INNOVARE JOURNAL OF AGRICULTURAL SCIENCE

Vol 10, Issue 6, 2022

ISSN - 2321-6832

Research Article

GENETIC RELATEDNESS AMONG MAIZE INBRED LINES BASED ON ISSR MARKERS AND ITS ASSOCIATION WITH HETEROSIS AND HYBRID PERFORMANCE

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Received: 09 October 2022, Revised and Accepted: 16 October 2022

ABSTRACT

The objectives of the present study were to measure the genetic diversity among eight inbred lines of maize using ISSR markers and the correlation coefficients between genetic diversity and each of heterosis and mean performance of hybrids for grain yield. Ten ISSR primers were used in the detection of polymorphism of the eight inbred lines in a laboratorial experiment. Heterosis and mean performance of grain yield/ha in their F_1 diallel crosses were measured in a 2-year field experiment using a randomized complete blocks design with three replications. Based on ISSR markers, the genetic similarity coefficients among the eight maize inbred lines ranged from 0.798 (between L17 and IL53) to 0.943 (between IL80 and IL84) with an average of 0.869. Unique bands associated with maize inbred lines were identified. The results revealed that the genetic diversity among the inbred lines based on ISSR markers showed a significant, and negative relationship with mid-parent heterosis and mean performance of grain yield/ha. Further, intensive investigation of a large set of maize inbred lines from diverse populations using a large number of ISSR primers is required for proper understanding of genetic diversity of maize crop. Findings will be valuable for maize breeder, to practice effective selection of parental inbred lines for obtaining maximum heterosis and high mean grain yield/ha in their hybrids.

Keywords: *Zea mays*, Inter-simple sequence repeats, Genetic diversity, Heterosis.

 DOI: http://dx.doi.org/10.22159/ijags.2022v10i6.46548. Journal homepage: https://innovareacademics.in/journals/index.php/ijags

INTRODUCTION

Maize (*Zea mays* L.) ranks as the second cereal crop in Egypt with respect to production and cultivated area, after wheat. On 1,458,881 hectares of maize cultivation, Egypt produced 7.50 million tonnes of grain in 2020, with a yield of 5.14 tonnes per hectare. The domestic production of maize is insufficient to satisfy domestic demand because Egypt imports around 7.88 million tonnes of maize grains for $1,880,862,000 US in 2020 (FAOSTAT, 2022). The grain yield productivity of the recently produced hybrids should be raised to reduce the disparity between local production and consumption of maize grains in Egypt. Efforts have been intensified by the breeders in this direction to develop potential inbred lines for yield which could be utilized in hybridization programs for better exploitation of heterosis.

Crop plant development is greatly influenced by knowledge of genetic diversity among breeding materials, and this knowledge has been successfully applied for effective germplasm management and genotype selection for various breeding goals (Fufa *et al*., 2005). According to Pajic *et al*. (2010), the degree of genetic divergence between two inbred lines affects the performance of the ensuing hybrids because when parents are more divergent, heterosis is higher, and vice versa (Duvick, 1999). Any breeding program's ability to produce novel and adaptive cultivars with the best possible heterosis expression depends on the parental lines' various genetic backgrounds (Hallauer *et al*., 2010).

Genetic divergence was generally measured in the past through the use of phenotypic, cytogenetic, and biochemical attributes, including isozyme patterns (Govindaraj *et al.,* 2015). Phenotypic data are useful as a preliminary assessment of maize genetic diversity but present many limitations, such as low polymorphism and the influence of the environment on morphological expressions (Beyene *et al*., 2005; Al-Naggar *et al*., 2020). In recent years, molecular markers have been employed to detect genetic diversity. Molecular marker analyses overcome many of the limitations of morphological traits (Gupta *et al*., 1999). Microsatellite sequences border areas in the genome known as inter-simple sequence repeats (ISSRs). Multiple amplification products are produced when these areas are amplified by PCR using a single primer, and these products can be employed as a dominant multilocus marker system for the study of genetic variation in many organisms. Compared to other prevalent markers, ISSR markers are less methodologically demanding, inexpensive, and simple to employ (George *et al*., 2011). Several studies concluded that ISSR markers are useful in evaluating genetic variability because of their high reproducibility and power for detecting polymorphism (Balestre *et al*., 2008, Legesse *et al*., 2008; Al-Naggar *et al*., 2017).

