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Development and characterization of a core set of SSR markers for fingerprinting analysis of Chinese maize varieties

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Abstract

A core set of 60 SSRs was selected and modified using 231 Chinese and USA maize (*Zea mays* L.) inbred lines from more than 2000 SSRs for DNA fingerprinting analysis. All 60 SSR markers met the following criteria: (1) amplification of a single locus; (2) distinct amplification products; (3) adequate intervals between adjacent alleles; (4) suitable PCR fragment size; (5) reasonable discrimination power (DP); and (6) even distribution across the maize genome. Furthermore, the 60 SSR primers were re-designed to adjust the PCR product size. Together with the application of four different fluorescent dyes, a high-throughput 10-plex capillary electrophoresis platform was explored. The 60 core SSR markers were further divided into three groups (20 SSRs per group) according to peak morphology and DP value. Groups I, II and III were used in DNA fingerprinting analysis as a basic core, an expanded core and a candidate core set respectively. The allele number per locus varied from three to 22 with an average of 8.95; the average number of alleles per group I, II and III was a respective 7.35, 7.8 and 11.4. The DP values ranged from 0.366 to 0.913, with an average of 0.718 among all loci; the average group DP values were 0.697, 0.718 and 0.737 for groups I, II and III respectively; and the cumulative values of discrimination power (CDP) approached 1 for all groups. Cluster analysis results using 60 selected loci divided the Chinese inbred lines into six groups, including Luda Red Cob, P, Improved Reid, Tang-si-ping-tou, Waxy and Lancaster. The USA inbred lines were segregated into four groups, including SSS, Lancaster, Iodent and Oh43/Oh07Mid mixed.

Keywords: Maize, SSR, core primers, DNA fingerprinting

Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops in the agricultural economy. Maize is widely cultivated throughout the world with broad applications, including human and animal food, biofuel, chemicals, and medicines, among others. In China, maize has become the second largest crop in terms of total production and growing area, and plays a key role in the agricultural structure of the country (Yu et al, 2007). The number of maize varieties has rapidly increased in China since 1980. To date, over 5,500 maize varieties have been authorized (http://www.newcorn.com.cn); more than 2,000 varieties have been given variety protection rights (http://www.cnpvp.cn), and over 1,000 varieties are inspected in national and regional trials each year. However, maize hybrid germplasm bases in China are quite narrow, with only a few inbred lines having played a central role in hybrid development i.e. Mo17, Huangzaosi, 330, E28, Dan340, and 478 (Li, 1998; Yu et al, 2007). In an attempt to breed high-yield corn hybrids, breeders prefer to use a very limited elite germplasm. Consequently, it becomes a challenge to identify established and new varieties and protect 'variety rights'. On the other hand, it is almost impossible to distinguish closely related inbred lines based on morphological characteristics, because morphology is highly dependent on environmental conditions and therefore variable. In addition, homonyms and synonyms further complicate identification among varieties. Therefore, DNA-fingerprinting is one of the most effective approaches to distinguish different maize varieties.

The extensive application of molecular markers to genetic studies has provided a foundation for its use in DNA fingerprinting analysis. In the last decade, many maize studies have been performed applying molecular markers to assess the levels of genetic diversity, QTL mapping, and marker-assisted selection (MAS) breeding. The markers used include random amplified polymorphic DNA (RAPD) (Khampila et al, 2008), restriction fragment length polymorphisms (RFLP) (Bernardo, 1997), inter-simple sequence repeats (ISSR) (Barcaccia et al, 2003), amplified fragment length polymorphisms (AFLP) (Hartings et al, 2008), simple sequence repeats (SSR) (Lu and Bernardo, 2001; George et al, 2004; Clerc et al, 2005), and single nucleotide polymorphisms (SNP) (Jones et al, 2009; Pozar et al, 2009). However, RAPD, ISSR, AFLP and RFLP marker systems are not suitable for DNA fingerprinting analysis because of the markers are of a

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Table 1 - Samples used in this study include 135 Chinese inbred lines and 96 (from ID136 to ID 231) USA inbred lines.

