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ASSESSMENT OF THE UNIFORMITY OF CHINESE MAIZE VARIETIES BY A SET OF SSR MARKERS

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ASSESSMENT OF THE UNIFORMITY OF CHINESE MAIZE VARIETIES BY A SET OF SSR MARKERS

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Abstract

The aim of this study was to evaluate the availabilityof SSR markers for variety uniformity testing in maize and to try to bring forward a standard for variety uniformity testing using SSR markers. For this purpose, a set of 13 SSR primers were used to analyze 213 maize varieties with 20 individuals per variety from national regional trial in 2004, containing 16 duplicates. One SSR marker per chromosome was selected, except that there were 2 markers located on chromosome 6 and 3 markers on chromosome 8.

Seven different types of variation between normal and anomalous bands were found with the frequency of each type varied. Uniformity distribution was quite uneven among different varieties and different SSR loci; a comparison of uniformity between SSR loci at the same chromosome and at different chromosomes was conducted and the results showed that there was correlation between different loci of the same chromosome, which was especially high at the same chromosome bin because of linkage between the loci. After taking account of both uniformity value at single SSR loci and average uniformity value at all the SSR loci, a standard of uniformity (divided into 5 levels) in maize variety was suggested, which was different from that currently used in morphological uniformity testing. We also compared three sampling methods - mixed sampling, single individual sampling and multiple individual sampling. For variety identification work, multiple individual sampling was recommended.

Introduction

1. In countries with Plant Breeders' Rights (PBR), a new variety must be Distinct from all other varieties, and also sufficiently Uniform and Stable with respect to the characteristics used to demonstrate distinctness, in short DUS. Varieties that meet these DUS criteria are eligible for the granting of PBR. The UPOV (International Union for the Protection of new Varieties of Plants) suggests a set of characteristics for DUS testing, which are traditionally morphological characters. Because many of them are multigenic, quantitative or continuous characters and easily altered by environmental conditions, field test in multiple locations and years is necessary. Moreover, both the reduced genetic variability and the increased number of new varieties are encouraging PVPOs around the world (Plant Variety Protection Offices) to find new approaches to DUS testing which keep the quality and scope of protection under PBR schemes.

2. Molecular markers have been viewed as additional tools for variety identification, which have many advantages over morphological characters, e.g. their independence from environmental influences, generally high polymorphism and almost unlimited availability. Molecular markers which have been employed in variety identification include RFLPs (Dillmann et al. 1997), RAPDs (Zhao et al. 1999), AFLPs (Roldan-Ruiz et al. 2000), ISSRs (Talhinhas et al. 2003) and SSRs (James et al. 2002; Heckenberger et al. 2002). SSR markers, also called microsatellites, have proven to be particularly useful in maize because of their codominance, high polymorphism, ready availability (more than 1800 primer pairs have been published in Maize Database) and ease of automation (Pejic et al. 1998; Matsuoka et al. 2002; Jones et al. 1997).

3. Uniformity is important in the DUS testing and PBR contexts. One of the reasons that SSRs or other molecular markers are not currently accepted for DUS testing by the registration authorities of most countries is the lack of information about the uniformity of varieties with regards to molecular markers. Although research on the application of SSRs and other molecular markers has increased greatly nowadays, only a few reports are related to the question of variety uniformity and molecular markers. Cooke et al.(2003) analyzed the uniformity of 45 wheat varieties at between 7-9 different SSR loci and 10 tomato varieties at six SSR loci, the number of individuals screened was 20 or 38 individuals per wheat variety and 36 individuals per tomato variety. Their results showed that there was variation both between varieties and between SSRs in the degree of uniformity observed. Djé et al. (2000) analyzed 25 sorghum accessions using 5 SSR loci and 10 individuals per accessions. They observed high genetic polymorphism of SSR loci within accessions as more than two-thirds of loci were polymorphic for a given accession and none of the accessions was fixed at all five loci. However, the number of varieties and primer loci analyzed in these reports is relatively low, which means they are unlikely to give a thorough and complete analysis of the uniformity of varieties at the molecular level. Additionally, there have been no such reports in maize varieties, especially maize hybrids so far.