For selecting suitable parents in heterosis breeding, information on the genetic diversity is necessary for a more effective hybridization scheme (Bhusal and Lal, 2017). A better understanding of the relationship between genetic diversity and hybrid performance would help to determine the heterosis and mean performance of hybrids. Several studies attempted to investigate the relationship between genetic diversity and heterosis, as well as to predict hybrid performance based on genetic distance (Devi and Singh, 2011; Dhliwayo *et al*., 2009; George *et al*., 2011; Ndlela *et al*., 2015), but the problem remained unsolved. Some researchers found a link between marker-based genetic distance and heterosis (Spooner *et al*., 1996; Amorim *et al*., 2005; George *et al*., 2011), while others found no link (Balestre *et al*., 2008; Legesse *et al*., 2008; Dhliwayo *et al*., 2009; Devi and Singh, 2011; Bhusal and Lal, 2017). The objectives of the present study were: (1) To assess the extent of genetic diversity among eight maize inbred lines, using ISSR markers, (2) to measure the genetic distance among these genotypes using cluster analysis, and (3) to establish the associations between distance matrix of ISSR markers and each of heterosis and mean grain yield of the hybrid.

METHODS

Plant material

Seeds of eight diverse maize (*Zea mays* L.) inbred lines in the ninth self-pollination generation derived from different populations (Table 1) and their 28 F_1 hybrids developed through diallel mating design in 2019 season were used in this study.

Field experiment

The field experiment was carried out at the Agricultural Experiment and Research Station of the Faculty of Agriculture, Cairo University, Giza, Egypt (30°03'N latitude and 31° 13'E longitude with an altitude of 18.60 meters above sea level) in the 2020 and 2021 seasons. The experimental design was a randomized complete block design (RCBD) with three replications. Each genotype was allotted to two rows of 4 m long and 60 cm apart, with 20 cm between hills (one plant per hill). Each plot size was 4.8 m^2. All other agricultural practices were followed according to the recommendations of ARC, Egypt, for obtaining healthy plants. Grain yield per ha (GYPH) data were collected.

Biometrical analyses

A separate analysis of the variance of RCBD was carried out for each year. A combined analysis of variance of RCBD across the 2 years was also performed if the homogeneity test was non-significant. The data were analyzed by the SAS software package. For each cross combination (P1 × P2), heterosis for GYPH was calculated as follows: MPH = [(F_1-MP)/MP] 100, where F_1 is the mean performance of the F_1 hybrid and MP is the mean performance of two parental inbred lines. Better parent heterosis (BPH) was also calculated as: BPH = [(F_1-BP)/BP] × 100, where BP is the mean of the better parent.

Laboratorial experiment

The ISSR analysis was carried out in the Molecular Markers Laboratory of the Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza, Egypt. Fresh leaves have been collected from 10-day-old seedlings of the eight maize inbred lines and stored at –80°C for DNA isolation.

DNA isolation

Extraction and purification of genomic DNA

DNA was extracted by the DNeasy Mini Kit (Qiagen Santa Clarita, CA) according to the manufacturer's instructions. DNA quality and quantity were detected using both NANODROP 2000 (Thermo Scientific, USA) and a 1% agarose gel (ethidium bromide staining).

ISSR analysis

ISSR-PCR reactions

Ten ISSR primers were used in the detection of polymorphism (Table 2). The amplification reaction was carried out in a 25 µl reaction volume containing 12.5 µl Master Mix (sigma), 2.5 µl primer (10 pcmol), 3 µl template DNA (10 ng), and 7 µl dH2O, according to Ibrahim *et al*. (2019).

Table 1: Parental source and origin of the eight inbred lines used in this study

Inbred line designation	Parental source	Origin
L 14-Y	Population Gemmeiza	ARC-Egypt
L 17-Y	SC30 N 11	DuPont Pioneer
L 21-Y	SC 72012	GWS-Egypt
L28-Y	Population 59	ARC-Thailand
IL51W	L-296 A locally developed	ARC-Egypt
IL53W	Rg-8 G.S. [(Sanjuan×Ci64) (SC.14)]	ARC-Egypt
IL80W	Rg-37 G.S. [(PI221866×307A) (SC.14)]	ARC-Egypt
IL84W	Rg-41 G.S. [(Sanjuan×307) (SC.14)]	ARC-Egypt

Y: Yellow, W: White, ARC: Agricultural Research Center

Thermocycling profile PCR

The PCR amplification was carried out on a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to complete 40 cycles after a 5-min denaturation cycle at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 45°C for 1 min, and an elongation step at 72°C for 1.5 min. The primer extension segment was extended to 7 min at 72°C in the final cycle.