ID	Inbred Line	Pedigree	ID	Inbred Line	Pedigree
1	Ye478	U8112 × Shen5003	117	335	Waxy corn inbred line
2	1141	Selected from American hybrid "78599"	118	Zinuo5B	Waxy corn inbred line
3	8001	488 × 3189	119	Xiangnuo8	Waxy corn inbred line
4	C8605-2	7922 × 5003	120	9902	Waxy corn inbred line
5	H21	Huangzao4 × H84	121	Shuangjin-11	Selected from Japanese hybrid "Jinyinsui"
6	K12	Huangzao4 × Weichun	122	SH-251	Selected from Chaotian-1
7	P138	Selected from American hybrid "78599"	123	P12	Selected from American hybrid "78599"
8	Chang7-2	(Huangzao4 × Wei95) × S901	124	Ai311	Unknown
9	Dan340	Baigulü9 × Pod corn	125	Luyuan92	Yuanqi122 × 1137
10	HuangC	((Huangxiao162 × Zi330) × O2) × Tuxepeno-1	126	Qi318	Selected from American hybrid "78599"
11	Huangzao4	Selected from Tangsipingtou	127	434	466 × Hua94
12	Lian87	5003 × Dan340	128	Su80-1	Jinhuang55 × Yuanwu02
13	Shen I37	Selected form American hybrid "6JK111"	129	Ji846	Ji63 × Mo17
14	Shen5003	Selected form American hybrid "3147"	130	D375	02428 × Nannongxian2
15	Tie7922	Selected form American hybrid "3382"	131	He344	Baitoushuang × Mo17
16	XingK36	Selected from Jiku-6	132	CA335	Selected form Pool 33
17	Ye107	Selected form hybrid "XL80"	133	P25	Introduced from CAU
18	Ye502	Dan340 × Huangzao4	134	Cheng18	Dingshangyumi × (Gong70 × 60-22)
19	1145 (A)	Selected form American hybrid "78599"	135	1145 (B)	Selected from American hybrid "78599"
20	Tie9010	Dankang1 × Dan340	136	B73	BSSSC
21	Ji853	Huangzao4 × Zi330	137	Mo17	187-2 × 103
22	X178	Selected form American hybrid "78599"	138	PH4CV	PH7V0 × PHBE2
23	J0045	478 × P78599	139	PH6WC	PH01N × PH09B
24	Jing501	Selected from a population of 10 hybrids	140	PHZ51	PH814 × PH848
25	Jing5237	Huangzao4 × Dan340	141	PB80	Unknown
26	D9046	Tie7922 × Shen5003	142	PHW65	PH861 × PH595
27	Jin96	Unknown	143	PHG83	PH814 × PH207
28	Nongxi531	Unknown	144	792	Unknown
29	Dan598	(((OH43Ht3 × Dan340) × Danhuang02) × Danhuang11) × 78599	145	PHT55	A33GB4 × A34CB4
30	Zi330	Oh43 × Keli67	146	NK790	NK235 × B73
31	420	(478 × Dan340) × 146	147	PHK76	PHAD18 × PHB02
32	Jing404	(Huangzao4 × Tuxepeno-2) × Huangzao4	148	PHG84	PH848 × PH595
33	Lx9801	Ye502 × H21	149	7831A	Unknown
34	Jing89	Ye478 × 78599	150	LH132	(H93 × B73) × B73
35	F349	Shen5003 × Dan340	151	IB014	Unknown
36	Jing24	Zaoshu302 × Huangyesi	152	LH123Ht	Pioneer Hyb 3535
37	Zheng58	Selected from Ye478	153	DKMBNA	Mo17Ht × MDA-28
38	SW1611	Introduced from Thailand	154	NS701	A632 × B73Ht
39	U8112	3382 × 3147	155	HBAI	Unknown
40	Ben7884-7	Ci7 × L289	156	PHT77	PH814 × PH995
41	MC0303	(9042 × Jing89) × 9046	157	NQ508	Unknown
42	Shennong92-67	popcorn inbred line	158	PHN11	PH207 × (PH207 × PH806)
43	502196	Huangzao4 × Dan340	159	NS501	Unknown
44	Ye515	(Huafeng100 × AiC103) × Huangzao4	160	LH39	Unknown
45	Ye52106	(Aijin525 × Ye107) × 106	161	LH1	(B37 × Holden line 644) × B37
46	Zong31	Selected from Zi330	162	B47	Unknown
47	444	A619 × Huangzao4	163	PHG35	PHG3BD2 × PH595
48	81162	(Aijin525 × Ye107) × 106	164	LH150	Unknown
49	Ji842	Ji63 × Mo17	165	LH52	[(Mo17 × Holden line 610) × Mo17] × Mo17
50	Longkang11	Mo17 × Zi330	166	ML606	Unknown
51	GY246	high oil inbred line	167	LH57	(Mo17 × H99) × LH53
52	Wu314	(Huangzao4 × Wu302D) × Huangbaoliao	168	PHG39	PHA33GB4 × PHA34CB4
53	zhong106	Yemen short corn × Synthetic	169	LH60	LH55 × LH47
54	Ji53	Selected from Ji synthetic 2Co-2	170	PHG71	A632Ht × PH207
55	Yuanfuhuang	Selected from Huangzao4	171	DKFBHJ	(FBAB × B84) × FBAB
56	3189	U8112 × Shen5003	172	NK740	Mo17 Backcross 3 × Mexican Deep Kernel
57	A801	Dan9042 × (Dan9046 × Mohuang9)	173	LH38	L120 × A619
58	CN1483	Introduced from China Academe of Agricultural Sciences	174	PHG47	PH041 × MKSDTE C10
59	DHuang212	D729 × Huangzao4	175	SG17	Unknown
60	Chang3	Selected from Yinglizi	176	PHG42	Unknown
61	Dabatang	Chinese landrace	177	LH51	Mo17 Backcross 5 recovery
62	Danhuang25	Selected from American hybrid “78599”	178	LH82	Holden line 610 × LH7
63	Dunbai	Chinese landrace	179	LH59	(Mo17 × H99) × LH53
64	Duo29	Selected from American hybrid “78599”	180	78371A	[(4726 × Iowa Long Ear) × 4726] × 4726
65	Ji7162	Unknown	181	Q381	Pioneer Hyb 3369
66	Jing123	Unknown	182	LH143	A632 derived
67	Jing186	Sizi × Jingdan841	183	LH143CMS	A632 derived
68	Jing594	Huangzao4 × P78599	184	DK78002A	B73 × A634
69	5872	Zheng58 × Mo17	185	NK807	W117 × B37
70	Yinglizi	Introduced from Europe	186	LP5	(Yugoslavian GLAMOS × B73Ht) × B73Ht
71	Zao673	Unknown	187	78004	B73 × A634
72	Zhe446	Unknown	188	PHG29	PH207 × (PH207 × PH806)
73	Zhe773-2	Ji63 × Huangzao4	189	LH156	Va85 × Pa91
74	Zhongchang7	Unknown	190	DJ7	B73 × BS16 Synthetic
75	835	V8112 × 718	191	NK764	NK235 × B73
76	Dan598-1	Selected from Dan598	192	LH74	A632 × B73

Table 1 - continued

77	895	Dan598 × Chang7-2
78	Ji1037	(Mo17 × Suwan1) × Mo17
79	Qi319	Selected from American hybrid "78599"
80	CT019	Qi319 × Shen137
81	Jun9058	6JK × 8085
82	F349(Disease Resistance)	(P25 × F349) × F349
83	P25(Disease Resistance)	Introduced from CAU
84	MC30	1145 × 1141
85	Huangye4	(Yejihong × Huangzao4) × Dunzihuang
86	4112	A619 × 8112
87	5005	Selected from 8147
88	A235	Unknown
89	Bjian8	(BC7321 × Jianduanqi) × 8112
90	zhonghuang64	Selected form Pioneer hybrid "64"
91	Dan341	5003 × 561-1•332-2•Men•B•330
92	K10	5003 × Chang3
93	135	Unknown
94	HOF2	Selected from American hybrid "78599"
95	Huotanghuang	Huobai × Tang203
96	BM	Unknown
97	352	Unknown
98	4F1	Selected from Mo17
99	E28	(A619Ht1 × Lû9Kuan) × Lû9Kuan
100	Chong72	3147 × B37Ht
101	Q126	Huangzao4 × Weichun
102	Fu80	lû9 × Pod corn
103	ZaG546	Unknown
104	Zhonghuang69	Introduced from China Academe of Agricultural Sciences
105	JN22	Unknown
106	673	Unknown
107	JN15	J0045 × Qi319
108	428	413 × Zi330
109	0020	Qi319 × short stalk 117B
110	Bainuo6	Selected from Zizuo-3
111	Paternal parent of Zinuo5	Waxy corn inbred line
112	Female parent of Zinuo5	Waxy corn inbred line
113	Zinuo3	Selected from Zinuo-3
114	Jingnuo6	Selected from Zhongnuo-1
115	9901	Waxy corn inbred line
116	Ziyu-3	Waxy corn inbred line
193	NK778	W117 × B37Ht
194	NK794	NK235 × B73
195	DK4676A	1067-1 × B-Line Composite
196	FR19	A635 × W438
197	DKFAPW	B14AHt × B37Ht
198	LP1CmsHt	A632Ht C cytoplasm male sterile
199	PHG50	PH848 × PH207
200	F42	B73 mutation selection
201	DKMDF-13D	H4101× Composite 800M
202	G103	Unknown
203	LH61	[(ASA × Mo17) × Mo17] × Mo17
204	PHG80	PH495 × PH331
205	PHG72	PH891 × PH207
206	PHG86	B64 × B73
207	PHB09	PH555 × PH031
208	LH145	A632Ht × CM105
209	LH146Ht	(B73 × CM105) × CM105
210	DK78010	B73 × A634
211	LH119	(H93 × B73) × B73
212	LH93	BS11(FR)C3
213	NSSS	Unknown
214	IODEET	Unknown
215	S07:61	Unknown
216	HPHR47	Unknown
217	2369	Unknown
218	PHM49	Unknown
219	OQ603	Unknown
220	11430	Unknown
221	NKH8431	(NK377 × NKB386) × NK347
222	PHW52	B73 × PHG39
223	DKMBPM	Composite 400M
224	LH149	[(A662 × B73) × B73] × B73
225	ZMA22	Unknown
226	SB326	Unknown
227	LH65	(Mo17 × LH18) × LH53
228	MBST	Unknown
229	PHR32	Unknown
230	NKS8324	(CH593-9 × B73) × B73
231	PHH93	Unknown