4. Hence, the main objectives of this study were to undertake a comprehensive evaluation of the availability of SSR markers for uniformity testing in maize varieties and to attempt to develop a standard for uniformity testing by SSR markers.

Materials and methods

Plant material

5. Seeds of the maize varieties were obtained from six groups of Chinese National Regional Trials in 2004, including groups from Northeast Early-mature region, Northeast & North region, Huanghuaihai region, Jingjintang region, Northwest region and Extremely Early-mature region. In total, 213 varieties were tested, of which 16 varieties were duplicated. The serial number K1-K197 represented accessions without duplication, and K198-K213 for duplicated accessions. All of the varieties were single hybrids. Table 1 lists the number of varieties from different groups before omission of duplicates. These varieties were basically representative of Chinese newest maize resources.

Table 1: Regional distribution of the investigated maize varieties

Region (group)	No. of accessions[a]	No. of subgroup
Northeast Early-mature	32	2
Northeast & North	70	4
Huanghuaihai	66	4
Jingjintang	17	1
Northwest	12	1
Extremely Early-mature	16	1
total	213	13

[a] No. of accessions while not omitting duplicates

DNA isolation and analysis

6. DNA was extracted as described in Guo et al. (1997). The standard PCR amplification reactions were preformed in a 20μl reaction volume containing 0.25 μM of each primer, 0.16 mM of each dNTP, 2.5 mM of $MgCl_2$, 1 unit of Taq polymerase, 1×PCR buffer (50mM KCl, 10mM Tris-HCl, pH 8.3) and approximately 20ng of genomic DNA. PCR protocol was as follows: one cycle of 94°C for 5 min; 35 cycles of 94°C for 40s, 60°C for 35s and 72°C for 45s; after the 35 cycles, one cycle of 72°C for 10 min was added. PCR products were detected by 4.5% denaturing polyacrylamide electrophoresis and fast Silver staining described in Wang et al (2004).

Selection of SSR primers

7. The SSR primers used are listed in Table 2. Some information can be found in the reference Wang et al (2003). The primers were selected on the basis that they had high degree of polymorphism, robust single-locus amplification, and easily scored products. The set of 10 SSR primers numbered P1-P10, which cover the whole genome with one SSR loci per chromosome, were used for uniformity analysis. Another three SSR loci were chosen for comparing correlation between linkage loci, one (numbered P6-1) was on the same chromosome bin with P6, two (numbered P8-1 and P8-2) were on the same chromosome with P8.

Table 2 Description of maize SSR markers employed

No.	**Locus**	**Bin**	**No. of alleles**	**PIC**
P1	bnlg439	1.03	6	0.58
P2	bnlg125	2.02-2.03	5	0.72
P3	phi053	3.05	4	0.56
P4	phi072	4.01	4	0.66
P5	umc1822	5.05	5	0.7
P6	Bnlg161	6.00	8	0.85
P6-1	phi126	6.00	8	0.82
P7	umc1944	7.04	6	0.65
P8	bnlg162	8.05	6	0.68
P8-1	bnlg240	8.06	5	0.77
P8-2	Phi080	8.08	6	0.79
P9	phi065	9.03	4	0.52
P10	umc1084	10.07	6	0.72