Detection of the PCR products

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 ug/ml) in 1X TBE buffer at 95 volts. PCR products were visualized under UV light and photographed using a Gel Documentation System (BIO-RAD 2000).

ISSR data analysis

For ISSR analysis, only clear and unambiguous bands were visually scored as either present (1) or absent (0) for all samples, and final data sets included both polymorphic and monomorphic bands. Then, a binary statistic matrix was constructed. Dice's similarity matrix coefficients were then calculated between genotypes using the unweighted pair group method with arithmetic averages (UPGMA). This matrix was used to construct a phylogenetic tree (dendrogram). It was performed according to the Euclidean similarity index using the PAST software Version 1.91 (Hammer *et al*., 2001). The nature and magnitude of association between genetic distance based ISSR markers and each of the MPH and BPH of hybrids were evaluated using Pearson's correlation coefficients.

RESULTS AND DISCUSSION

Polymorphism as revealed by ISSR markers

Ten ISSR primers revealed discernible amplification profiles and were employed to investigate the genetic polymorphism among the eight maize inbred lines (Tables 3 and 4, Figs. 1 and 2). The 10 ISSR primers produced 117 amplicons, out of which 58 were polymorphic, and the average percentage of polymorphism was 49.6% (Table 3). The number of amplicons per primer ranged from 7 (ISSR-03) to 20 (ISSR-20) with an average of 11.7 fragments/primer across the different genotypes. However, the number of polymorphic amplicons varied from 0 (ISSR-06) to 13 (ISSR-12), with an average number of polymorphic amplicons of 5.8 fragments/primer. One out of the 10 primers exhibited 90% polymorphism (ISSR-05), while two primers (ISSR-07, ISSR-03) showed low polymorphism (27 and 29%, respectively). The size of the amplified fragment varied with the different primers, ranging from 100 to 1100 bp (Fig. 1). The number of bands and the percentages of polymorphism found in this study are suitable for estimating genetic diversity when compared with other investigations that used ISSR markers (Lenka *et al*., 2015; Soliman *et al.*, 2021). ISSRs are polymorphic markers that are useful for the discrimination of closely related maize genotypes (Valdemar *et al*., 2002; Berilli *et al*., 2011).

Genotype identification by unique ISSR markers

Unique markers are defined as bands that specifically identify one accession from the other by their presence or absence. The bands that are present in one accession but not found in the others are termed

Table 2: Primer's numbers, codes, and sequences

Primer No.	Primer code	Repeat motif	Sequence
ISSR-1	UBC835	$(AG)_8YC$	5'-AGAGAGAGAGAGAGAGYC-3'
ISSR-2	UBC834	$(AC)_8YT$	5'-ACACACACACACACACYT-3'
ISSR-3	UBC856	$(AC)_8YA$	5'-ACACACACACACACACYA-3'
ISSR-4	UBC857	$(AC)_8YG$	5'-ACACACACACACACACYG-3'
ISSR-5	UBC855	$(GT)_8YG$	5'-GTGTGTGTGTGTGTGTYG-3'
ISSR-6	UBC806	$CGC(GATA)_4$	5'-CGCGATAGATAGATAGATA-3'
ISSR-7	UBC807	$GAC(GATA)_4$	5'-GACGATAGATAGATAGATA-3'
ISSR-9	UBC809	$GAT(GATA)_4$	5'-GATAGATAGATAGATAGC-3'
ISSR-10	UBC810	$(AGAC)_4AAT$	5'-GACAGACAGACAGACAAT-3'
ISSR-12	UBC812	$(AC)_4YA$	5'-ACACACACACACACACYA-3'

A: Adenine, T: Thymine, G: Guanine, C: Cytosine, Y: (C or T)

positive unique markers, in contrast with the negative unique markers, which are absent in a specific genotype. These bands could be used for genotype identification (Al-Naggar *et al*., 2017). As shown in Table 4, the ISSR assay permitted the identification of six out of eight maize inbred lines by unique positive and/or negative markers. The data showed a total number of unique ISSR markers of 23; 14 of them were positive and nine were negative.