dominant nature, are not highly informative, and provide inadequate exchange of data among different studies. In contrast, SSR markers are a suitable technique for DNA fingerprinting analysis. SSRs exhibit simple banding pattern, are informative, the markers are of a co-dominant nature, the method is highly repeatable and provides comparable data among different studies. Single SNP makers have lower information than SSRs, but are amenable to high-throughput methods with lower genotyping error rates. Consequently, the International Union for the Protection of New Varieties of Plants (UPOV) (2007, 2010) recommends SSRs for current construction of DNA fingerprint databases that have been well-defined and tested, and suggests future use of SNPs.

Selection of a universal SSR set is important for DNA fingerprinting analysis. Although a large number of SSRs are available in the maize GDB or PANZEA database, each SSR marker is not suitable for fingerprinting analysis. The SSRs must be evaluated and some primers re-designed; only SSR markers with high repeatability, distinct PCR bands, reasonable polymorphism, and known chromosomal loci are suitable for constructing a fingerprinting database. Furthermore, for a core SSR set, all SSRs should be evenly distributed across the genome. In recent years, construction of SSR-based fingerprinting databases have been completed in several crops, including rice (Nandakumar et al, 2004), wheat (Rôder et al, 2002; Li et al, 2006), potato (Coombs et al, 2004; Reid, 2004), and tomato (Bredemeijer et al, 2002), and each study reported a suitable set of SSRs. In maize, several sets of SSR markers have been published (George et al, 2004; Clerc et al, 2005; Kahler et al, 2010) based on the standardization of fingerprinting analysis technology, genetic diversity of French varieties, or identification of North American varieties. However, a core set of SSR markers has not been established for DNA fingerprinting analysis based on Chinese maize varieties. Therefore, the objectives of this study were to establish a core set of SSR markers for use in Chinese maize varieties to construct a DNA fingerprinting database that serves to accurately distinguish existing maize varieties and establish the identity of new varieties.

Materials and Methods

Plant materials

A total of 231 maize inbred lines were selected to evaluate SSR markers and primers, including 135 Chinese and 96 USA inbred lines (Table 1). Table 1 shows the inbred lines and their pedigrees. The 135

Table 2 - Characterization of the 60 SSR loci for Chinese maize DNA fingerprinting analysis based on 231 inbreds. The map coordinates of the 60 loci are from the genetic map IMB2 2004 neighbors frame.