Uniformity analysis

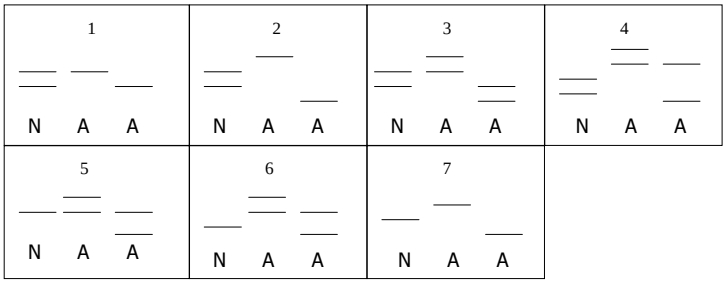
8. For each accession, 20 individual seeds were taken randomly and ten SSR primers (P1-P10) were used for uniformity analysis. The uniformity ratio of single SSR locus was calculated using the formula r =1-m/s, where m was the number of anomalous individuals, s was the total number of individuals in a variety detected. The average uniformity ratio of all the SSR loci was calculated using the formula $R=\sum r_i/t$, where r_i was the uniformity ratio of SSR locus Pi, t was the total loci analyzed.

Results

Different types of variation in uniformity analysis

9. Seven types of variation between normal and anomalous bands appeared in the uniformity identification of the 213 varieties (fig.1). The occurrence frequency varied greatly for most varieties and most SSR loci. The types where normal and anomalous bands shared one common allele (types 1, 3, 5) accounted for a higher proportion than those which shared no common alleles (types 2, 4, 6, 7). Although the anomalous bands in a variety/locus combination probably had several types, generally only one of them (called the main anomalous band) had a high proportion, that was, the sum of individuals with normal bands and with the main anomalous band accounted for the predominant proportion for most variety/locus combinations. In some cases, normal and anomalous bands were not easily distinguished. For instance, the accession K185 had three band types at loci P3, the number of individuals with each type was 7, 6, 6, respectively, making it difficult to determine which type was the normal type.

Fig.1 Band types in uniformity analysis. N represents the normal band and A represents the anomalous band.



Distribution character of variety uniformity among different varieties and different SSR loci

10. The uniformity data of the 213 accessions at ten SSR loci were analyzed and part results were listed in table 3. It manifested that: (1) Distribution of uniformity ratio was quite uneven for different varieties and different SSR loci. Some accessions had a high uniformity ratio at all loci (for instance, K1, K103); some accessions had a high uniformity ratio at most loci and a low ratio only at one or two loci (for instance, K76, K153); some accessions had a low uniformity ratio at most loci (for instance, K29, K185). (2) For many accessions, non-uniformity was detected at more than one locus, but the anomalous individuals among those loci were usually different (data not listed), for instance, in the 20 individuals of K75, the anomalous individuals at P1 were sample 8, at P3 were sample 7, 15, 17 and 20, at P10

were sample 9, 10, 18. Of course, there were also a few accessions whose anomalous individuals were common at all non-uniformity loci, for instance, the anomalous individuals of K197 were sample 14 and 17 at all the 7 non-uniformity loci. (3) For different SSR loci, the amount of accessions distributed in different uniformity ratio interval varied greatly (table 4). The highest number of accessions were detected in r=1 for all of the ten loci, varied from 180 at P7 to 129 at P8. Only at five loci (P1, P2, P3, P5, P7), accessions in r< 0.5 were detected and only at loci P3, one accession in r=0.35 was detected. There were no accessions detected in r< 0.35 for all of the loci. (4) The number of non-uniformity loci in the different individuals of each accession varied greatly from 0 to 7 and the number of non-uniformity individuals of each accession also varied much from 0 to 20 (table 5). For instance, there were 16 non-uniformity individuals in K8 and the number of non-uniformity loci was 1 (9 individuals), 2 (4 individuals), 3 (2 individuals) and 7(1 individual).

11. Furthermore, we also compared the two sets of SSR loci, one set (P6 and P6-1) on the same chromosome 6 and the same bin 6.0, another set (P8, P8-1 and P8-2) on the same chromosome 8 but different bins 8.05, 8.06 and 8.08 (data not listed). 187 of the 213 accessions had the same uniformity ratio between P6 and P6-1. More than 70% of the accessions shared the same or approximate uniformity ratio among P8, P8-1 and P8-2, the similarity among which were much higher than that among P1-P10. It indicated that there was correlation between the different loci of the same chromosome, especially high at the same chromosome bin because of linkage between the loci. We might easily deduce that the SSR loci for uniformity detection should be evenly distributed so as to avoid or decrease the correlation between them.

