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SELECTION AND DEVELOPMENT OF REPRESENTATIVE SIMPLE SEQUENCE REPEAT PRIMERS AND MULTIPLEX SSR SETS FOR HIGH THROUGHPUT AUTOMATED GENOTYPING IN MAIZE

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Abstract: In the current study, 1,900 maize simple sequence repeat (SSR) primers published in MaizeGDB were screened utilizing reference literature, 15 representative Chinese maize inbred lines and 15 Chinese maize hybrids from national regional testing. In total, 500 highly polymorphic primers were identified and used to construct a genetic map. 100 evenly distributed primers, 10 primers per chromosome, were further selected as a set of universal SSR core primers, recommended as preferred primers for general studies. These core primers were then redesigned and used to construct a high throughput multiplex PCR system based on a five-color fluorescence capillary detection system. We report here that two sets of ten-plex PCR combinations have been constructed, each consisting of 10 primers, with one primer per chromosome.

Keywords: maize, core primer, SSR, multiplex PCR, capillary electrophoresis, fluorescent-labelled.

SELECTION AND DEVELOPMENT OF REPRESENTATIVE SIMPLE SEQUENCE REPEAT PRIMERS AND MULTIPLEX SSR SETS FOR HIGH THROUGHPUT AUTOMATED GENOTYPING IN MAIZE

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Introduction

1. Development and selection of a set of universal core SSR primers is important in germplasm certification, heterosis group division, genetic diversity analysis, variety purity and identity detection, and DNA fingerprint database construction in maize. Maize was one of the first crops used in the development of simple sequence repeat (SSR) primers, initially developed from genomic or microsatellite enriched libraries, a costly and time-consuming process[1]. The large-scale development of SSR primers has benefited from the implementation of the Maize Genome Sequencing project and the publication of a large number of DNA sequence databases[2-4]. More than 2,000 SSR primers have been developed in maize. Their use, however, is limited by great variability among markers in polymorphism, amplification quality, whether codominant inheritance, number of amplification sites, and location and distribution in the genome, and should be thoroughly assessed to determine their usefulness. A lack of systematic evaluations of SSR primers in maize has hampered their widespread use.

2. A high throughput, automated detection platform is helpful for large-scale use of SSR primers. Compared to other markers such as AFLP, RAPD and ISSR, the throughput of SSR primer analysis is relatively low as it yields genotype information at only one site per primer. To increase SSR analysis information content, multiplex PCR has been explored in some crops. Multiplex PCR combined with automated fluorescence detection may significantly increase SSR analysis throughput and reduce costs associated with large-scale SSR genotyping. The few recently published studies investigating multiplex PCR in maize, however, were limited by the fact that the constructed multiplex primer combinations were research-specific, i.e. not universal. Additionally, the number of primers used in a combination was low, typically ranging from two to four primers[5-7].

3. The purpose of the current study was to thoroughly screen and assess published maize SSR primers to establish a set of universal core SSR primers in maize. Core primers were assessed and redesigned to construct a high throughput multiplex PCR amplification system using automated capillary electrophoresis fluorescence detection.

Materials and methods

(a) Materials

4. Representative maize inbred lines (n=15) and hybrids (n=15) from national regional testing in China were used for primer screening, comparison between newly designed and original primers and laboratory verification of the multiplex PCR combination system. Inbred lines were 'Huangzhao4', 'Chang7-2', 'Jing24', 'Jing89', '87-1', 'X178', 'Qi319', 'Jing501', 'Dan341', 'Tai411', 'HuangC', 'SW1611', 'B73', 'Mo17' and 'E28'. Hybrids were 'Ben2204', 'Changcheng303', 'Q2104', 'Danke1', 'Aoshi3111', 'Dong0201', 'Hengfeng1', 'M3309', 'ND5598', 'CM10', 'ND311', 'Dong4326', 'Jingpin202', 'Tangyu16' and 'DH3702'. The inbred lines were purified through continuous selfing; hybrids with the greatest genetic variation between lines and the minimum genetic variation within lines were selected using morphological identification in the field and uniformity detection in the laboratory [8].

