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Full Length Research Paper

Genetic diversity assessment of *Guzoita abyssinica* using EST derived simple sequence repeats (SSRs) markers

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***Niger (Guzoita abyssinica)* is traditionally very important in the production of oil and as an oilseed crop in Ethiopia. The level of *Niger* genetic diversity is not studied at molecular level particularly with microsatellite markers which are cost effective, informative and co-dominant. In this study, contrasting samples of 65 Ethiopian *Niger* germplasm accessions collected from diversified eco-geographic regions were studied with eleven polymorphic EST derived SSR markers. Based on dissimilarity matrix ranging from 0.056 to 0.75, the accessions were grouped into three major and six sub clusters, showing the wealth of genetic diversity for exploitation in future breeding programs. The EST derived SSR markers used in this study also revealed high polymorphic information content (PIC) ranging from 0.2624 to 0.3677, the average being 0.3308 which indicate the usefulness of the primers in *Niger* germplasm characterization in the future by providing basic breeding information for breeders. The mean number of major allele frequency, gene diversity and heterozygosity using power marker v3.25, showed a value of 0.69, 0.42 and 0.50, respectively. The number of private alleles using GenAIEx 6.41 is 66 alleles with mean of 9 alleles per locus. Thus, utilizing EST SSR marker for diversity study lays basic foundation to understand the genetic distance of crop verities and greatly contributes for further improvement and preservation.**

Key words: Alleles, dendrogram, diversity, EST-SSR markers, heterozygosity, locus, *Niger* and polymorphic information content (PIC).

INTRODUCTION

Niger (Guzoita abyssinica, 2n=30) is belongs to the plant family "Asteraceae". It is one of the most valuable oil-seed crops in Ethiopia (Getinet and Sharma, 1996). *Niger* grows in water-logged soils where other oil-seed crops fail, and its cultivation is beneficial for soil conservation and rehabilitation. Scientific community gives a little

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attention for *Niger* and hence *Niger* is renowned as "neglected and underutilized oil crops" (Getinet and Sharma, 1996). It is widely cultivated in Ethiopia today but has received only limited attention from agricultural scientists and modern breeding techniques have yet to be applied to its improvement. *Niger* (*G. abyssinica*) is traditionally very important in the production of oil and as an oilseed crop in Ethiopia, less systematically in India, Africa and Asia (Getinet and Sharma, 1996). It has been grown in Ethiopia since at least the 2nd millennium BC and probably much earlier (Boardman, 1999; Boardman, 2000). It is indigenous to Ethiopia, a center of its diversity.

In most places, *Niger* is widely cultivated in an intercropping condition which may render an opportunity for morphological variation. Consequently, it hides from improvement using modern breeding techniques. Any crop improvement program starts with identification of variability among the genotypes. *Niger* has been characterized morphologically (Bulcha et al*.,* 2005). It was hypothesized that the domesticated crop, *G. abyssinica* probably evolved from the wild type Guizotia *scabra ssp. Schimperi* (Petros et al*.,* 2007). Besides the morphological based characterization of *Niger*, studies using molecular markers such as ISSR (Petros et al*.,* 2007) and RAPD (Geleta et al*.,* 2007) have also been conducted to assess the genetic diversity existing among *Niger* accessions. According to (Dempewolf et al*.,* 2010a) identification of SSR from EST library of *G. abyssinica* was in place. This led to the first insight in developing SSR, which was not known before.

Microsatellites (SSRs) are quite many in eukaryote genomes. They offer supreme information about the genetic diversity of the crop; they are hypervariable, locus specific, multi-allelic, co-dominant, rapid, low cost and independent from environmental factors (Pejio et al*.,* 1998). They have wide genetic application in the study of genetic diversity, genotyping, finding connections among collections emanating from diverse geographical origin and gene pool, and identifying core collections to launch easy management and effective utilization of genetic resources (Alemayehu, 2007).

