279 and phi233376) had relatively low PIC values (0.14, 0.10, 0.19 and 0.14, respectively) and thus they may not be as informative as the other 16 loci for diversity assessment and fingerprinting purposes within this genotype set. The mean PIC value across the 20 SSR was 0.44, lower than previously detected within Vietnamese maize line nurseries (Phan et al., 2004 and Bui et al., 2007a, b). A similar study among Chinese elite inbred lines revealed a PIC average of 0.60 among 50 SSR loci assessed, also slightly higher than revealed among the 20 loci in the current study (Li et al., 2002) and closer to a mean of 0.62 revealed in the much larger study of Smith et al (1997) and mean 0.69 PIC of loci used to assess relationships among collections of Japanese

(Enoki et al., 2002) and Thai (Phumichai et al., 2008) inbred lines. An even higher mean PIC range of 0.53 to 0.99, with a mean of 0.83, was previously detected among 22 SSR loci (132 alleles) screened across 45 Indian inbred lines (Ranatunga et al., 2009). The differences in numbers and levels of informative alleles among studies may also be due to differences in sample sources and number, specific selection *a priori* imposed as well as different SSR loci assessed.

In the current study, 45% of the loci analysed comprised tetra-repeats, indicating their informativeness for estimating genetic diversity among maize inbred lines. Indeed, the mean number of observed alleles per tetra-repeat locus was 4.89, compared to 3.00 and 3.25 for tri- and penta-/complex- repeats, respectively. Congruent to this, the mean PIC value for tetra-repeat type loci was 0.60 compared to 0.25 and 0.40 for tri-, and penta- /complex-repeats, consistent with a previous report (Phan et al., 2004). Conversely, Bantte and Prasanna (2003) reported that di-repeats represented 38.9% of the analyze loci and had a relatively higher mean PIC value (0.62) than tri- (0.44), tetra- (0.55), penta- (0.40) or complex repeats (0.53). This was consistent with the di-repeat hypervariability previously reported in maize (Smith et al., 1997; Li et al., 2002). Thus, a future optimal strategy may be to target di- and tetra-repeat SSR loci for molecular discrimination among very closely related lines within the Vietnamese program.

3.1 Current and Future Maintenance of Genetic Diversity

The similarity coefficient among the Vietnamese elite lines ranged from 0.32 to 0.91, indicating substantial genetic breadth among some of lines (0.68 maximum dissimilarity) and similar to the range from 0.34 to 0.72 (0.66 maximum dissimilarity) that was observed between loci screened on Indian inbred lines (Ranatunga et al., 2009). This was also similar to the 0.57 maximum dissimilarity observed with 259 alleles from 50 SSR loci among 59 inbred Chinese lines (Li et al., 2002).

A loose cluster was observed among the Vietnamese elite inbreds, comprising lines sourced from Charoen Pokphand Seeds as well as a Syngenta (VNL13) and Monsanto (VNL11) line. This may highlight the sources of elite germplasm used within this commercial breeding program (Figure 1). However, the majority of lines did not cluster closely, indicating genetic distinction and heterogenic representation within the program. Indeed, the three lines representative of those bred within the NMRI program (VNL14, VNL15 and VNL16) were scattered throughout the dendrogram and PCA (data not shown), indicating substantial diversity among them at the loci assessed (Figure 1). Although 62 % of the variation among all 20 lines was explained by the three largest principal coordinates (49.07, 7.08 and 6.00 %, respectively), a far more comprehensive sampling is required to determine the detailed structure of the program. Structure on a much larger scale has previously been shown within maize inbred breeding programs using SSR markers, for indicating heterotic groupings and/or pedigree (Choukan et al., 2006; Lui et al., 2003; Sales et al., 2004).