(b) Screening of primer polymorphism

(i) Reference literature screening

5. Eight hundred total candidate primers were selected based on the amplification of more than 1,900 SSR primers found in MaizeGDB (http://www.maizegdb.org/ssr.php), location of primers on chromosomes, as well as an extensive literature review[9-17]. Primer inclusion criteria were as follows:

(1) polymorphism: the number of alleles (amplification band number)≥3 or polymorphism information content (PIC) value ≥0.65;
(2) banding pattern distribution: distinct band size with no intensive or locally intensive band distribution;
(3) chromosome distribution location: primers located exclusively on the chromosomes and all candidate primers nearly evenly distributed throughout the genome. Primer amplification efficiency was not a criterion for candidate primer selection.

(ii) Laboratory rescreening

6. A total of 800 candidate primers were re-screened for polymorphism utilizing 15 inbred lines. Eligible candidate primers were further assessed for heterozygosity at the primer site using 15 hybrids. Primers were assessed based on both the reference literature and laboratory results; 500 highly polymorphic candidate primers were identified.

(c) Candidate primer chromosome distribution and determination of a set of universal core primers

7. The Maize molecular genetic map IBM2 2004 neighbors frame covering most SSR primer sites was downloaded from MaizeGDB and used to identify the location of the highly polymorphic candidate primers. The framework was plotted using a Marco program compiled by the China Agricultural University.

8. The location of primers on the integrated map, as well as the principle of even distribution were used to select 100 primers, 10 per chromosome, as a set of universal core primers.

(d) Primer redesign and construction of a universal PCR amplification program

9. To facilitate multiplex PCR combination, the 100 candidate core primers were redesigned according to uniform requirements. We used NCBI (http://www.ncbi.nlm.nih.gov), MaizeGDB (http://www.maizegdb.org) and PlantGDB (http://www.plantgdb.org) to retrieve the original genomic sequences corresponding to candidate primer loci. These were then redesigned and assessed using Primer Premier 5.0 and Oligo 6.22. Nomenclature of newly designed primers at the same polymorphic site was specified as 'name of the original primer + code of the designer + serial number'.

10. Given that the TM value for the newly designed primers was relatively high and the optimal annealing temperature was 68˚C, a two-step amplification program was constructed: one cycle (94˚C, 5 min); 35 cycles (94˚C, 40 s; 68˚C, 80 s) followed by a last cycle (72˚C, 10 min).

11. The original and newly designed primers at the same sites were detected using the previously-mentioned amplification program; we investigate differences between original and newly designed primers in amplification banding patterns and amplification efficiency. If the location of the amplified bands was not as expected or the amplification efficiency was low, primers were removed or new primers redesigned. Eligible new primers (at the same microsatellite site) were used as candidate primers for further multiplex PCR combination.

(e) Construction of fluorescence multiplex PCR combination

(i) Design requirements for multiplex PCR combination primers

(1) Each primer set was comprised of 10 primers, one per chromosome.
(2) The combination pattern was 3+3+2+2. Each set of primers was divided into four groups based on fragment length. No overlap of amplification products of primers labeled with the same fluorescence was allowed. Primers of the same group were labeled with the same fluorescence (FAM, NED, PET, or VIC).
(3) Interaction between sets of primers was as weak as possible, $\triangle G<13$.

(ii) Construction of ten-plex PCR combination.

12. According to the location of candidate primers on maize chromosomes, 10 primers, one primer per chromosome, were selected. The expected location of the primer was roughly determined based on the distribution characteristics of primer amplification banding patterns. Primers were designed and evaluated with Primer Premier 5.0 and Oligo 6.22. Multiplex PCR assessment of primers was conducted with Primer Premier 5.0 and by experiment to exclude primer combinations that could potentially result in significant primer interactions.

13. PCR amplification was similar to that of new single primers. Electrophoresis detection were conducted on a DNA analyser ABI3730XL. Fluorescent primers were synthesized with reagents from ABI company.

Results and analyses

(a) Determination of highly polymorphic primers

14. In total, 500 highly polymorphic primer sites were selected. Primer amplification efficiency was not considered during the first step of screening since primer amplification efficiency is directly associated with the quality of primer design and weak amplification efficiency can be improved through redesigning primers according to conservative sequences lateral to simple repeat sequences.

15. As none of the selected primers met all primer candidate criteria in some chromosome regions, primers meeting a compromised criterion were used in these regions, such as primer phi041. Primers were excluded based on low polymorphism (umc2259), intensive band distribution making statistical analysis difficult (nc030), or discrepancy in the chromosome location (umc1593, located at maize chromosome 3.05 and 7.03).