Set	No. of Loci	Loci name	Chr BIN	Motif sequence	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')	No. of alleles	Allele range	DP
	N01	bnlg439w1	1.03	(TC)	AGTTGACATCGCCATCTTGGTGAC	GAACAAGCCCTTAGCGGGTTGTC	12	319-369	0.801
	N02	umc1335y5	1.06	(AG)	CCTCGTTACGGTTACGCTGCTG	GATGACCCCGCTTACTTCGTTTATG	4	233-257	0.375
	N03	umc2007y4	2.04	(TC)	TTACACAACGCAACACGAGGC	GCTATAGGCCGTAGCTTGGTAGACAC	13	233-300	0.822
	N04	bnlg1940k7 [a]	2.08	(CT)	CGTTTAAGAACGGTTGATTGCATTCC	GCCTTTATTTCTCCCTTGCTTGCC	13	324-388	0.805
	N05	umc2105k3	3.00	(AG)	GAAGGGCAATGAATAGAGCCATGAG	ATGGACTCTGTGCGACTTGTACCG	6	280-350	0.675
	N06	phi053k2 [b]	3.05	(GTAT)	CCCTGCCTCTCAGATTCAGAGATTG	TAGGCTGGCTGGAAGTTTGTTGC	4	333-363	0.663
	N07	phi072k4 [b]	4.01	(TGTT)	GCTCGTCTCCTCCAGGTCAGG	CGTTGCCCATACATCATGCCTC	4	408-432	0.485
	N08	bnlg2291k4 [a]	4.06	(AG)	GCACACCCGTAGTAGCTGAGACTTG	CATAACCTTGCCTCCCAAACCC	6	362-421	0.705
	N09	umc1705w1	5.03	(CT)	GGAGGTCGTCAGATGGAGTTCG	CACGTACGGCAATGCAGACAAG	9	254-349	0.792
I	N10	bnlg2305k4	5.07	(GA)	CCCCTCTTCCTCAGCACCTTG	CGTCTTGTCTCCGTCCGTGTG	12	240-312	0.860
	N11	bnlg161k8	6.00	(AG)	TCTCAGCTCCTGCTTATTGCTTTCG	GATGGATGGAGCATGAGCTTGC	13	154-216	0.876
	N12	bnlg1702k1	6.05	(CT)	GATCCGCATTGTCAAATGACCAC	AGGACACGCCATCGTCATCA	13	260-347	0.821
	N13	umc1545y2 [b]	7.00	(AAGA)	AATGCCGTTATCATGCGATGC	GCTTGCTGCTTCTTGAATTGCGT	7	180-249	0.721
	N14	umc1125y3	7.04	(CTCG)	GGATGATGGCGAGGATGATGTC	CCACCAACCCATACCCATACCAG	5	149-175	0.756
	N15	bnlg240k1	8.06	(GA)	GCAGGTGTCGGGGATTTTCTC	GGAACTGAAGAACAGAAGGCATTGATAC	7	220-239	0.807
	N16	phi080k15 [b]	8.08	(GGAGA)	TGAACCACCCGATGCAACTTG	TTGATGGGCACGATCTCGTAGTC	6	202-238	0.689
	N17	phi065k9 [b]	9.03	(GTGAA)(GTGCA)	CGCCTTCAAGAATATCCTTGTGCC	GGACCCAGACCAGGTTCCACC	4	391-415	0.685
	N18	umc1492y13	9.04	(GCA)	GCGGAAGAGTAGTCGTAGGGCTAGTGTAG	AACCAAGTTCTTCAGACGCTTCAGG	4	270-290	0.467
	N19	umc1432y6 [a]	10.02	(TC)	GAGAAATCAAGAGGTGCGAGCATC	GGCCATGATACAGCAAGAAATGATAAGC	5	211-259	0.366
	N20	umc1506k12 [a]	10.05	(TTTG)	GAGGAATGATGTCCGCGAAGAAG	TTCAGTCGAGCGCCCAACAC	6	163-196	0.771
	Average						7.35		0.697
	N21	umc1147y4	1.07	(CA)	AAGAACAGGACTACATGAGGTGCGATAC	GTTTCCTATGGTACAGTTCTCCCTCGC	6	149-172	0.429
	N22	bnlg1671y17	1.10	(CT)	CCCGACACCTGAGTTGACCTG	CTGGAGGGTGAAACAAGAGCAATG	14	173-255	0.896
	N23	phi96100y1 [b]	2.00	(AGGT)	TTTTGCACGAGCCATCGTATAACG	CCATCTGCTGATCCGAATACCC	7	231-287	0.786
	N24	umc1536k9	2.07	(GT)(TA)	TGATAGGTAGTTAGCATATCCCTGGTATCG	GAGCATAGAAAAAGTTGAGGTTAATATGGAGC	13	216-238	0.890
	N25	bnlg1520k1 [a]	2.09	(CT)(AC)(GA)(TA)	CACTCTCCCTCTAAAATATCAGACAACACC	GCTTCTGCTGCTGTTTTGTTCTTG	6	156-204	0.722
	N26	umc1489y3 [a]	3.07	(GCG)	GCTACCCGCAACCAAGAACTCTTC	GCCTACTCTTGCCGTTTTACTCCTGT	4	231-265	0.428
	N27	bnlg490y4	4.04	(TA)	GGTGTTGGAGTCGCTGGGAAAG	TTCTCAGCCAGTGCCAGCTCTTATTA	11	245-331	0.766
	N28	umc1999y3	4.09	(TGC)	GGCCACGTTATTGCTCATTTGC	GCAACAACAAATGGGATCTCCG	8	167-200	0.682
	N29	umc2115k3	5.02	(GCCAT)	GCACTGGCAACTGTACCCATCG	GGGTTTCACCAACGGGGATAGG	6	265-295	0.749
II	N30	umc1429y7	5.03	(AGC)	CTTCTCCTCGGCATCATCCAAAC	GGTGGCCCTGTTAATCCTCATCTG	4	125-145	0.524
	N31	bnlg249k2 a	6.01	(AG)	GGCAACGGCAATAATCCACAAG	CATCGGCGTTGATTTCGTCAG	9	259-313	0.671
	N32	phi299852y2 [a, b]	6.07	(CTG)	AGCAAGCAGTAGGTGGAGGAAGG	AGCTGTTGTGGCTCTTTGCCTGT	6	200-254	0.828
	N33	umc2160k3	7.01	(AG)	TCATTCCCAGAGTGCCTTAACACTG	CTGTGCTCGTGCTTCTCTCTGAGTATT	10	198-244	0.799
	N34	umc1936k4	7.03	(TG)	GCTTGAGGCGGTTGAGGTATGAG	TGCACAGAATAAACATAGGTAGGTCAGGTC	6	153-176	0.637
	N35	bnlg2235y5	8.02	(TG)	CGCACGGCACGATAGAGGTG	AACTGCTTGCCACTGGTACGGTCT	7	174-198	0.804
	N36	phi233376y1 [b]	8.09	(CCG)	CCGGCAGTCGATTACTCCACG	CAGTAGCCCCTCAAGCAAAACATTC	8	180-222	0.737
	N37	umc2084w2	9.01	(CTAG)	ACTGATCGCGACGAGTTAATTCAAAC	TACCGAAGAACAACGTCATTTCAGC	7	184-214	0.800
	N38	umc1231k4 [a]	9.05	(GA)	ACAGAGGAACGACGGGACCAAT	GGCACTCAGCAAAGAGCCAAATTC	10	239-283	0.675
	N39	phi041y6 [a, b]	10.00	(CAGC)	CAGCGCCGCAAACTTGGTT	TGGACGCGAACCAGAAACAGAC	6	296-334	0.749
	N40	umc2163w3	10.04	(AG)	CAAGCGGGAATCTGAATCTTTGTTC	CTTCGTACCATCTTCCCTACTTCATTGC	8	280-352	0.794
	Average						7.80		0.718
	N41	bnlg1025y4	1.07	(AG)	CTCTCCTCACGCCAACTTAATCTGTG	GTGACTCCTAAGCTCGCCGAATAA	11	141-201	0.804
	N42	umc1538y3	1.11	(AG)	CTCGAAACAGGTGGTACAGTGCG	AGCAGCTTTTACCCCTGATTTTTCC	20	131-194	0.838
	N43	umc1261k10 [a]	2.02	(GT)	TGGTAATGGTATGTAGAAGAAGTGCGTATG	CAGCGACAAGAGCAGCGTG	5	231-249	0.692
	N44	bnlg1175k1	2.04	(AG)	GACACTTGCACGGTCTCGCTTAT	ATCCCAAGCACCACGGTCAAG	17	261-340	0.880
	N45	bnlg1523k3 [a]	3.02	(CT)	GTTTCGGACGAAAGCCTAATAACCC	AGCCGCGTAGTGGATAGGAGC	17	183-263	0.585
	N46	umc1136y2 [a, b]	3.10	(CAG)	CCTCTCGTCTCATCACCTTTCCC	GCTGCATACAGACATCCAACCAAAG	7	122-162	0.673
	N47	phi021y8	4.03	(AG)	CCAAGTGTAAAGAGTGCGAAGAAGCAG	CCATCACGAAAGGTGGAGGTAGAAGA	10	167-208	0.580
	N48	umc1051k2	4.08	(CT)	GGGATCGGAGTAGCGCAAAGTAG	GCCATCAAACCCTCAACTCTGC	14	226-286	0.852
	N49	umc1496y3 [a]	5.00	(GCA)	GATTACAACCCACCGGAGTTACAGG	CCAACATGAAGGGAGGGTGC	9	146-184	0.630
III	N50	mmc0081k1	5.05	(CT)	GAAAACCATTCCCAGTTAGGAGCAG	GGTTGTTTGCCTCTTCTGTACTCTGTTG	6	167-215	0.706
	N51	umc1859k1	6.06	(AG)	AATCTCCAGGTTGGTGTTCAAAGG	AAAGATGACTTTGTGGGCAGTGG	14	140-194	0.871
	N52	umc1127k1 [a]	6.08	(GA)	CCCCCCTCCCTAATTTTGCTTC	GCACATCTTACGGATCTAGCTGGACTG	13	153-261	0.797
	N53	phi328175y4 [b]	7.04	(GAG)	CTGGAACACCTTCACGCCCTCT	CGGCGACCCACTCATCTCATT	4	302-346	0.723
	N54	phi116k1 [b]	7.06	(GTCA)(TGTA)	GTACTTCAACACCGACCTCACCG	CGGCCATGGATGGGATACAA	5	240-262	0.588
	N55	umc1741w1	8.03	(AG)	CATGCGCTTGGCATCTCCATGTATATC	CTGGTGCAGCAATGGCCGAG	13	144-215	0.680
	N56	bnlg162k2 [a]	8.05	(CT)	GGCTCACGTCCGTATCCAAACC	TCAGTTCAGGTCCGTCGTCCAG	17	239-284	0.840
	N57	bnlg1191k7 [a]	9.06	(TC)	GCCGATCCAGAGGGTCCATTC	CGTCGCATTGCATTGCATAGC	13	240-299	0.814
	N58	umc1366y1	9.06	(CCT)	TGTTTATGGTGGGAAGAACGGGAC	CTTCACGACGAATGCCTAACTCTGC	3	226-238	0.482
	N59	bnlg1712k17	10.03	(GA)	CGATTTCACGGCTCGTGGC	GAAAATACCCTTGGTTCTCCTTCCTGG	8	256-294	0.799
	N60	bnlg1450A1 [a]	10.07	(TC)	TCTCCCATCATGTACGAGAAATCATG	CGTCGAACTCATCCAGCAGATG	22	232-352	0.913
	Average						11.4		0.737

