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Full Length Article

SNP Markers Potential Applied in DUS Testing of Maize

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Abstract

Single nucleotide polymorphisms (SNPs) are abundant and evenly distributed throughout the maize (*Zea mays* L.) genome. In this research, 348 SNPs were selected to explore the possibility of utilization in maize distinctiveness, uniformity and stability (DUS) testing. Mantel test indicated no missing information, by selecting the 384 SNPs. We examined 333 Chinese maize varieties with data from DUS test carried out for 42 characteristics and SNP genotyping data. The distance between varieties was calculated and we found no significant correlation between molecular and phenotypic distances. The phenotypic distances showed incredible differences, which are strongly influenced by environmental and statistical methods. It is necessary to set relative weightage for those characteristics which are easy to be affected by these factors in DUS testing. The application models for molecular markers in DUS testing should also go back to Model 1 (molecular characteristics as a predictor of traditional characteristics or gene specific markers) and look for more genetic linkage markers. © 2020 Friends Science Publishers

Keywords: Maize variety; DUS testing; SNP; Plant variety protection

Introduction

Maize (*Zea mays* L., Family Poaceae) is an important crop, and is grown around the world as a food, feed and fuel crop. Maize genotypes or varieties are vital for agricultural production. Breeding new and high yielding varieties need a huge investment and hard task (Yadav and Singh, 2010). The newly bred varieties have solved the livelihood of several million people. As well as the plant breeders' intellectual property rights have also been protected, and by commercial returns the breeding work by the breeders have been supported and encouraged. The International Union for the Protection of New Varieties of Plants (UPOV) is an intergovernmental organization. Encouraging innovation in plant breeding is the purpose of UPOV (Jones *et al.*, 2013). The uniqueness of a variety is established by tests for DUS. According to the UPOV treaty, the DUS test is a necessary condition to grant Plant Breeders Rights (PBR). At present, DUS testing is mainly based on assessment of morphological and physiological characters about new and existing varieties. The traditional method of DUS testing is time-consuming and expensive. It requires large areas of land and skilled personnel and often needs subjective decisions. Moreover, many of the morphological characters are multigenic or quantitative, and their expression is affected by environmental factors (Kuang *et al.*, 2016).

Maize is the first crop with maximum planting area and gives highest total yield in the People's Republic of China. The crop plays an important role in the agricultural economic structure of the country. From 1972 to 2013, 6,291 maize varieties were approved by national and provincial governments (Yang *et al.*, 2014). By 2019, the number of maize varieties had grown sharply to more than 40,000. Due to the extensive size of the maize varieties, DUS testing needed a rapid and highly reliable method of plant variety identification system. This is likely to reduce cost and promote efficiency. With the development of high throughput genotyping technology, molecular marker technology has been widely used. DNA markers were implicated in plant breeding, such as marker-trait association, genomic prediction and selection, germplasm characterization and seed purity monitoring (Tian *et al.*, 2015). DNA marker techniques are also used to protect intellectual property of varieties. The UPOV guidelines allow for the use of markers as proxy for traits so long as there is a reliable marker-trait association. This could serve marker data as a surrogate for specific phenotypic characteristics including disease resistance (UPOV, TGP/15 "Guidance on the Use of Biochemical and Molecular Markers in the Examination of Distinctness, Uniformity and Stability (DUS)") in crop plants (Maton *et al.*, 2014; Yan-Fang *et al.*, 2016). The UPOV has suggested three application models for molecular markers in variety registration (UPOV document INF/18/1, 2011). These are: 1) molecular characteristics as a predictor of traditional characteristics *i.e.*, use of molecular characteristics which are directly linked to traditional characteristics (gene specific

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markers), 2) calibration of threshold levels for molecular characteristics against the minimum distance in traditional characteristics and 3) the development of a new system.

Many markers are available in maize but for most of these markers, the genetic linkage with other agronomical or morphological characteristics is unknown. There are not enough fully diagnostic markers to adjudge distinctness in the UPOV Model 1. So, the researchers focused on the UPOV Model 2, which has been investigated in grapevine, maize, oilseed rape and in wheat and barley crops (Jones and Mackay, 2015). But the outcomes of these investigations were unsatisfactory. UPOV's Biochemical and Molecular Techniques (BMT) Working Group has established a distinctness threshold in maize. Its work was based on the analysis of the phenotypic data of a set of varieties that have been identified as unique lines (Maton *et al.*, 2014). As shown in the Fig. 1, the threshold for the molecular distances is 0.2 and for morphological data it is two traits. Thus, a secure system is implemented to ensure that the varieties sustain certain difference, which is enough to make an estimate without comparison in the field.