16. Hybrid amplification results were used to assess heterozygosity of primers, including heterozygosis rate of the primer loci and whether or not abnormal amplification occurred (three bands or asymmetric amplification of heterozygotic bands). Primers with low heterozygosis rates or abnormal amplification were excluded.

17. A total of 500 highly polymorphic candidate primers with high levels of polymorphism (mean PIC value, 0.74; mean number of alleles, 5.3) were identified (Table1) and suitable primers could then be selected from this primer set, significantly decreasing the screening work during early stages of relevant research.

Table 1: Polymorphism information and chromosome distribution of 500 highly polymorphic candidate primers

Chromosome	Length (cM)	Primer number	Mean density	PIC value (maximal; minimal; mean)	Mean number of alleles
1	1137	59	19.3	0.70;0.83;0.76	5.6
2	770	54	14.3	0.64;0.78;0.73	5.0
3	842	58	14.5	0.63;0.83;0.74	5.3
4	804	50	16.1	0.64;0.84;0.74	5.1
5	676	53	12.8	0.64;0.81;0.72	5.5
6	579	46	12.6	0.66;0.84;0.77	5.7
7	644	52	12.4	0.60;0.77;0.70	4.4
8	632	47	13.4	0.60;0.75;0.75	5.8
9	805	46	17.5	0.64;0.84;0.73	5.9
10	533	35	15.2	0.64;0.81;0.73	5.0
all	7422	500	14.8	0.64;0.81;0.74	5.3

(b) Determination of a set of universal core primer loci

18. A genetic map (named maize HP-SSR composite map) was created to adjust the list of candidate primers according to the location of primers on the chromosomes (Fig. 1). Of the

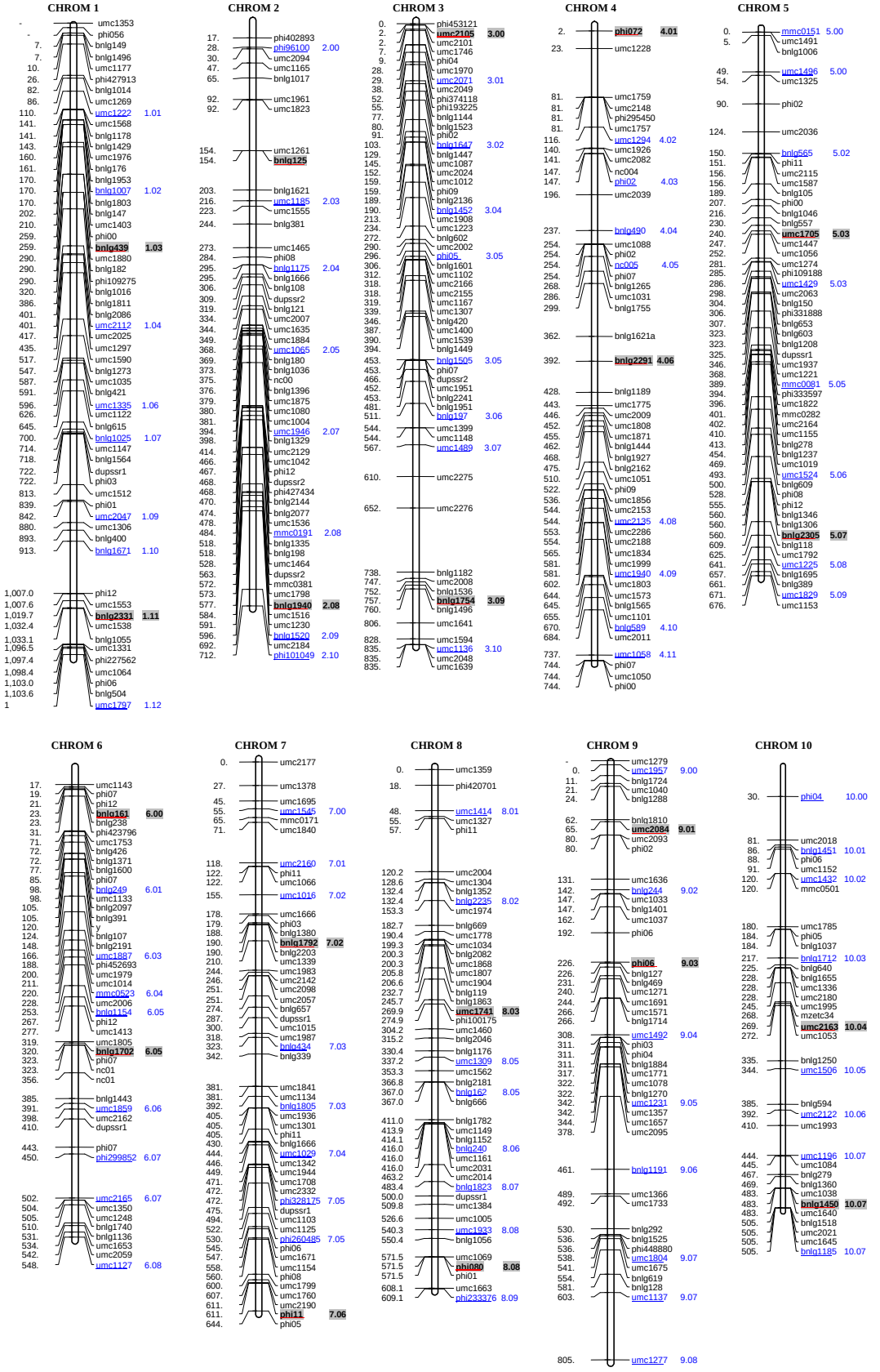
500 highly polymorphic candidate primers 460 were located on the IBM2 2004 neighbors frame map and the remaining 40 were integrated onto the IBM2 2004 neighbors frame map based on their location on other genetic maps. This map was used to demonstrate the chromosomal distribution of the candidate primers and to provide a reference for core primer selection.