[a] Locus was used in Kahler et al (2010); [b] Locus was used in George et al (2004)

Chinese inbred lines cover a broad germplasm resource and have been frequently used to develop maize hybrids in China. The inbred lines belong to the following heterotic groups: Tang-si-ping-tou (TSPT), Luda Red Cob (LRC), Lancaster (Lan), Improved Reid, P groups, and Waxy corn (Table 1). The 96 USA inbred lines (from ID136 to ID 231, Table 1) were obtained over the terms of variety protection rights in the USA. The germplasm background includes Lancaster, SSS, Iodent, Oh43, and Oh07Mid heterotic groups (referred to Mikel, 2006; Mikel and Dudley, 2006).

DNA extraction and SSR analysis

Sixty seeds of each inbred were grown in an incubator for approximately five days. Subsequently, approximately fifty young leaves representing each inbred were selected from different plants and ground into a fine powder. Total genomic DNA was extracted from 50-pooled leaf (4.5-5.0 g) using the CTAB procedure according to the CIMMYT (2005) laboratory protocols. DNA quality and quantity were estimated using a BioPhotometer Plus (Eppendorf, Hamburg, Germany).

PCR was performed in a 20μl reaction volume containing approximately 4μl of total genomic DNA, 0.25μM of each primer (one primer was dye labeled), 0.15mM dNTP, 2.5mM $MgCl_2$, 1 unit of Taq polymerase (Tiangen, Beijing, China), and 1×PCR buffer. PCR amplification parameters were as follows: 94°C for 5 min; followed by 35 cycles at 94°C for 40 sec, 60°C for 35 sec, and 72°C for 45 sec; with a final extension at 72°C for 10 min. PCR products were run on an AB 3730xl DNA Analyzer (Applied Biosystems, USA). Subsequently, 1.5μl of the 10-plex PCR product, 8.5μL Hi-DiTM formamide, and 0.1μl GeneScanTM-500 LIZ (Applied Biosystems, USA) as an internal standard was loaded into each well of a 96-well optical reaction plate. The samples were denatured at 95 °C for 5 min, and the sample plates were spun at 1000 rpm for 1 min. Finally, electrophoresis was carried out on an AB 3730xl DNA Analyzer, and the resulting data were analyzed using Data Collection Ver. 1.0.

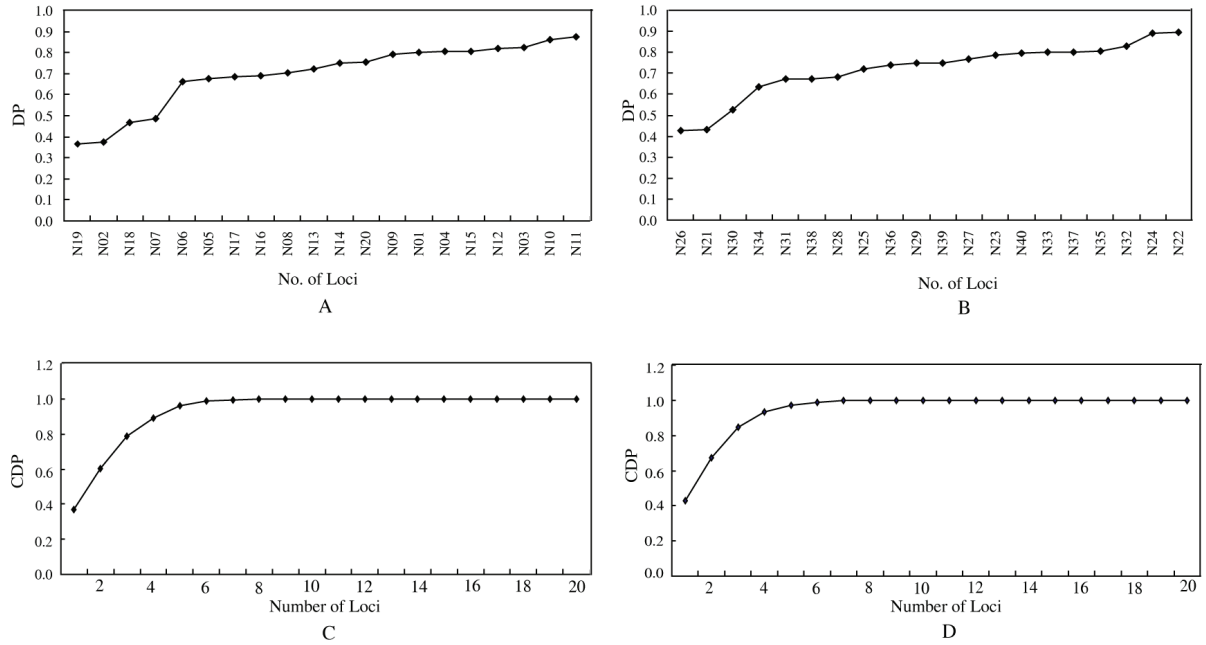

Figure 1 - Discrimination power (DP) curve and cumulative discrimination power (CDP) value of groups I and II primers.
A: DP value curve of group I primers (the order of primers displayed on the abscissa is according to the DP value)
B: DP value curve of group II primers (the order of primers displayed on the abscissa is according to the DP value)
C: CDP value curve of group I primers
D: CDP value curve of the group II primers