This study was, therefore, aimed at studying the genetic diversity of 65 *Niger* accessions using 11 EST-SSR markers to improve its production and productivity, and conservation as assessing genetic diversity of *Niger* at molecular level is a paramount importance for future breeding program. Previous efforts to characterize diversity of *Niger* populations using anonymous genomic SSR markers was not informative to reveal the levels of intra- and inter-population diversity (Geleta et al., 2007) and the major disadvantage associated with the anonymous genomic SSR is the high cost of development and time- consuming process. In the present study, 11 EST derived SSR markers were used to trace the genetic diversity and evolutionary patterns of 65 *Niger* accessions to reach at better understanding of the origin.

MATERIALS AND METHODS

The detailed material and methods used to evaluate the genetic diversity of 65 *Niger* germplasms using 11 EST- SSR markers is clearly described as follows.

Germplasm collection/study area

In this study, 65 accessions of *Niger* were collected from different agro ecologies of Ethiopia (Table 1). The places of sampling area were systematically selected. Most of the samples were collected from North Gondar, South Gondar, West Gojjam, East Gojjam, West Showa, and North showa, South Wello, Bale, West Wellega, East Wellga, Awi and West Harergia (Table 1).

Selection of markers

There is inconsistency between the choice of markers in terms of cost, time, inheritance of marker, quantity of DNA required, technical labour and degrees of polymorphism, precision of genetic distance estimates and the statistical power of tests. The SSRs were still the preferable markers in assessing genetic diversity, genetic mapping and marker-assisted selection of important traits as SSRs are multi-allelic, easily detectable by PCR, abundantly distributed in genome and codominantly inherited (Gupta and Varshney, 2000).Thus, the certain number and length of SSRs were probable to reflect the evolutionary history in particular species and its relatives. EST derived SSRs markers have higher rate of transferability across related species and genera than anonymous genomic SSRs (Guo et al., 2006, Mishra et al., 2012), and hence are preferable for phylogenetic studies and QTL mapping. In this study, based on the above mentioned promising property SSR markers are selected. Markers that are used in this study are described below in the table.

Sample preparation

The collected planting materials were planted in green house for two weeks. Fresh young leaf materials from three to five leaf numbers were selected to extract DNA. The leaves that were targeted for DNA extraction were taken from young seedlings. The leaf sample was taken from the most upper part, which are active regions undergoing mitosis. The dirty materials and fungal infection or any other contaminant on the surface of leaf materials were removed using 70% ethanol.

DNA extraction

After ten days of collection, DNA was extracted from each fresh and dried leaf following modified CTAB method (Doyle and Doyle, 1990). The dried leaf was grinded using Geno Grinder (MM-200, Retsch). Based on the procedures, cells were fractionated and DNA was extracted. The presence of the genetic material was checked via running the mixes of 5 μL of gDNA and 3 μL of 6X loading dye containing gel red in 0.8% of agarose gel for 30 min at 100 volt, in 1xTAE buffer using Junyl electrophoresis apparatus. The presence and absence of extracted genomic DNA was checked via transilluminator (3uv bench top, M-20 transilluminator). The quantity and purity of DNA was confirmed by Nano drop spectrophotometer (ND-8000, Thermoscitnific). Following the amount purified, proper

Table 1. List of geographical area for sample collection.