19. The full length of the maize HP-SSR composite map was 7167 cM, covering 97% of IBM2 2004 neighbors frame map (full length, 7422 cM) with a mean primer density of 14.8 cM. Primer numbers varied from one chromosome to another (maximum, 59 on chromosome 1; minimum, 35 on chromosome 10). Additionally, five vacant regions with a length >70 cM were identified, including chromosome 1 (81.5 cM, 90.9 cM, 93.6 cM), chromosome 2 (95.8 cM), and chromosome 9 (201 cM). Vacancies resulted from the lack of developed primers or highly polymorphic primers. Multiple candidate primers were identified in certain chromosomal regions (for instance, 11 primers in bin 6.00 and bin 6.01), all of which met the primer candidate criteria and would be further screened by comparing their design difficulty level.

20. A total of 100 primers, 10 from each chromosome, were selected as a set of universal core primers (Fig. 1). This set of highly polymorphic and evenly distributed universal core primers may be preferentially used in general research, including variety certification, heterosis group division, genetic diversity analysis and gene mapping.

Fig.1: A maize HP composite map-distribution of 500 highly polymorphic candidate primers on maize chromosomes

Underline = 100 primers selected 10 from each chromosome. Bold = 20 primers used in the two ten-plex PCR compositions.



(c) Redesign of core primer sites and establishment of multiplex PCR combinations

21. Original genomic sequences corresponding to primer sites must be obtained for primer redesign. Of the 500 candidate primers, 432 original genomic sequences (390 from NCBI, 36 from Maize GDB and 6 from Plant GDB) were downloaded and primers were redesigned for these sites. No original genomic sequences for the remaining 68 primers were found, so additional effort is still needed to obtain their original genomic sequences by searching other websites for these primers or by sequencing positive clones registered in maize genomic BAC.

22. The construction of two sets of ten-plex PCR combinations used 20 of the 100 universal core primers, two from each chromosome (Table 2).