3.2 Development of an SSR Database for NMRI

The alleles identified in this study will be useful to maintain true-to-type and hybrid identification within the Vietnamese NMRI maize in-bred breeding program, saving time and money in future crossing and reselection. The database is also a tool for breeders elsewhere who will use the Vietnamese hybrid lines as parental germplasm in future material exchange programs (Table 3).

4. Conclusions

The genotyping of the 20 representational elite inbred lines with the 20 SSR loci has indicated the diversity existing across the Vietnamese program. The inclusion of a greater number of loci and individuals is predicted to greatly improve the information available. This will enable breeders to choose loci to track to ensure maximum diversity is maintained within their breeding program through future strategic introductions, crosses and selections.

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Table 1. The twenty elite inbred lines from the Vietnamese breeding program that were assessed in the study

Name of line	Source
VNL01	Cargill777 hybrid,-Cargill Seeds, India
VNL02	Syn70224 hybrid,-Syngenta, USA
VNL03	NK67 hybrid, Syngenta, USA
VNL04	NK67 hybrid, Syngenta, USA
VNL05	LNS222 hybrid, Luong Nong Seeds, Vietnam
VNL06	MB69 hybrid, Mien Bac Seeds, Vietnam
VNL07	CP999 hybrid, Charoen Pokphand Seeds, India
VNL08	CP999 hybrid, Charoen Pokphand Seeds, India
VNL09	CP999 hybrid, Charoen Pokphand Seeds, India
VNL10	CP999 hybrid, Charoen Pokphand Seeds, India
VNL11	C919 hybrid, Monsanto, USA
VNL12	CP999 hybrid, Charoen Pokphand Seeds, India
VNL13	NT6621 hybrid, Syngenta Seeds, USA
VNL14	LVN61, NMRI, Vietnam
VNL15	LVN10, NMRI, Vietnam
VNL16	LVN154 NMRI, Vietnam
VNL17	C919 hybrid, Monsanto, USA
VNL18	C919 hybrid, Monsanto, USA
VNL19	NK4300 hybrid, Syngenta Seeds, USA
VNL20	Pacific747 hybrid, Pacific Seeds, Australia

Table 2. SSR markers that produced stable and multiallele profiles among the twenty elite inbred lines

No.	Marker	Bin No. ^a	Repeat Type	Size range (bp)	OPC (nM) ^b	na ^c	ne ^d	PIC value
1	umc1109	4.10	ACG	137-145	10	3	1.36	0.27
2	umc1161	8.06	GCA	175-187	10	3	1.35	0.26
3	phi448880	9.05	AAG	213-216	10	2	1.11	0.10
4	phi227562	1.12	ACC	342-354	10	4	1.42	0.29
5	phi308707	1.10	AGC	149-163	20	3	1.96	0.49
6	phi233376	8.03	CCG	174-181	10	3	1.16	0.14
7	umc1279	9.00	(CCT) 6	124-130	20	3	1.23	0.19
8	phi109188	5.00	AAAG	177-200	10	4	2.54	0.61
9	phi96100	2.00	ACCT	297-326	10	6	2.08	0.52
10	phi063	10.02	TATC	196-241	10	7	2.23	0.61
11	phi093	4.08	AGCT	316-325	10	5	2.78	0.64
12	phi102228	3.04-.05	AAGC	155-158	10	2	1.28	0.22
13	phi114	7.03	GCCT	168-198	10	7	3.31	0.70
14	phi083	2.04	AGCT	159-167	15	4	2.64	0.62
15	phi072	4.00	AAAC	175-187	15	5	4.08	0.83
16	umc1399	3.07	(CTAG)5	142-156	10	4	2.81	0.64
17	phi423796	6.01	AGATG	155-166	10	3	1.16	0.14
18	phi065	9.03	CACTT	164-183	10	2	1.47	0.32
19	umc1136	3.10	GCTGGG	163-188	10	5	2.20	0.55
20	phi029	3.04	AG/AGCG***	178-182	10	3	2.41	0.59
	Mean					4	2.07	0.44