The inbred line L14 was characterized by three unique positive markers amplified by the primer ISSR-12 (340bp) and the primer ISSR-2 (620 and 200bp) and three unique negative markers amplified by the primers ISSR-9 (310 bp), ISSR-1 (530 bp) and ISSR-2 (330 bp). The inbred line L17 was characterized by four unique positive markers amplified by the primer ISSR-5 (650, 450, 330 and 230 bp) and three unique negative markers amplified by the primerISSR-5 (210, 170 and 150 bp). The inbred line L21 was characterized by two unique positive markers amplified by the primer ISSR-7 (650 bp) and the primer ISSR-1 (100 bp). The inbred line L28 was characterized by only one unique negative marker amplified by the primer ISSR-9 (420 bp). The inbred line L51 was characterized by two unique positive markers amplified by the primer ISSR-12 (850 and 670 bp). The inbred line L53 was characterized by three unique positive markers amplified by the primer ISSR-10 (200 and 170 bp) and the primer ISSR-12 (540 bp) and two unique negative markers amplified by the primer ISSR-10 (460 and 420 bp).

Table 3: The list of primers, total number of bands, monomorphic bands, polymorphic bands, and percentage of polymorphism, as revealed by inter-simple sequence repeat analysis of eight inbred lines of maize

Primer	Size fragment (bp)	TB	MB	PB	Percentage of polymorphism
ISSR-01	100–750	14	9	5	35.7
ISSR-02	150–620	11	6	5	45.5
ISSR-03	220–570	7	5	2	28.6
ISSR-04	170–450	9	5	4	44.4
ISSR-05	150–650	10	1	9	90.0
ISSR-06	160–500	8	8	0	0.0
ISSR-07	230–1400	11	8	3	27.3
ISSR-09	220–1350	15	6	9	60.0
ISSR-10	170–1000	12	4	8	66.7
ISSR-12	210–1100	20	7	13	65.0
Total		117	59	58	-
Average		11.7	5.9	5.8	49.6

TB: Total number of bands, MB: Monomorphic bands, PB: Polymorphic bands, ISSR: Inter-simple sequence repeat

The size of these unique markers ranged from 100 to 850 bp. Using ISSR analysis, we were able to identify 23 unique bands associated with eight maize inbred lines. Further experiments need to be achieved to determine the linkage between the ISSR markers used in the present study and gene(s) of maize genotypes. The present results support the idea that ISSR analysis can provide a fast detection of ISSR markers linked to maize genotypes. These markers would help in breeding programs of maize.

Genetic relationships among maize inbred lines

The scored data from the ISSR analysis in this study were used to compute the similarity matrices. As shown in Table 5, the genetic similarity ranged from 0.789 (between L17 and IL53) to 0.943 (between IL80 and IL84) with an average of genetic similarity of about 0.869. The results of this investigation indicated that all the eight maize inbred lines differ from each other at the DNA level.

In this context, some investigators reported wide genetic dissimilarity based on ISSR markers among maize genotypes (Santos *et al.,* 2017; Tanvir *et al.,* 2018). Genetic dissimilarity could be utilized by corn breeders in hybridization programs for using heterosis phenomenon.

Cluster analysis as revealed by ISSR

The ISSR-based coefficients of genetic similarity among the eight maize inbred lines were employed to develop a dendrogram using the UPGMA method (Fig. 2). The dendrogram separated the maize inbred lines from each other. The inbred L17 was separated into one cluster and the rest of the inbreds (7) into another cluster. The inbred L14 was separated into one sub cluster and the rest of the inbreds (6) into another sub cluster. The inbred L28 was separated into one sub-sub cluster and the rest of the inbreds (5) into another sub-sub cluster. The inbred IL53 was separated into one group and the rest of the inbreds (4) into another group. The last group was divided into two sub-groups; the first sub-group included one inbred (L21), and the second sub-group was divided into two sub-sub groups. The first sub-sub group included one inbred (IL51) and the second sub-sub group included two inbreds (IL80 and IL84); these two inbreds were the most genetically similar inbred lines. In conclusion, the use of ISSR markers can increase the efficiency of conventional plant breeding by identifying markers associated with the quantitatively inherited traits controlled by several genetic loci and their genetic components are difficult to measure.