S/N	Acce.	Zone	Latitude	Longitude	Altitude
1	241142	Mirab Harerge	08-52-33-N	40-39-90-E	1660
2	243793	Agew Awi	11-19-00-N	36-47-00-E	1890
3	208391	Bahir Dar Special	11-30-00-N	37-18-0 -E	1920
4	243789	Debub Gondar	12-08-00-N	37-50-00-E	2135
5	208391	Bahir Dar Special	11-30-00-N	37-18-0 -E	1920
6	243778	Semen Wello	11-50-00-N	39-32-00-E	1835
7	243796	Agew Awi	10-56-00-N	36-36-00-E	1835
8	208384	Debub Gondar	12-00-00-N	37-39-00-E	1850
9	237518	Mehakelegnaw	14-10-00-N	38-45-00-E	2200
10	243772	Debub Wello	11-24-00-N	39-11-00-E	2060
11	235781	Semen Gondar	12-07-00-N	37-01-00-E	2080
12	238286	Mirab Gojam	10-09-00-N	36-09-00-E	2000
13	243784	Semen Gondar	13-59-00-N	37-47-00-E	2555
14	208946	Misrak Wellega	09-33-00-N	37-12-00-E	2450
15	215681	Debub Wello	11-19-00-N	39-44-00-E	2050
16	238292	Semen Shewa	10-01-00-N	38-02-00-E	2500
17	243775	Debub Wello	11-29-00-N	39-22-00-E	2390
18	243785	Semen Gondar	12-21-00-N	37-31-00-E	1920
19	208394	Agew Awi	11-20-00-N	37-00-00-E	1950
20	243777	Debub Wello	11-45-00-N	39-41-00-E	2020
21	237515	Mehakelegnaw	14-12-00-N	38-56-00-E	2010
22	208947	Misrak Wellega	09-30-00-N	37-03-00-E	2470
23	243786	Semen Gondar	12-21-00-N	37-31-00-E	1920
24	243791	Mirab Gojam	11-38-00-N	37-20-00-E	1870
25	243771	Debub Wello	10-57-00-N	39-47-00-E	1780
26	243783	Semen Gondar	13-02-00-N	38-05-00-E	1310
27	212492	Semen Shewa	09-56-00-N	38-54-00-E	1610
28	243770	Debub Wello	11-10-00-N	39-54-00-E	2070
29	235780	Semen Gondar	12-02-00-N	45-11-00-E	1830
30	243773	Debub Wello	11-20-00-N	39-15-00-E	2400
31	238291	Semen Shewa	10-01-00-N	38-02-00-E	2490
32	15191	Agew Awi	10-58-00-N	36-33-00-E	2080
33	15034	Mirab Shewa	09-01-00-N	38-25-00-E	2340
34	15059	Mirab Shewa	09-00-00-N	38-17-00-E	2140
35	238288	Misrak Gojam	10-02-00-N	37-09-00-E	2180
36	229989	Bale	06-36-00-N	39-35-00-E	1430
37	15136	Mirab Shewa	10-05-00-N	39-00-00-E	1800
38	15135	Semen Shewa	10-02-00-N	38-52-00-E	1600
39	15125	Mirab Shewa	08-37-00-N	38-12-00-E	2240
40	234144	Misrakawi	14-11-00-N	38-37-00-E	2210
41	15002	Mirab Gojam	10-42-00-N	37-03-00-E	2100
42	15162	Debub Wello	11-15-00-N	39-45-00-E	1964
43	15196	Mirab Shewa	08-58-00-N	37-36-00-E	2100
44	15055	Mirab Shewa	08-58-00-N	37-52-00-E	2091
45	15167	Mirab Shewa	09-04-00-N	38-30-00-E	2390
46	15154	Mirab Wellega	09-47-00-N	35-08-00-E	1650
47	234135	Mirabawi	14-09-00-N	38-16-00-E	1900
48	15062	Mirab Shewa	09-09-00-N	37-10-00-E	1700
49	234136	Mirabawi	14-07-00-N	38-20-00-E	2930
50	15198	Mirab Shewa	09-00-00-N	38-09-00-E	2240
51	234133	Mirabawi	14-06-00-N	38-14-00-E	1850

Table 1. Contd.

52	15031	Mirab Shewa	09-01-00-N	38-20-00-E	2160
53	15159	Misrak Wellega	08-50-00-N	36-29-00-E	2250
54	235399	Semen Gondar	36-32-00-N	37-20-00-E	1850
55	15134	Semen Shewa	07-33-00-N	37-35-00-E	2350
56	15010	Bahir Dar Special	11-34-00-N	37-23-00-E	2000
57	15161	Debub Wello	11-18-00-N	39-40-0 -E	2000
58	15144	Mirab Shewa	08-58-00-N	37-36-00-E	2340
59	15079	Bale	07-01-00-N	39-24-00-E	2430
60	15142	Mirab Shewa	08-58-00-N	37-52-00-E	2010
61	15081	Mirab Shewa	08-59-00-N	37-22-00-E	1800
62	15004	Mirab Gojam	10-34-00-N	37-29-00-E	2050
63	15129	Mirab Shewa	09-01-00-N	38-20-00-E	2200
64	15037	Mirab Shewa	08-08-00-N	38-01-00-E	2400
65	235877	Semen Gondar	12-32-00-N	37-32-00-E	2050