^aLocation of SSR marker within 10 chromosomes from the Maize GDB (<http://www.maizegdb.org>)

^bOptimized primer concentration (OPC)

^cObserved number of alleles (na)

^dEffective number of alleles (ne)

Table 3. A genotype database constructed for the twenty elite lines within the Vietnamese breeding program

Loci	Lines	VNL01	VNL02	VNL03	VNL04	VNL05	VNL06	VNL07	VNL08	VNL09	VNL10	VNL11	VNL12	VNL13	VNL14	VNL15	VNL16	VNL17	VNL18	VNL19	VNL20	
		umc1109	A1	137	137	137	137	137	145	137	137	137	137	137	137	137	137	137	137	137	140	140
	A2	137	137	137	137	137	145	137	137	137	137	137	137	137	137	137	137	137	140	140	137	
phi029	A1	178	178	182	178	179	179	182	182	182	182	182	182	182	178	182	179	182	179	178	182	178
	A2	178	178	182	178	182	179	182	182	182	182	182	182	182	178	182	182	182	179	178	182	178
phi423796	A1	161	161	161	161	161	161	161	161	155	161	161	161	161	161	161	161	161	161	161	161	161
	A2	161	161	161	161	161	161	161	161	155	161	161	161	161	161	166	161	161	161	161	161	161
phi109188	A1	197	177	193	197	197	197	193	193	193	193	193	193	193	197	200	193	193	200	197	200	
	A2	197	177	193	197	197	197	193	193	197	193	193	193	193	197	200	193	193	200	197	200	
phi96100	A1	314	320	320	297	320	305	320	320	320	320	320	320	320	320	320	309	305	326	305	320	
	A2	314	320	320	297	320	305	320	320	320	320	320	320	320	320	320	309	320	326	305	320	
phi065	A1	164	164	164	164	183	164	164	164	164	164	164	164	164	164	164	164	164	183	164	164	
	A2	164	164	183	164	183	183	164	164	164	164	164	164	183	165	183	164	164	183	164	164	
phi063	A1	197	205	205	205	205	205	205	205	197	197	205	205	205	196	205	205	205	196	196	204	
	A2	197	205	222	205	222	205	205	205	203	203	205	241	205	205	205	205	205	222	196	204	
phi093	A1	324	316	317	321	316	325	316	316	324	316	316	316	316	316	316	324	317	316	321	321	
	A2	324	316	317	321	316	325	316	316	324	316	316	316	316	316	316	324	317	316	321	321	
phi102228	A1	155	155	155	155	155	155	155	155	158	155	155	155	155	155	155	155	155	155	155	158	155
	A2	155	155	155	155	155	155	155	155	158	155	155	155	155	155	158	155	155	155	155	158	155
phi114	A1	198	168	177	195	198	195	195	195	195	195	195	198	198	198	191	198	195	192	186	191	
	A2	198	168	195	195	198	195	198	195	195	195	195	198	198	198	191	198	195	192	195	192	
umc1399	A1	152	142	152	152	152	152	147	147	147	152	147	147	147	147	152	152	142	147	156	152	
	A2	152	142	152	152	152	152	147	147	147	152	147	147	147	147	156	152	142	147	156	152	
umc1161	A1	181	175	175	175	175	175	175	175	175	175	175	175	175	181	175	175	175	175	175	175	
	A2	181	175	187	175	175	175	175	175	175	175	175	181	175	175	181	175	175	175	175	175	
umc1136	A1	185	185	185	182	163	185	185	185	185	179	185	179	185	163	185	188	185	185	182	185	
	A2	185	185	185	182	163	185	185	185	185	179	185	179	185	163	185	188	185	185	182	185	
phi448880	A1	216	216	216	213	216	216	216	216	216	216	216	216	216	216	216	216	216	216	216	216	
	A2	216	216	216	213	216	216	216	216	216	216	216	216	216	216	216	216	216	216	216	216	
phi083	A1	159	167	159	165	167	163	167	167	165	167	165	167	159	159	159	-	159	159	165	167	
	A2	167	167	167	165	167	167	167	167	165	167	165	167	167	167	167	-	159	159	165	167	
phi072	A1	179	183	186	183	186	187	-	175	175	175	175	175	186	183	183	179	186	183	183	187	
	A2	186	183	186	183	186	187	-	175	175	175	175	175	187	183	183	186	186	183	183	187	
phi227562	A1	342	342	342	342	342	342	-	342	342	342	342	342	342	342	342	-	342	342	348	345	
	A2	354	342	342	342	342	342	-	342	348	342	342	342	342	342	342	-	342	342	348	345	
umc1279	A1	127	127	127	127	124	127	127	127	127	127	127	127	127	130	127	127	127	127	127	127	
	A2	127	127	127	127	124	127	127	127	127	127	127	127	127	130	127	127	127	127	127	127	
phi308707	A1	160	160	149	163	163	163	163	163	163	163	163	163	160	163	149	163	160	160	163	149	
	A2	163	163	163	163	163	163	163	163	163	163	163	163	160	163	149	163	160	160	163	149	
phi233376	A1	181	181	181	181	181	181	181	181	181	181	181	181	181	181	174	181	181	181	180	181	
	A2	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	180	181	