Our results indicated that the ISSR technique is useful in the establishment of genetic fingerprinting and estimation of genetic relationships among maize inbred lines. Furthermore, this technique could detect enough polymorphism in the studied maize genotypes

Table 4: Unique positive and negative inter-simple sequence repeat markers generated for eight maize genotypes, marker size (bp), and total number of markers identifying each genotype

Maize inbred	Positive unique markers		Negative unique markers		Grand total
	Primer (band size bp)	Total number	Primer (band size bp)	Total number	
L14	ISSR-12 (340) ISSR-2 (620) ISSR-2 (200)	3	ISSR-9 (310) ISSR-1 (530) ISSR-2 (330)	3	6
L17	ISSR-5 (650) ISSR-5 (450) ISSR-5 (330) ISSR-5 (230)	4	ISSR-5 (210) ISSR-5 (170) ISSR-5 (150)	3	7
L21	ISSR-7 (650) ISSR-1 (100)	2			2
L28			ISSR-9 (420)	1	1
IL51	ISSR-12 (850) ISSR-12 (670)	2			2
IL53	ISSR-10 (200) ISSR-10 (170) ISSR-12 (540)	3	ISSR-10 (460) ISSR-10 (420)	2	5
Total		14		9	23

ISSR: Inter-simple sequence repeat

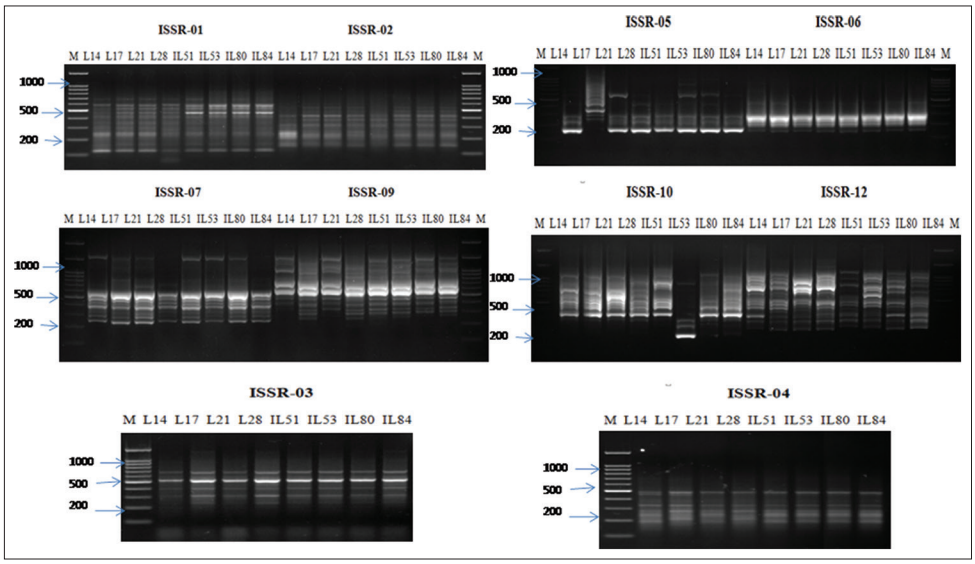

Fig. 1: Banding patterns of eight maize inbred lines amplified with the ISSR primers "ISSR-01, ISSR-02, ISSR-03, ISSR-04, ISSR-05, ISSR-06, ISSR-07, ISSR-09, ISSR-10, and ISSR-12 M: 100bp DNA ladder, Lanes "L14, L17, L21, L28, IL51, IL53, IL80, and IL84