Table 2: Primers and combination modes of ten-plex PCR combination

Combination [a)]	BIN	Primer name [b)]	Amplification range	Primer sequence	Fluorescence labeled
1-1	4.01	phi072k4	413-433	GCTCGTCTCCTCCAGGTCAGG; CGTTGCCCATACATCATGCCTC	VIC
1-1	5.03	umc1705w1	273-330	GGAGGTCGTCAGATGGAGTTCG; CACGTACGGCAATGCAGACAAG	VIC
1-1	6.00	bnlg161k8	152-208	TCTCAGCTCCTGCTTATTGCTTTCG; GATGGATGGAGCATGAGCTTGC	VIC
1-2	9.03	phi065k9	399-419	CGCCTTCAAGAATATCCTTGTGCC ; GGACCCAGACCAGGTTCCACC	NED
1-2	2.03	bnlg125k1	219-327	GGGTACGGTTTCGTTTCCTTTGG ; TGCATCTAACAGCATCCCTTGAGC	NED
1-2	10.04	umc2163k5	154	GATGCAAGCGGGAATCTGAATC; CGACGAAATTGCTGGGGTTC	NED
1-3	1.03	bnlg439w1	319-385	AGTTGACATCGCCATCTTGGTGAC; GAACAAGCCCTTAGCGGGTTGTC	FAM
1-3	7.02	bnlg1792k8	192-250	CCCCAAAATTCCAGGTGCC ; CCTCGTCGTCTCCTACCAGAATG	FAM
1-4	8.08	phi080k15	203-233	TGAACCACCCGATGCAACTTG; TTGATGGGCACGATCTCGTAGTC	PET
1-4	3.09	bnlg1754w3	145	GGACGTCGGTACTGGCAATGG; CCACCACGCTGTCGTAGTGCTC	PET
2-1	1.11	bnlg2331k1	377-432	TTCCTTTCCTCGGTTAGGCAACAG; CCAAAGCTGCCAGTTCCTAGATGAG	PET
2-1	3.00	umc2105k3	286-330	GAAGGGCAATGAATAGAGCCATGAG; ATGGACTCTGTGCGACTTGTACCG	PET

2-1	7.06	phi116k3	150-171	AACTCCCTGCCGGGACTCCT; CGGCCATGGATGGGATACAAATAC	PET
2-2	2.08	bnlg1940k9	407	GGCTCGTTTAAGAACGGTTGATTGC; GCACTAGACGGCTGGCATTGG	NED
2-2	5.07	bnlg2305k4	292	CCCCTCTTCCTCAGCACCTTG; CGTCTTGTCTCCGTCCGTGTG	NED
2-2	9.01	umc2084w2	188-211	ACTGATCGCGACGAGTTAATTCAAAC; TACCGAAGAACAACGTCATTTCAGC	NED
2-3	10.07	bnlg1450k2	288-376	GCACTGAAATCTCCCATCATGTACG; TACAGCTCTTCTTGGCATCGTCG	FAM
2-3	8.03	umc1741k7	142-183	GCGCTTGGCATCTCCATGTATATC; GACCATCATCTTTCCCTCGTGC	FAM
2-4	4.06	bnlg2291k4	384	GCACACCCGTAGTAGCTGAGACTTG; CATAACCTTGCCTCCCAAACCC	VIC
2-4	6.05	bnlg1702k1	265	GATCCGCATTGTCAAATGACCAC; AGGACACGCCATCGTCATCA	VIC

a) two sets of ten-plex combination, each with four groups;

b) newly designed primers at the same microsatellite locus were named as “original primer name + designer code + serial number ”.

23. Chosen primers were redesigned, and each ten-plex PCR combination was constructed in the 3+3+2+2 mode, which has the following advantages: (a) only one ten-plex PCR amplification and one electrophoresis detection are needed to detect 10 primers using capillary electrophoresis and a five-color fluorescence detection system; and (b) even if a conventional denaturing polyacrylamide gel system were used, compared with single amplification, the detection efficiency is still increased 2.5-fold using only four PCR amplifications and four electrophoresis detections.

24. Size ranges of amplification fragments of the 14 primers were determined by the statistical results of DNA fingerprints of 96 maize inbred lines and 1300 maize hybrids (*data in press*). Size ranges of the remaining seven primers were not determined as their DNA fingerprint database has not been constructed, but the size of an allelic fragment of the primers was estimated by oligo 6.22. Nevertheless, polyacrylamide gel electrophoresis results demonstrated there was no overlap of amplification fragments in these combinations.