Table 3 Uniformity ratio of the maize accessions (part results)

Accession No.	r[a]										R[b]	level
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10		
K1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.000	1
K2	0.95	1.00	1.00	1.00	0.95	0.95	1.00	0.95	0.95	1.00	0.975	1
K8	0.90	0.55	0.95	0.80	0.95	0.95	1.00	0.55	0.84	1.00	0.849	5
K12	0.95	1.00	1.00	0.95	1.00	0.90	1.00	0.58	0.95	1.00	0.933	3
K27	1.00	0.80	0.60	0.95	1.00	1.00	0.95	0.90	1.00	1.00	0.920	3
K29	0.74	0.85	0.83	0.70	0.85	0.50	1.00	0.85	0.95	1.00	0.827	5
K33	1.00	1.00	0.77	1.00	0.75	1.00	1.00	0.67	1.00	1.00	0.919	3
K36	0.90	0.70	0.90	0.95	0.95	1.00	1.00	0.95	0.90	1.00	0.925	3
K50	1.00	0.81	0.78	1.00	0.75	0.94	0.90	0.80	1.00	0.94	0.892	4
K75	0.95	1.00	0.80	1.00	1.00	1.00	1.00	1.00	1.00	0.85	0.960	2
K76	1.00	1.00	1.00	1.00	1.00	0.60	1.00	1.00	1.00	1.00	0.960	2
Accession No.	r[a]										R[b]	level
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10		
K88	0.85	1.00	0.80	1.00	1.00	1.00	1.00	1.00	1.00	0.95	0.960	2
K103	1.00	0.95	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.995	1
K132	0.60	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.70	0.930	3
K147	1.00	0.88	0.70	1.00	1.00	0.75	1.00	1.00	0.55	1.00	0.888	4
K151	0.55	1.00	1.00	1.00	0.95	1.00	0.90	0.55	1.00	1.00	0.895	3
K153	1.00	0.47	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.947	3
K186	0.90	0.79	0.85	0.95	0.47	0.70	0.85	0.95	0.90	1.00	0.836	5
K193	0.55	1.00	1.00	1.00	1.00	1.00	0.75	1.00	1.00	1.00	0.930	3
K197	0.90	0.90	0.90	1.00	1.00	0.90	0.95	1.00	0.90	0.90	0.935	3

[a] uniformity ratio of single SSR locus; [b] average uniformity ratio of all the ten SSR loci

Table 4 Amount of accessions distributed in different SSR loci and different uniformity ratio interval

SSR loci	Different uniformity ratio interval													
	1	0.95	0.9	0.85	0.8	0.75	0.7	0.65	0.6	0.55	0.5	0.45	0.4	0.35
P1	144	32	11	7	3	1	1	3	4	5	0	1	0	0
P2	147	28	6	10	7	4	3	1	1	1	2	1	1	0
P3	148	20	8	9	10	6	3	0	2	5	0	0	0	1
P4	164	27	7	3	6	2	1	1	0	1	0	0	0	0
P5	154	19	8	8	6	5	4	1	0	3	2	2	0	0
P6	143	29	12	7	2	6	5	0	3	2	3	0	0	0
P7	180	14	7	2	3	2	1	1	1	0	0	0	1	0
P8	129	32	18	7	8	4	1	1	3	7	2	0	0	0
P9	160	22	9	5	3	4	4	0	2	3	0	0	0	0
P10	159	22	14	8	5	0	2	0	1	1	0	0	0	0

Table 5 Distribution of individuals in different number of non-uniformity loci for each accession (part results)