A1= Allele 1; A2 = Allele; "-" =Missing data

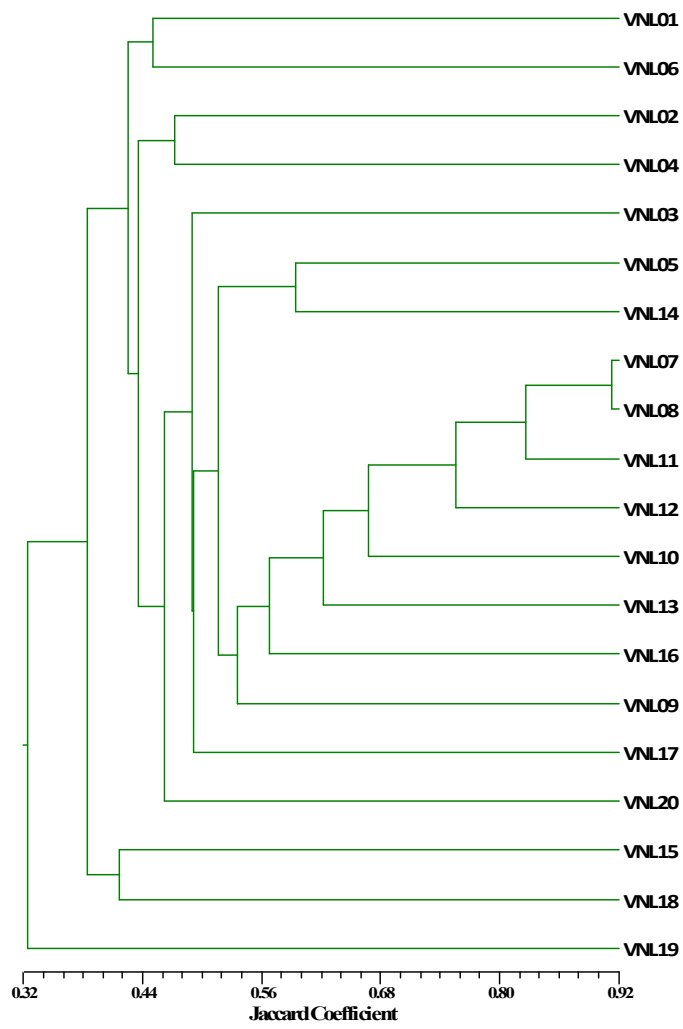


Figure 1. A dendrogram showing relationships among the 20 maize inbred lines based on 20 SSR loci