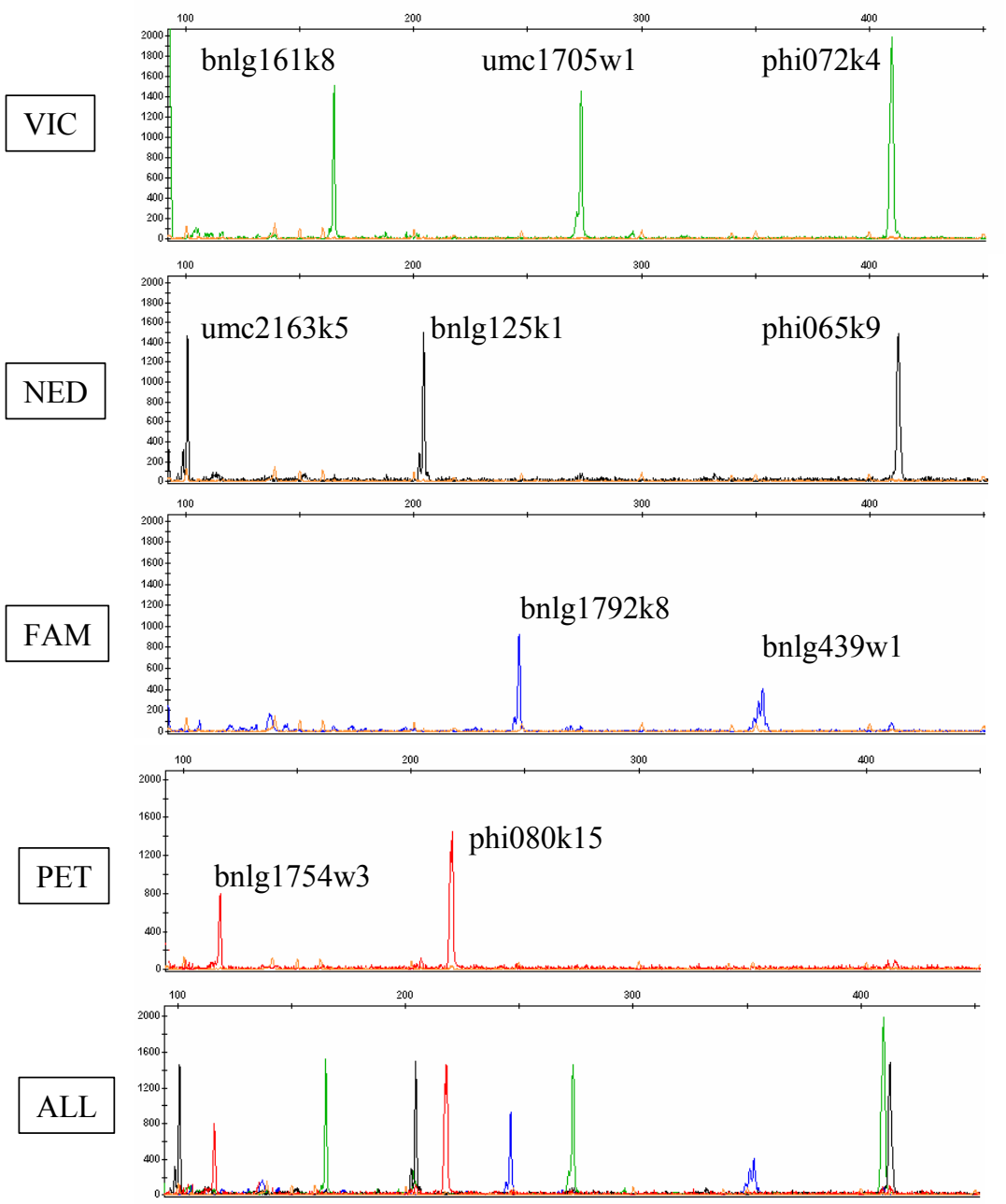
25. Compared with original primers, redesigned primers showed significant improvements in every parameter studied. Different primers had similar sequence features and were readily amplified under the same conditions (Table 3). Comparison of newly designed and original primers demonstrated that the amplification bands of the new ones were located as expected and the amplification efficiency was improved. Software assessments demonstrated that the maximal ΔG value was 11.4 while experimental assessments of primer interactions also demonstrated weak primer interactions（fig.2）.