Accession No.	Sum of non-uniformity loci							Non-standard individuals [a]	Standard individuals [b]	Proportion of standard individuals
	1	2	3	4	5	6	7			
K1	0	0	0	0	0	0	0	0	20	1.00
K2	1	0	0	1	0	0	0	2	18	0.90
K26	12	5	0	1	0	0	0	18	2	0.10
K55	7	0	0	0	0	0	0	7	13	0.65
K60	6	3	0	0	0	0	0	9	11	0.55
K108	4	0	0	0	0	0	0	4	16	0.80
K116	10	4	0	0	0	0	0	14	6	0.30
K120	8	4	0	0	0	1	0	13	7	0.35
K128	6	1	1	0	0	0	0	8	12	0.60
K147	12	5	0	0	0	0	0	17	3	0.15
K150	10	0	0	0	0	0	0	10	10	0.50
K183	6	2	0	0	1	1	1	11	9	0.45
K184	0	1	0	0	0	0	0	1	19	0.95
K185	3	7	4	5	0	1	0	20	0	0.00
K187	1	4	0	1	0	0	0	6	14	0.70

[a] individuals that had anomalous bands at one or more SSR loci; [b] individuals that had normal bands at all the ten SSR loci.

Standard for maize variety uniformity testing by SSR markers
Based on the analysis of the results above mentioned, the standard for evaluating uniformity of maize variety was suggested as follows:

(1) Uniformity at single SSR locus (r): high ($r \geqq 95\%$); middle ($85\% < r < 95\%$; low ($r \leqq 85\%$);

(2) Average uniformity at all of the ten SSR loci (R): high ($R \geqq 95\%$); middle ($85\% < R < 95\%$; low ($R \leqq 85\%$);

(3) After taking account of both the criteria above, a comprehensive standard for uniformity of maize variety were brought forward, in which the uniformity levels were divided into five (Table 6).

Table 6 Standard for evaluating uniformity level of maize varieties

Level	Standard
1 (highest)	(i) R≧99%; or (ii) r≧95% at all of the ten SSR loci
2 (higher)	95%≦R<99% and no more than 2 SSR loci with r≦85%
3 (middle)	(i) 95%≦R<99% and no less than 3 SSR loci with r≦85%; o≦ (ii) 90%≦R<95%; or (iii)85%<R<90% and no more than 2 SSR loci with r≦85%
4 (lower)	85%<R<90% and 3-4 SSR loci with r≦85%
5 (lowest)	(i)R≦85%; or (ii) no less than 5 SSR loci with r≦85%

12. According to the standard, the results about uniformity levels of the 213 accessions were obtained and summarized in table 7. It showed that the uniformity levels varied among different groups, on the whole, the uniformity in Extremely Early-mature group was the lowest, the next lowest in the Northeast Early-mature group.

Table 7 Summary of uniformity levels of each group of the 213 accessions

Group	Level					Sum on group
	1	2	3	4	5	
Northeast Early-mature group	10(31%)	12(38%)	5(16%)	2(6%)	3(9%)	32
Northeast & North group	17(24%)	34(49%)	15(21%)	4(6%)	0(0%)	70
Huanghuaihai group	30(45%)	19(29%)	12(18%)	4(6%)	1(2%)	66
Jingjintang group	10(59%)	3(18%)	3(18%)	1(6%)	0(0%)	17
Northwest group	3(25%)	6(50%)	3(25%)	0(0%)	0(0%)	12
Extremely Early-mature group	5(31%)	3(19%)	4(25%)	1(6%)	3(19%)	16
Sum on level	75(35%)	77(36%)	42(20%)	12(6%)	7(3%)	213

Discussion

Comparison of different sampling method

13. There are three main sampling methods for variety detection, including mixed sampling (mixing several individuals into one sample), single individual sampling (selecting one individual with 'typical' characters of a variety) and multiple individual sampling (selecting several individual with typical characters of a variety). The mixed sampling and individual sampling methods have been commonly used in many studies (Röder et al. 2002; Bredemeijer et al. 2002; Gethi et al. 2002), while multiple individual sampling was rarely used because of its much greater workload. However, either mixed sampling or single individual sampling might lead to a wrong result unless the varieties have high uniformity. How to balance efficiency and accuracy was a relevant question. In our study, mixed sampling and single individual sampling were compared. One or two specific bands were amplified when individual DNA were used, there rarely appeared more than two specific bands in a single reaction (fig.1). When bulked DNA of five seed individuals of an accession

was used for amplification, several bands different from individual DNA at some SSR loci often appeared. The probably reason was as follows: for a maize variety with low level of uniformity, if DNA of the individuals with different bands was mixed, the amplified bands may be different from that of individuals. So it would not be appropriate for distinctness testing of maize varieties to use mixed DNA of several individuals, especially when the maize variety had a low level of uniformity.