Among many DNA markers, the SNP marker became popular. It is a broadly sampled genome having high quality and publicly available and collectively has a high capability to distinguish different varieties. So far, a lot of maize SNP markers have been developed (Jones *et al.*, 2009; Chai *et al.*, 2012; Mammadov *et al.*, 2012; Unterseer *et al.*, 2014; Tian *et al.*, 2015; Xu *et al.*, 2017); in addition, high-density platforms such as Affymetrix and Infinium platforms have also been developed. These techniques have been triumphantly applied to the mapping of genome-wide association and quantitative trait locus (QTL) in maize (Zwonitzer *et al.*, 2011; Li *et al.*, 2012). In addition, those techniques are also used to investigate the genetic structure of maize germplasm (Yan *et al.*, 2010; Semagn *et al.*, 2012; Wu *et al.*, 2014; Hao *et al.*, 2015; Zhou *et al.*, 2016).

In the present research, using Chinese maize varieties, we selected and assessed SNP loci for the analysis of maize DNA fingerprinting from the maize SNP50 array. We also examined the applicability of this SNP array to maize DUS testing based on genotypic and phenotypic data analysis of 333 varieties. The feasibility of UPOV Model 2 in the DUS testing of Chinese maize was also evaluated.

Materials and Methods

Materials and SNP Genotyping

A total of 449 samples were selected to evaluate the SNP array, including 341 hybrids and 108 inbred lines. Total genomic DNA was extracted from young tissues of each cultivar using the CTAB procedure according to Wang *et al*. (2011). The maize SNP50 BeadChip was used to genotype DNA samples, which contained 56,110 SNP loci (Ganal *et al.*, 2011).

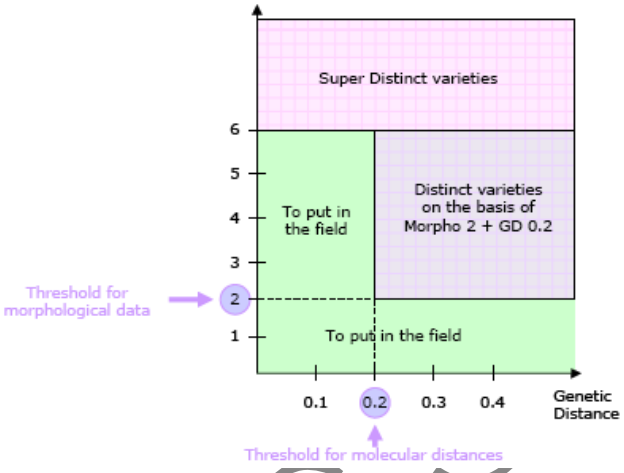

Fig. 1: Application model proposed by the BMT Working Group, it suggests the distinctness threshold for morphological and molecular distances

DUS Phenotyping

The hybrids were grown in the field situated at the variety testing division of the Kunming DUS station. A total of 42 agronomic traits were measured in each variety. These traits were the ones usually recommended by UPOV. They are also used by the national authorities in China for assessing DUS (Table 1). Following screening, varieties with missing phenotypic data of more than 10% and genotypic data of more than 20% were excluded. Finally, 333 varieties with both phenotypic and genotypic data were selected for the present study. These hybrid samples are commercial varieties that have been widely promoted in large areas.

SNP Data Analysis

Firstly, SNP rejecting was performed using parameters like Genome Studio Gen Train scores (<0.6), call rate (<95%), monomorphic, missing value (>5%) and minor allele frequency (MAF <0.05). From the 56,110 SNPs, a total of 40,890 (73%) SNPs were designated as candidate loci. Secondly, deleting the SNPs with MAF values under 0.2 and copy numbers greater than or equal to 2. The remaining 5532 SNPs were used for further analysis. The statistical values, minor allele frequency (MAF), gene diversity, heterozygosity and Polymorphic Information Content (PIC) for each SNP were estimated using PowerMarker v3.25 (Liu and Muse, 2005). Thirdly, in order to minimize number of SNPs to cut costs, 384 SNPs were selected based on the quality of the flanked regions, with PIC value more than 0.35 and even distribution on each chromosome.

Statistical Analysis

We calculated the genotypic and characteristic phenotypic distances. The genetic distances across the genotypes and UPGMA tree were calculated based on Nei's genetic distance.