Selection and evaluation of SSR markers

One hundred SSRs, with 10 markers per chromosome, were selected from over 2000 in the Maize GDB public database. The location of the 100 SSRs on the integrated genetic map IBM2 2004 neighbors frame was known to exhibit an even distribution along the maize chromosomes. The screening procedure is reported in Wang et al (2007). Fifteen primer pairs of each locus were redesigned for the 100 candidate loci using Primer Premier 5.0 and Oligo 6.22 to develop the multiplex set. Nomenclature for the newly designed primers was specified using the “name of the original primer”, the “code of the designer”, and the “serial number”. The original and newly designed primers at the same locus were detected using the above described amplification program and electrophoresis method. Based on the banding patterns,

efficiency of amplification, and the score estimates from Primer Premier 5.0 and Oligo 6.22, one optimized primer pair was selected per locus.

The 100 primers with the 231 maize inbred lines were used to screen a core SSR set for fingerprinting analysis using fluorescent dye labeled primers. Sixty core SSRs were subsequently selected for fingerprinting analysis on the basis of the following criteria (referred to Macaulay et al, 2001; Vardhney et al, 2008; UPOV, 2007, 2010): (1) the amplification fragment included a single locus not multiplex loci; (2) the fragment was easily amplified; (3) appropriate intervals between adjacent alleles; (4) a suitable fragment range; (5) reasonable discrimination power (DP); and (6) genomic distribution. In order to avoid linkage among loci, and fully reflect the genetic information, the selected loci were of known genetic location and distributed throughout the genome. The genetic location of all loci was based on the genetic IBM2 2004 neighbors frame map (Table 2). For the selected loci, we calculated linkage disequilibrium (LD) among all loci using GENEPOP Ver. 3.4 (Raymond and Rousset, 1995) based on the 231 inbred lines. If there was significant linkage disequilibrium (P<0.01) between two loci, one locus was deleted.

The selected 60 core SSRs were further divided into three groups, groups I, II, and III as a basic core set, an expanded core set and a candidate core set respectively, according to three criteria: the stability of the amplification product, peak morphology and discrimination power (Table 2).

Construction of a 10-plex capillary electrophoresis system using dye-labeled primers

Based on the fragment size range and the type of dye labeled, a 10-plex electrophoresis set was constructed. The combination pattern was 3+3+2+2, each set of primers was divided into four groups based on the four dyes types (VIC, NED, PET, FAM), and markers with the same label were separated by more than 10 bp (Table 3).

Data analysis

SSR loci were scored using GeneMapper ver. 3.7 (Applied Biosystems, USA). The software PowerMarker ver. 3.25 was used to estimate the number of alleles and genotypic frequencies (Liu and Muse, 2005). The discrimination power (DP) for an individual SSR locus and the cumulative discrimination power (CDP) for multiple loci were estimated as follows (Tessier et al, 1999): $DP=1-\sum(pi)^2$ and $CDP=1-C1\times C2\times C3\ldots\times Ci$, where pi represents the frequency of each genotype, and Ci represents the confusion probability of each locus ($Ci=\sum(pi)^2$). The neighbor-joining (NJ) tree for the Chinese and USA inbred lines was obtained respectively using Rogers (1972) genetic distance, based on data from the selected 60 SSR loci using Power-Marker ver. 3.25 software.

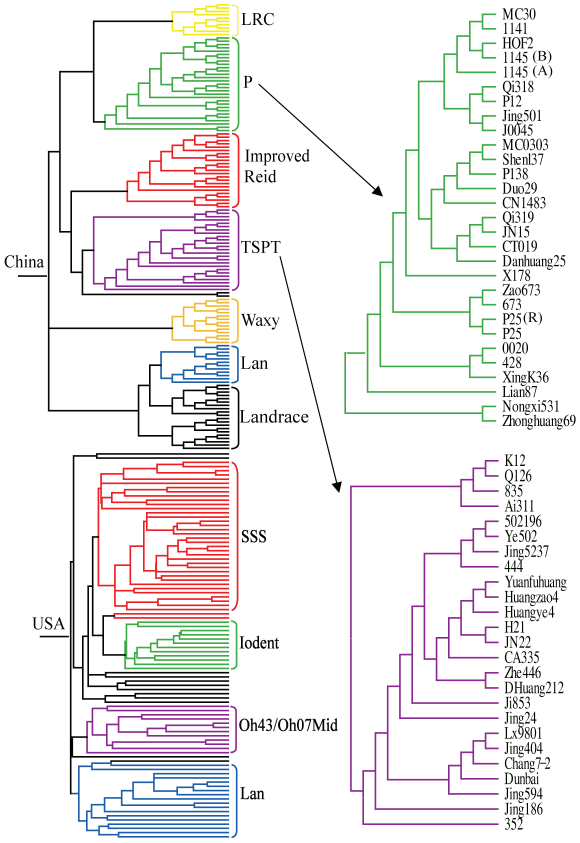

Figure 2 - Neighbor-joining (NJ) trees for the Chinese and USA inbred lines based on Roger's genetic distance.

Results

Characterization of the 60 SSR primers for construction of the maize fingerprinting database

The 231 inbred lines were used to characterize the 60 primers. Table 2 provides detailed information for the 60 primers, including genetic map location, motif sequence, redesigned primer sequence, allelic numbers, PCR product range, and DP value. All inbred lines were successfully amplified using the 60 primers, and PCR products were clearly observed and unambiguously scored. Each of the 60 SSR primers displayed widely polymorphic PCR products across all samples. In total, the 60 SSR primers generated 537 alleles. The number of alleles per locus varied from three to 22, with an average of 8.95. The average number of alleles for groups I, II, and III were respectively 7.35, 7.8, and 11.4. The number of alleles detected should reflect the actual number of alleles within the 60 loci, as the samples spanned a range of resources. PCR fragment size at each locus varied from 12 bp at locus N58 to 120 bp at locus N60. In addition, we calculated DP and CDP values based on genotypic frequency. DP values varied among loci, and exhibited a range from 0.366 (N19) to 0.913 (N60), with an average DP of 0.718 over all 60 loci; the average DP values of groups I, II, and III were

Table 3 - An example of the combination patterns of the 20 primers of I group (two 10-plex sets) and the 20 primers of II groups (two 10-plex sets).