14. Single individual sampling and multiple individual sampling were also compared. The quantity distribution of standard individuals that had normal bands at all SSR loci was analyzed (Table 5 & Table 8). There were only 28 varieties (accounting for 14% of all the 197 no-duplicated accessions) whose proportion of standard individuals was 100%. 116 varieties (accounting for 59%) whose proportion of standard individuals was ≤80%., in which 47 varieties (accounting for 25%) whose proportion of standard individuals was ≤25%. Furthermore, the individuals with normal bands at the 10 SSR loci still might have anomalous bands in the new locus if more primers were used. It was difficult to find true standard individuals of a variety. Therefore, selecting only one individual of a variety easily led to an erroneous DNA fingerprint result.

15. On the whole, there was poor accuracy in variety distinctness testing when using one individual or mixed several individuals. In order to reduce error, multiple individual sampling is recommended.

Table 8 Varieties' quantity distribution in different proportion of standard individuals

Proportion of standard individuals	1	0.95	0.9	0.85	0.8	0.75	0.7	0.65	0.6	0.55	0.5
Varieties' number and proportion	28 (0.14)	23 (0.12)	20 (0.10)	10 (0.05)	13 (0.07)	15 (0.08)	8 (0.04)	12 (0.06)	13 (0.07)	8 (0.04)	13 (0.07)
Proportion of standard individuals	0.45	0.4	0.35	0.3	0.25	0.2	0.15	0.1	0.05	0	
Varieties' number and proportion	10 (0.05)	5 (0.03)	4 (0.02)	3 (0.02)	6 (0.03)	2 (0.01)	2 (0.01)	1 (0.005)	0 (0.00)	1 (0.005)	

Standard for uniformity testing by SSR markers

16. Why should the SSR loci used for uniformity testing be evenly distributed over the whole genome? Why should both uniformity at single locus and average uniformity at all of the SSR loci be considered when evaluating variety uniformity? Why is the suggested standard for uniformity testing by SSR markers different from the standard currently applied for DUS testing by morphological traits? The reasons are as follows:

(1) Only when the loci are evenly distributed could the linkage among the loci be reduced in most extent. Therefore, if 10 loci are used, one locus per chromosome is the best choice in maize.

(2) In contrast to morphological traits, breeders have not used a long-term selection for uniformity at SSR loci before. Residual variation at some loci may remain and result in low uniformity, which may not be reflected directly in morphological traits. Additionally, variations at SSR loci are neutral and are not affected by human selection, and they have no

direct linkage with morphological traits. Therefore, rational evaluation on variety uniformity would not be drawn if only based on uniformity at single locus. However, we may get a comprehensive evaluation to variety uniformity by randomly scanning evenly distributed SSR loci in the whole genome to detect both uniformity at single loci and average uniformity at all the loci.

(3) Compared with the suggested standard for uniformity by SSR markers, a higher level of morphological uniformity is required in Test Guidelines for DUS testing in maize, in which 3% population criteria and 95% acceptance probability are used, that is, no more than 3 untypical individuals are permitted in a sample of 40 individuals. Since SSR loci do not experience long-term selection for uniformity and some of them may have high mutation, the suggested standard by SSR markers is lower than that used in morphological traits. Of course, after gathering mass related information, we could fix a set of SSR markers for uniformity identification by gradually discarding SSR markers with high mutation. These markers may follow the same criteria as morphological traits at a single loci do now.

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