Table 1: Characteristics of maize used in DUS testing

No.	Characteristics	No.	Characteristics
1	First leaf: anthocyanin coloration of sheath	20	Stem: anthocyanin coloration of brace roots
2	First leaf: shape of apex	21	Leaf: width of blade
3	Tassel: time of anthesis	22	Foliage: intensity of green color
4	Ear: time of silk emergence	23	Leaf: anthocyanin coloration of sheath
5	Upside leaf: angle between blade and stem	24	Plant: height of insertion of peduncle of upper ear
6	Lower leaf: angle between blade and stem	25	Plant: length
7	Leaf: curvature of blade	26	Plant: ratio height of insertion of peduncle of upper ear to plant length
8	Tassel: anthocyanin coloration at base of glume	27	Peduncle: length
9	Tassel: anthocyanin coloration of glumes excluding base	38	Ear: length
10	Tassel: anthocyanin coloration of anthers	29	Ear: diameter
11	Tassel: density of spikelets	30	Ear: number of rows of grain
12	Tassel: angle between main axis and lateral branches	31	Ear: shape
13	Tassel: curvature of lateral branches	32	Ear: number of colors of grains
14	Ear: anthocyanin coloration of silks	33	Ear: type of grain
15	Tassel: length of main axis above lowest lateral branch	34	Ear: color of top of grain
16	Tassel: length of main axis above highest lateral branch	35	Ear: color of dorsal side of grain
17	Tassel: number of primary lateral branches	36	Ear: shap of grain
18	Tassel: length of lateral branch	37	Ear: anthocyanin coloration of glumes of cob
19	Stem: degree of zig-zag		

For the purpose PowerMarker v3.25 was used and the UPGMA tree was visualized using MEGA5. For phenotypic data, Euclidean distance was calculated by the statistical software package R v2.9.0., the matrix of all features was standardized.

Results

Selection SNPs for Corn Variety DNA Signature Development

A total of 56,110 SNPs were removed having a Genome Studio Gen Train scores <0.6, call rate <95%, MAF <0.05 leaving 40,890 SNPs for downstream analysis. Out of these, 27.10% (11,083) SNPs showed >5% missing values. In addition, 24,275 (26.39%) SNPs with a MAF of <0.20 were also present. These SNPs were removed from the dataset and the remaining 5,532 (13.52%) SNPs were included for further analysis. From this large amount of markers, 384 SNP markers were selected. This singled out each of the 449 genotypes used in the present study. The selection factors included the minor allele frequency score, the quality of the flanked regions and their distribution on the genome (Fig. 2).

For the total 5,532 SNPs, the allele frequency of SNPs ranged from 0.20–0.57. The gene diversity across 5,532 loci ranged from 0.4282–0.5 with an average 0.4876. The mean polymorphism information content (PIC) value of SNPs was 0.3876 with a range of 0.3365–0.375. The allele frequency of SNPs ranged from 0.20–0.57. The gene diversity across 384 loci ranged from 0.4282–0.5 with an average 0.4856. The mean PIC value of SNPs was 0.3680 with a range of 0.3365–0.375. The correlation between 5532 and 384 SNPs is positive and statistically significant at genetic distance matrices ($r = 0.983$; $p<0.001$). The study revealed that no information is lost by selecting the 384 SNPs (Fig. 3).

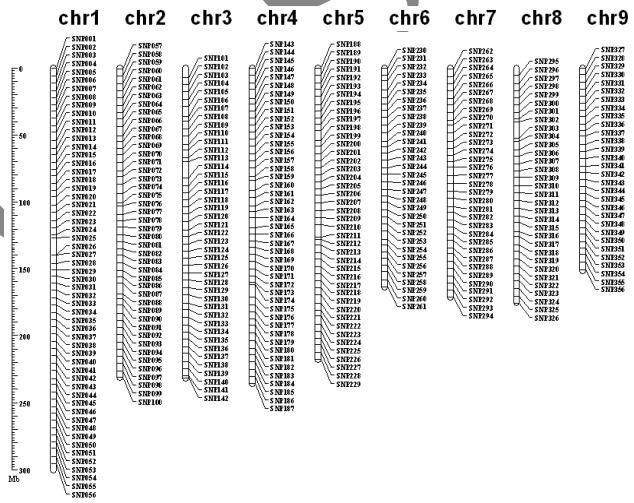

Fig. 2: Relative position based on the framework markers position of 384 SNP across the nine chromosomes

Genetic Distance and Diversity Analyses by SNP Marker

In the analysis of molecular fingerprints of 384 SNP markers, genetic distances coefficients among 333 varieties ranging from 0.0047–0.5287, with an average of 0.27. Of the varieties, 1.06, 10.90, 62.43, 24.67, 0.93 and 0.01% had genetic distances <0.1, 0.1–0.2, 0.2–0.3, 0.3–0.4, 0.4–0.5 and >0.5, respectively. Among them, 88.04% of the varieties had a genetic distances coefficient greater than 0.2 (Fig. 4).