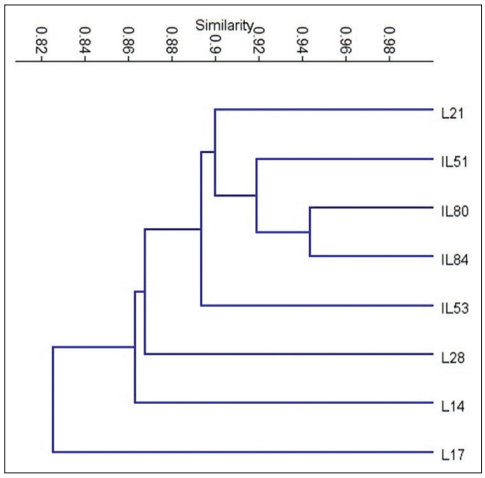

Fig. 2: Dendrogram of eight maize inbred lines using ISSR markers as per the average method of clustering

to distinguish each genotype from the others by at least one specific fragment. Furthermore, the use of these results in the future is important for maize germplasm improvement as well as for the selection strategies of parental lines that facilitate the prediction of crosses to produce hybrids with higher performance, as was indicated by several investigators (Adetimirin *et al*., 2008; Bhusal and Lal, 2017).

In general, the overall results indicated the possible use of ISSR analyses to detect some species-specific markers for the eight maize inbred lines that can be used to discriminate among these maize genotypes and also, to detect genetic relationships among these genotypes that can be used in breeding programs. The molecular genetic results of these eight maize genotypes are efficient tools for the characterization of these genotypes. Similar results have been reported in other studies of genetic diversity in maize using ISSR markers (Santos *et al*., 2017; Tanvir *et al*., 2018; Soliman *et al*., 2021).

Correlation between genetic diversity and heterosis and hybrid mean performance

Genetic dissimilarity based on ISSR markers showed negative and significant ($p \leq 0.05$) correlation coefficient with mid-parent heterosis and negative and significant ($p \leq 0.01$) correlation coefficient with hybrid mean performance, but did not show a significant correlation

Table 5: Genetic similarity coefficients based on inter-simple sequence repeat analysis among eight maize inbred lines

Inbred	L14	L17	L21	L28	IL51	IL53	IL80
L17	0.802						
L21	0.884	0.802					
L28	0.842	0.864	0.866				
IL51	0.882	0.823	0.871	0.852			
IL53	0.821	0.798	0.879	0.860	0.866		
IL80	0.857	0.856	0.891	0.874	0.925	0.920	
IL84	0.890	0.831	0.936	0.880	0.912	0.908	0.943

Table 6: Pearson correlation coefficients between molecular genetic dissimilarity and each of mid-parent heterosis, better parent heterosis, and hybrid mean performance (mean) for grain yield/ha

Parameter	MPH	BPH	Mean
GD (mol)	−0.39*	−0.27	−0.63**

GD: Genetic dissimilarity, MPH: Mid-parent heterosis, BPH: Better parent heterosis ,*,** indicate significant at 0.05 and 0.01 probability level, respectively

with better-parent heterosis (heterobeltiosis) for grain yield/plant (Table 6).

Our results on the genetic diversity based on ISSR markers for maize inbred lines concluded that the more dissimilarity of parents, the lower mid-parent heterosis, and the lower mean grain yield/ha can be produced. This conclusion contradicts the belief of some researchers that the greater the divergence of the parents, the more heterosis can be produced (Spooner *et al*., 1996; Amorim *et al*., 2005; George *et al*., 2011). Other investigators (Balestre *et al*., 2008; Legesse *et al*., 2008; Dhliwayo *et al*., 2009; Devi and Singh, 2011; Bhusal and Lal, 2017), reported non-significant or no association with heterosis. Further, intensive investigation of a large set of maize inbred lines from diverse populations using a large number of primers is required for proper understanding of the genetic diversity of the maize crop.

CONCLUSION

Results indicated that the ISSR technique is useful in the establishment of genetic fingerprinting and estimation of genetic relationships among maize inbred lines. Furthermore, this technique could detect enough

polymorphism in the studied maize genotypes to distinguish each genotype from the others. Using ISSR analysis, we were able to identify unique bands associated with maize inbred lines. These bands might also be used in breeding programs for differentiating among maize inbred lines. The results revealed that ISSR markers showed a significant and negative relationship with heterosis and mean performance of grain yield/ha. Further, intensive molecular investigation of a large set of maize inbred lines from diverse populations is required for proper understanding of the genetic diversity of this important crop using a large number of primers.

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