Table 3 Comparison of original and newly designed primers

Primer name	Product length	TM value (U;L;P) [a)]	GC content (U ;L ;P)	initiation efficiency (U;P)	False initiation (U;P)	immer (U;L; UL)	Hair pin (U;L)	3'-terminal ΔG
bnlg439	201-253	79.2;77.9;82.2	50;41.4;41.5	492;483	91;131	4;8;5	3;3	7.9;8.5
bnlg439w1	319-385	75;75.4;85.6	50;56.5;47.9	469;522	117;99	3;3;4	3;3	6.1;6.7
bnlg125	323-422	66.6;67.9;83.7	47.6;43.5;43.7	377;388	81;146	2;2;2	0;0	6.7;6.6
bnlg125k1	219-327	74.9;75.1;83.9	52.2;50.0;45.7	465;445	31;64	3;4;3	3;3	8.8;8.2
bnlg1754	215	68.5;68.6;87.9	50.0;55.0;55.8	450;401	86;136	4;6;3	0;3	8.8;8.2
bnlg1754w3	145	75.4;75.9;88.0	61.9;63.6;56.6	447;431	88;58	6;3;3	0;3	8.4;8.2
phi072	142-162	78.6;78;78	50;42.9;34.5	533;484	103;113	6;6;4	3;0	6.4;9.7
phi072k4	413-433	74.3;;75.2;83.7	66.7;54.5;42.9	432;463	69;90	3;4;3	3;3	8.2;9.4
umc1705	57-114	70.7;76.4;82.9	50;50;49.5	411;480	193;81	4;6;3	0;3	6.4;6.4
umc1705w1	273-330	73.9;74.4;87.4	59.1;54.5;52.6	425;464	98;92	2;6;3	0;0	8.4;6.7
bnlg161	129-185	70.6;70.4;82.7	41.7;39.1;45.1	379;410	115;82	2;4;4	0;3	6.9;6.3
bnlg161k8	152-208	75.2;74.8;83.3	48.0;54.5;45.4	462;428	38;111	4;4;5	3;3	9.0;8.5
bnlg1792	113-171	68.0;68.4;81.9	50.0;45.0;44.4	431;444	120;97	4;2;4	0;0	6.4;7.0
bnlg1792k8	192-250	73.1;72.8;84.4	57.9;56.5;47.2	471;426	0;65	4;2;4	0;3	9.4;6.9
phi080	243-273	75.9;75.1;87.9	54.5;54.5;58	464;407	78;69	4;4;3	3;4	5.5;6.3
phi080k15	203-233	74.9;74.0;89.4	52.4;52.2;59.4	458;449	71;71	4;4;3	0;3	6.7;5.5
phi065	132-153	70.8;72; 83.6	50;50;47	379;415	33;76	4;4;3	3;3	5.5;6.7
phi065k9	399-419	75.2;75.7;88.4	50.0;66.7;54.2	497;436	94;88	4;3;3	4;3	9.4;9.4
umc2163	145	72.2;72.1;81.7	41.7;41.7;43.4	496;462	86;83	3;5;3	3;5	6.7;7.0
umc2163k5	154	73.6;73.4;82.5	50.0;55.0;44.8	495;462	83;63	4;4;4	3;3	6.6;7.9

[a)] U: forward primer sequence; L: reverse primer sequence; P: amplification products.

Fig.2: The first set of ten-plex PCR combinations detected in the ABI3730XL DNA sequencer



Discussion

(a) Polymorphism of SSR primer sites

26. Previous studies have demonstrated that the polymorphism level of SSR primers is associated with the number and the type of repeat units, the genomic region, the database source for primer development and the materials used for detection.

27. Sharopova *et al* [3] (2002) developed maize SSR primers and found that primer polymorphism was positively correlated with the number of repeat units. Of the 1,051 primers developed, polymorphism levels increased significantly with an increase in the number of repeat units. Masi *et al* [18](2003) studied bean SSR and demonstrated that the

polymorphism level was higher in repeats of two or three bases than in repeats of more than three bases, and was also higher in long repeat sequences compared to short ones.

28. Additional studies revealed differences primer density among various chromosomal regions. Sharopova *et al* [3](2002) demonstrated that there was higher SSR marker density in the centromere region than in the terminal region for chromosomes 2, 3, 5, 7, 8 and 10, contributing to differences in varied recombination rates in different chromosomal regions. Through sequence analysis of BAC clones of two maize centromeres, Nagaki *et al* [19] (2003) found mainly C-rich satellite sequences and centromere-specific retrotransposon sequences in the centromeres. Macaulay *et al*[20] (2001) studied SSR primers in barley and found uneven SSR distribution in the genome, with SSR marker density higher in the centromere region than in other regions. In the current study, a greater number of highly polymorphic primers were selected from regions adjacent to the centromere than from other regions. In addition, few or no primers were selected from the terminal regions of the long arm of chromosomes 2, 9 and the short arm of chromosomes 4, 5 and 10. SSR marker-rich and SSR marker-free sites were also identified in other regions, consistent with previous studies. Uneven distribution of SSR primers throughout the genome was an obstacle for the selection of a set of universal primers; as a consequence we reduced the stringency of the selection criteria for primer polymorphism in some regions with low primer density so that an even distribution of primers on chromosomes was ensured.