Set	Loci name	Chr BIN	Allele range	Fluorescence labeled	Set	Loci name	Chr BIN	Allele range	Fluorescence labeled
I-1	umc1432y6	10.02	211-259	VIC	I-1	umc1429y7	5.03	125-145	VIC
I-1	umc2105k3	3.00	280-350	VIC	I-1	umc1999y3	4.09	167-200	VIC
I-1	phi072k4	4.01	408-432	VIC	I-1	umc1489y3	3.07	231-265	VIC
I-1	umc1545y2	7.00	180-249	NED	I-1	umc1147y4	1.07	149-172	NED
I-1	bnlg1702k1	6.05	260-347	NED	I-1	phi299852y2	6.07	200-254	NED
I-1	phi065k9	9.03	391-415	NED	I-1	umc2163w3	10.04	280-352	NED
I-1	bnlg240k1	8.06	220-239	PET	I-1	phi233376y1	8.09	180-222	PET
I-1	bnlg439w1	1.03	319-369	PET	I-1	bnlg490y4	4.04	245-331	PET
I-1	bnlg2305k4	5.07	240-312	FAM	I-1	umc2084w2	9.01	184-214	FAM
I-1	bnlg1940k7	2.08	324-388	FAM	I-1	phi96100y1	2.00	231-287	FAM
I-2	umc1125y3	7.04	149-175	VIC	I-2	umc1936k4	7.03	153-176	VIC
I-2	umc1335y5	1.06	233-257	VIC	I-2	umc2160k3	7.01	198-244	VIC
I-2	phi053k2	3.05	333-363	VIC	I-2	phi041y6	10.00	296-334	VIC
I-2	bnlg161k8	6.00	154-216	NED	I-2	bnlg2235y5	8.02	174-198	NED
I-2	umc2007y4	2.04	233-300	NED	I-2	umc1536k9	2.07	216-238	NED
I-2	bnlg2291k4	4.06	362-421	NED	I-2	bnlg249k2	6.01	259-313	NED
I-2	umc1506k12	10.05	163-196	PET	I-2	bnlg1520k1	2.09	156-204	PET
I-2	umc1705w1	5.03	254-349	PET	I-2	umc1231k4	9.05	239-283	PET
I-2	phi080k15	8.08	202-238	FAM	I-2	bnlg1671y17	1.10	173-255	FAM
I-2	umc1492y13	9.04	270-290	FAM	I-2	umc2115k3	5.02	265-295	FAM

respectively 0.697, 0.718, and 0.737; and the cumulative values of discrimination power (CDP) approached 1 for all groups (Table 2, Figure 1).

Multiplex capillary electrophoresis system

Grouping SSRs into a multiplex electrophoresis system depends on the labeled dye and the fragment size range. The 60 primers used in this study could be designed into multiplex system applying four dyes and the different size range. Table 3 represents a combination pattern of four 10-plex sets, comprised of 40 primers for groups I and II.

Clustering analysis

The neighbor-joining analysis method was used to construct a dendrogram from 60 loci of the 135 Chinese and 96 USA inbred lines (Figure 2). The clustering results were nearly consistent with the heterotic groups established based on pedigree information. The 135 Chinese inbred lines were clustered into six groups, including Luda Red Cob (LRC), P, Improved Reid, Tang-si-ping-tou (TSPT), Waxy, and Lancaster (Lan) groups. The LRC group was represented, in part, by inbreds Dan340, Tie9010, and Fu80. Twenty-nine Chinese inbreds, including P138, X178, and Qi319 comprised the P group, most of which were derived form USA hybrid P78599. The Improved Reid group consisted of 23 Chinese inbreds, which included Ye478 and Zheng58 etc, most of which were derived form Shen5003 and U8112. The TSPT group included 25 inbreds, which were mainly derived from the Chinese local germplasm Huangzao4. Fourteen Waxy inbreds were clustered into one group. The Lancaster group was comprised of inbreds Ji1037, Ji846, and Ji842, among others, which have Mo17 in their pedigree. The 96 USA inbreds formed the following groups based on the NJ tree and pedigree data: SSS, Lancaster, Iodent, and Oh43/Oh07Mid. The SSS group comprised 36 inbreds containing the genetic composition of B73. The Lancaster group included 18 inbreds, and results indicated most lines were closely related to Mo17. The Iodent and Oh43/Oh07Mid groups were distinguished according to cluster results and genetic background (Mikel, 2006).

Discussion

Selection criteria for a core set of SSR primers in a DNA fingerprinting database

Common selection criteria are necessary to consider for a single locus primer, including product quality, high polymorphisms, and PCR fragment size. However, two additional points are important in selecting core primers. First, a few base pair intervals between adjacent alleles are required to easily differentiate alleles. Second, primers have to exhibit reasonable polymorphisms, because too high or low level of polymorphism is directly related to over complexity in data analysis. It is inefficient to construct a database using low polymorphic primers, and difficult to score alleles using high polymorphic primers. Figure 3 provides two representative loci, which are suitable to construct a fingerprinting database. On a core primer set, the following criteria are ideal: (1) All selected loci are distributed across the genome to avoid linkage among loci. (2) The primers are combined in a multiplex electrophoresis to increase throughput (for example, different size ranges). (3) Efficiency is improved when the candidate primers have the potential for multiplex amplification (for example, similar annealing temperatures). The primer set reported in this study might not meet all the above requirements, such as uniform distribution. SSR distribution is non-uniform in the maize genome (Sharopova et al, 2002), therefore, it is impossible to achieve a perfect primer set.