The general clusters of 333 varieties were analyzed adopting UPGMA tree based on Nei's genetic distance. Data showed that 384 SNP markers were effective enough to differentiate 333 commercial maize varieties. These markers were able to distinguish the cultivars derived from common lineage such as ZBS112, ZBS113 and ZBS114.

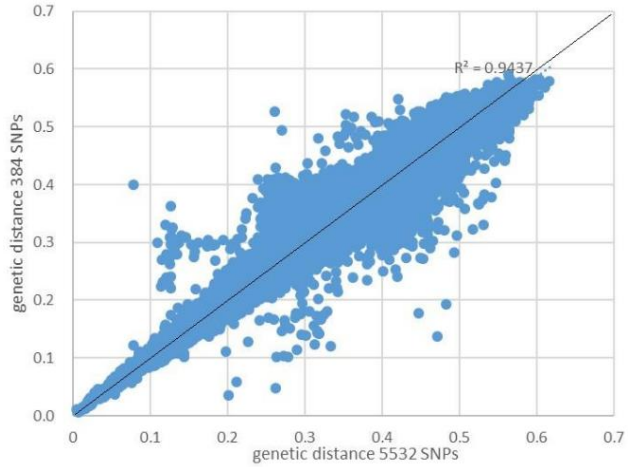

Fig. 3: Correlation analysis of genetic distance calculated from 5532 SNPs and 384 SNPs in 446 samples. Each point represents genetic distance between a pair of samples

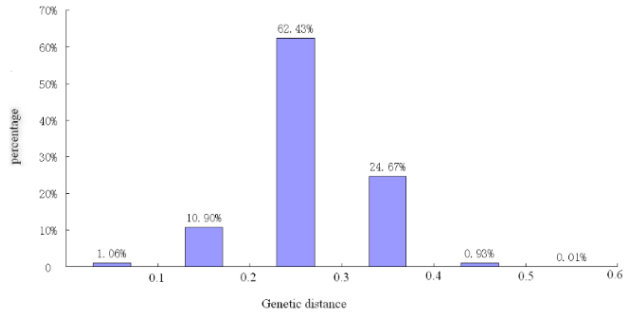

Fig. 4: Distribution of genetic distance coefficients for the 333 maize varieties

Phenotypic Analysis of DUS Traits

In 2017 and 2018, 333 varieties were planted side by side and tested with reference to the DUS test guideline. Significant differences were found between the 333 varieties except for two pairs of varieties (ZBS109 and ZBS449, ZBS204 and ZBS253). Among them, ZBS109 and ZBS449 are the same varieties from different sources and ZBS204 and ZBS253 have common lineage. The morphological distances ranged from 0.26–0.92, reflecting genetic variation.

Correlation between SNP Markers and Morphological Traits

The correspondence between the morphological traits and the SNPs markers genetic distances matrices was tested in a correlation analysis (Fig. 6). The correlation coefficient was 0.17. The morphological distances and molecular distances showed extremely low correlation. According to the model in Fig. 1, 12% of the varieties had a genetic distances coefficient greater than 0.2, but almost all varieties have >2 differences traits.