29. Studies in various crops have demonstrated that the polymorphism level was generally lower in primers developed from ESTs with unknown functions and genes with known functions compared to those developed from the entire genome [21,22]. That is, the polymorphism level of SSR primers was generally higher in non-coding regions than in coding regions. This may have been caused by a decrease in alleles in the breeding resource from selection for specific allelic combinations, resulting in decreases in the polymorphism level of linked SSR markers.

30. In addition, previous research has demonstrated that the evaluation of primer polymorphism levels is greatly influenced by the material type and the range of materials selected [12, 23]. Although the level of polymorphism for many primers is not high in cultivated varieties, the level may be higher in wild types or closely related species. The selection of highly polymorphic primers in this study was based on common maize varieties, making these primers significant to related studies on ordinary maize. Primers with low or no polymorphism in common maize may still be of use in future research investigating a wider variety of types.

(b) Primer selection criteria and multiplex PCR combination

31. A variety of selection criteria have been proposed for SSR primers in different species, such as potato[22], sunflower[24] barley[20], soybean[18], wheat[25], maize[26], rice[27], grape[28], rape[29] and tomato[30]. These criteria include high polymorphism, high amplification quality, even coverage of the entire genome and multiplex PCR amplification potential. In addition, multiplex PCR combination has been explored in other species, such as maize[5-7], sunflower[31], cotton[32], rice[33], and soybean[34]. However, it is still difficult to obtain a set of high-quality, universal core SSR primers and construct a multiplex PCR amplification system for a certain species, given the limited quantity and quality of primers that completely meet the aforementioned selection criteria.

32. Because the 1,900 primers published in Maize GDB were designed by different research institutes according to different criteria the quality of the designed primers and the suitable amplification conditions are variable. As a result, there are many limitations for multiplex PCR combination, such as differences in primer TM value, differences in the optimal annealing temperatures, and relatively narrow size ranges of amplification products of different primers. In spite of these limitations, strong primer interactions may exist resulting in greater difficulty in selecting proper multiplex combinations.

33. Previous research on multiplex PCR combination in maize (Gethi *et al*, 2002; Clerc *et al*, 2005; Wang *et al,* 2003) were limited by the fact that SSR primers were not systematically screened for polymorphism and the polymorphism level of some primers was relatively low. Moreover, the main considerations in those studies were the size range of amplification fragments and primer interactions; an even distribution of primers on chromosomes was not fully considered. Previously developed multiplex PCR primer combinations were consequently only suitable for specific studies and only duplex to tetraplex PCR combinations could be constructed, while stable, more than five-plex PCR combinations were not constructed.

34. In order to obtain a set of universal SSR core primers of maize, a stepwise screening strategy was adopted. First, polymorphism screening of the 1,900 SSR primer sites throughout the genome was performed and 500 highly polymorphic SSR primers of maize were obtained. Subsequently, 100 core primers were selected as a set of universal core primers according to the even distribution principle. The set of core primers were redesigned to improve the amplification quality so as to meet the requirements of ten-plex PCR primer combination. Each primer combination consisted of 10 primers with one primer per chromosome. Future research in our laboratory will construct 10 sets of ten-plex PCR primer combinations. Compared with previous studies, the present strategy was interested in that the screening of polymorphism and amplification quality was separated, and primer design and multiplex PCR combination was conducted simultaneously. The level of polymorphism is an inherent characteristic of microsatellite loci and is not associated with the quality of primer design. Only through thorough screening can highly polymorphic primer sites be selected in the entire genome of maize. Nevertheless, primer amplification quality was directly associated with the quality of primer design which can be improved by redesigning at the same microsatellite locus.

35. In this study, the ten-plex PCR combination established was based on highly polymorphic core primers, evenly distributed throughout the genome. The primers used were universal. For each set of ten-plex primer combinations, only one ten-plex PCR amplification and one ten-plex electrophoresis were were required using capillary electrophoresis and a five-color fluorescence system, resulting in high detection efficiency. Our data suggest the multiplex PCR primer combinations obtained in the present study could be widely applied.

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