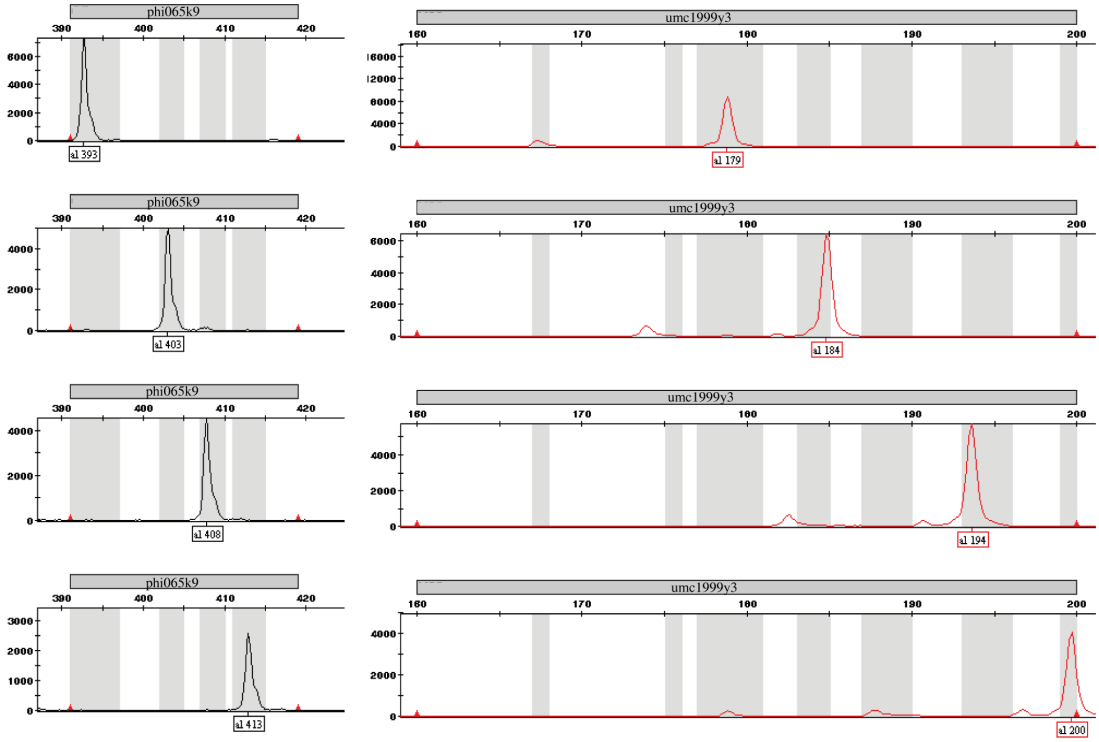

Figure 3 - Electrophoresis results of two representative loci suitable to construct a database and fingerprinting analysis (on AB 3730xl DNA Analyzer).

Comparison between published loci and loci reported in this study

The majority of previously published loci were included in the 2000 used in this study (i.e. reported in George et al, 2004; Clerc et al, 2005; Kahler et al, 2010). During the screening process, which resulted in selection of 100 markers, we made choices based on amplification efficiency, polymorphisms, and genetic location (see Wang et al, 2007 for details). The selection process for capillary electrophoresis reduced the core primer set from 100 to 60, which differed from the denaturing polyacrylamide (George et al, 2004; Clerc et al, 2005) and agarose (Kahler et al, 2010) gel electrophoresis platform. Therefore, some primers were not retained. Compared with reports of previously applied primer sets, 18 were consistent with Kahler et al, (2010), and 12 with George et al (2004), however we had no primers in common with those used by Clerc et al (2005) (Table 2). Therefore, some loci are more general and can be selected independent of materials and electrophoresis platform, and other loci are specific to materials and electrophoresis platform. In this study, we selected and evaluated the 100 best candidate SSRs systematically and comprehensively using 231 inbred lines representing a broad Chinese and USA maize genetic background for DNA fingerprinting analysis (Table 1 and Figure 2). Presently, the 60 core primers and in particular the 40 primers of groups I and II have been used to construct the Chinese maize database and are widely used in China to identify maize varieties. Overall, based on the current level of research and technology platforms available in maize, this primer set should be superior to any other primers in DNA fingerprinting analysis for Chinese maize varieties.

The optimal number of primers to construct a maize DNA fingerprinting database

Considering the differences in fingerprinting database scales, and the range of genetic backgrounds in maize varieties, the SSR primer numbers should be variable according to different database. For example, it was possible to discriminate 192 Eucalyptus samples using only three SSRs (Kirst et al, 2005), and establish a Chinese criminal DNA database using 13 SSR polymorphisms (Du et al, 2000). In this study, 10 basic core primers for group I was sufficient to differentiate 231 inbred lines (Figure 1), however, so few markers is not desirable in practice. Maize can be backcrossed an unlimited number of times and become infinitely close to the original variety. Consequently, there is little genetic difference among some maize varieties. Currently, plant variety protection law in China is based on the 1978 version of UPOV, and does not include the concept of an essentially derived variety (EDV). Therefore, a series of maize varieties exist with genetic similarity that exceeds

90%. For example, many new hybrids similar to Zhengdan958 were popularized during recent years. The 231 inbred lines used in this study are relatively easy to distinguish because the lines cover a broad genetic background and exhibit a wide range of genetic diversity. Although the 231 inbred lines could successfully be differentiated using 10 primers, it is not always feasible in practical applications. Therefore, we maintained the 60 core primer set, and these primers were further subdivided into three groups to select primers of any group or a combination of groups for different research purposes. Group I is comprised of 20 primers used as basic core primers. This group exhibits the highest evaluation scores to construct a large-scale database to identify varieties. Primers in group II show a slightly lower score than group I, and should be chosen as expanded core primers. Generally, a combination of groups I and II (a total of 40 primers) is sufficient to identify varieties with narrow genetic backgrounds or similar varieties. Compared with groups I and II primers, group III primers exhibit the lowest scores, but the highest polymorphisms. Consequently, group III primers are recommended as candidate core primers. All 60 core primers demonstrate utility in estimating maize germplasm resources (Figure 2).

Molecular markers for a maize DNA fingerprinting database

Until recently SSR markers were the choice for fingerprinting analysis, however, advances in technology have resulted in a shift toward SNPs, particularly in model plants with substantial genomic resources. UPOV has provided extensive recommendations for SSRs and future sequencing information for SNPs (UPOV 2007, 2010). SSR and SNP markers have been the most appropriate to construct DNA fingerprinting databases, but both methods have their own advantages and disadvantages.

The disadvantages of SSRs compared with the advantages of SNPs are as follows: (1) SSRs occur at a much lower genomic density and exhibit an uneven distribution relative to SNPs (Sharopova et al, 2002; Hamblin et al, 2007). (2) Compared with SNPs, SSRs have a slightly higher genotyping error rate. This is due to the multi-allelic nature of SSRs, and the subsequent variation in fragment size. Furthermore, comparison and integration issues exist between different SSR detection platforms. SNPs are bi-allelic, and represent the smallest units of genetic variation in the genome. Therefore, alleles are easily read, compared, and integrated between different data sets. (3) SSR detection throughput is far lower than SNPs. SSR throughput generally includes only one primer for polyacrylamide gels or as high as 10-plex primers using four-color fluorescence. However, SNPs are amenable to design a high-throughput platform. Based on the current technical level, the throughput can range from 48, to thousands, and even tens of thousands (using an Applied Biosystems, USA; Illumina BeadArray, USA platform). Therefore, it is likely SNPs will provide increased resolution. However, SSRs exhibit higher allelic diversity for each locus relative to SNPs. SSRs are well researched and the experimental technique is relatively mature. In addition, SSRs can be performed without the need for expensive instrumentation; therefore any laboratory can apply SSR research. Compared with SNPs, the key advantage of SSRs is a lower cost for fingerprinting analysis of individual samples.

In a summary, Both SSRs and SNPs are ideal marker systems to construct a DNA fingerprinting database. SSRs will continue to play an important role in the long term because of their higher information content and low cost. SNPs will be the marker of interest in the future due to data accuracy and higher throughput, along with improved SNP technology.

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