Fig. 5: UPGMA clustering analysis of 333 varieties by 384 SNPs markers

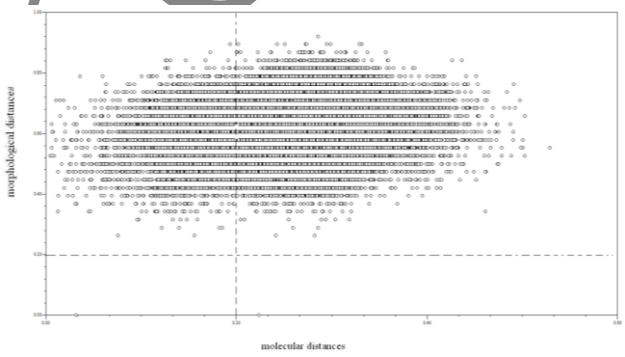

Fig. 6: Correlations between genetic distances and morphological distances

Discussion

It is particularly important to screen a set of SNP loci with high discriminatory ability, good stability and uniform distribution in fingerprint research based on SNP markers. In this study for the genotyping of the samples, the maize SNP50 Bead Chip containing 56,110 loci was used. According to the domestic and foreign screening requirements for SNP marker (Yang *et al.*, 2011; Blair *et al.*, 2013), this research filtered the SNP markers with genome studio gentrain scores less than 0.6. In the filtering process of the SNP markers call rate less than 95%, monomorphic, missing value greater than 5%, and MAF less than 0.2 were considered. The remaining 5,532 SNPs were left for further analysis. The SNP markers were then selected considering PIC value more than 0.35, and their even distribution on each chromosome.

Finally, 384 SNP loci were selected for maize DNA fingerprinting analysis. The diversity analysis indicated that there was no significant difference in the allele frequency, the gene diversity and the polymorphism information content between 5,532 SNPs and 384 SNPs. In addition, two genetic distance matrices calculated from 5,532 and 384 SNPs had obvious correlation. This has indicated that no information was lost by selecting the 384 SNPs. In the study of Wu *et al*. (2014), a total of 83,638 alleles were detected, the average PIC was 0.291, ranging from 0.091–0.375. For this study, the average PIC across 384 loci was 0.3680. Therefore, this set of SNP markers has high polymorphism and credibility and can be used for fingerprint construction and identification of maize varieties.

In the analysis of molecular fingerprints of 384 SNP markers, genetic distances coefficients among 333 varieties ranged from 0.0047–0.5287, with an average of 0.27. But the morphological distances ranged from 0.26–0.92 based on DUS testing. Furthermore, there was no correlation between the genetic distance matrices obtained by SNP and by the morphological data. The signs suggested that the number of phenotypic descriptors was large enough to separate the varieties, but the reliability of the calculated genetic relationship was poor. This may be due to the fact that the morphological traits were strongly affected by environmental condition, where all the phenotypic descriptors depend on the visual assessment of DUS test experts except for the measurement traits. Moreover, in statistical analysis of the data, the expression range for each feature in the DUS test guideline is divided into a number of states and the wording of each state is attributed to a digital annotation. These realities may expand the differences between varieties. So, these characteristics in the DUS Test Guidelines need to set a weight coefficient for variety identification to reduce error.

In the decision making for DUS testing, a comparison between correlation values and the error rate was performed. The results showed that when correlations are below 0.60, the discrepancy rate based on phenotypic and molecular distance exceeds 80% in made distinctness decisions (Jones and Mackay, 2015). However, there was a lack of correlation between the genetic distance matrices obtained by SNP and by the morphological data in our work. The essence of UPOV BMT Model 2 requires calibration of genetic distance measures to reproduce the decisions made using morphological distances. If a genetic threshold is set at too low level, the quality of protection by Plant Breeders' rights is diminished. If a novel genetic threshold is set at too high, the 'distinctness' needed to acquire protection of a new variety unreasonably diminished. Either of these situations poses a risk to breeders. These results of this study make the implementation of UPOV Model 2 infeasible. We consider that the application models for molecular markers in variety registration and DUS testing should also go back to the Model 1 and look for more genetic linkage markers. Fortunately, the costs of high-throughput DNA marker generation and sequencing are sharply reducing and the improvement of data processing efficiency is making its implementation achievable.

UPOV's Biochemical and Molecular Techniques (BMT) Working Group has confirmed and adopted the threshold of 0.2 in maize. In the present study, the genetic distances coefficient of 333 varieties' showed normal distribution. Where, 88.04% of the varieties had a genetic distances coefficient greater than 0.2. In this study, the molecular markers revealed useful genetic diversity. The genetic differences of most varieties are obvious with high discrimination and the same varieties can be clustered together. The varieties used in this study were a small sample representative of existing commercial hybrids. They typified the kind of diversity encountered by the testing authorities conducting registration tests. Therefore, even if the SNP markers used in the present study turn out not to be useful for DUS testing and registering varieties, they should be of value in other areas. These could be variety identification for consumer protection and seed authenticity and purity detection.

Conclusion

The molecular markers revealed useful genetic diversity, but proved not to be useful for DUS testing. The phenotypic distances showed incredible differences, which are strongly influenced by environmental and statistical methods. We suggest that it is necessary to set relative weightage for those characteristics which are easy to be affected by these factors in DUS testing. The models for molecular markers application in variety registration and DUS testing should also go back to the Model 1 and look for more genetic linkage markers.

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