Table 2. List and characteristics of EST-SSR markers used to assess the genetic diversity of *Niger.*

Locus	Repeat motifs	Forward primer(F, 5'-3') Sequence Versus Reveres primers (R, 3'-5') Sequence	Expected allele size	Linked Loci
GA003	$(gat)_2$	CGCCCTAAAGCTACTTTCTTCC-F CACACTCGCACTAGGA-R	399-402	GA127,GA238
GA012	$(gat)_2$	CAGTAAGCTCGGTATCTCCAAGTT-F AGAAGATCTCGTCAGCAGAAACAG-R	263-275	GA107,GA138
GA013	$(ctt)_9$	GGTAATGGTAATGGAGGTTCTGG-F CCTCATCAGAGTTCTTCGGGTTAT-R	424-455	None
GA018	$(agc)_2$	GTTCCAGCCCATGAGTCATAA-F CTATCTCTTATCTCGTGGGGTTTTG-R	353-358	GA 183,GA186
GA029	$(atc)_3$	CCATCATCAATGGCGTTACTC-F GTCTCGTTCTAGAAGCTTCATCCT-R	270-276	GA108,GA143,GA186
GA035	$(tga)_3$	GATTTCTCAGGTGAAGGA-F GCCCTCCCTACAACATACTTGATA-R	301-307	GA107,GA144,GA217
GA037	$(ta)_2$	GGTGTTTTTGTGTAGTGGTCTGTC-F GACTAGCCAGAAACCGAAGAATC-R	347-350	GA081
GA055	$(ct)_3$	CCTGAAACAAACCCCAACAA-F CAGTACATCGCGGAGAGAGG-R	194-200	GA191,GA205
GA127	$(cct)_2$	CAATCTGCAACTACTGCCATACC-F CCAGTCAGAACCCTTGATCACTA-R	213-216	GA003,GA117
GA138	$(aag)_5$	ATCAACTTCCCCATATACCTCTGG-F CTTCCTCTGTCACTTCTTTTGGAC-R	363-378	GA018,GA0035GA108, GA183
GA139	(gaa)7	GTACATCCCAACTTTACCATCCAC-F CTCTACAACCAACACCACTTTCC-R	223-241	GA077,GA238

Source: Dempewolf et al. (2010a).

concentration of DNA was determined for further use. DNA was stored in the refrigerator till the next use. The DNA extracted from different samples was coded carefully. DNA sample from the autonomous organelles, nuclear and chloroplast was included for the case of tracing evolutionary origin of *G. abyssinica.*

Polymerase chain reaction and acquisition of markers

The primers (Table 2) were obtained from published article (Dempewolf et al*.,* 2010a). It was ordered and purchased from companies. The SSR region was amplified using proper PCR condition and reaction mixtures by using master cycler (Pros, eppendorf). A proper 96 well PCR plate with an allowed reaction mixture was in place. The touchdown PCR techniques were used with a program of 94°C for 4 min, 94°C for 45 s, 60 to 55°C for 1 min and 72°C for 1 min 30 s running for 9 cycle, and 94°C for 45 s, 55°C for 1 min and 72°C for 1 min 30 s running for 26 cycle and eventually, 72°C for 5 min final extension was suggested for all

Table 3. List of polymorphic SSR markers, allele frequency, gene diversity and PIC.

Locus	Major allele frequency	Gene Diversity	Heterozygosity	PIC
GA003	0.5846	0.4857	0.7692	0.3677
GA012	0.6308	0.4658	0.6154	0.353
GA013	0.6769	0.4374	0.4923	0.3417
GAO18	0.6615	0.4478	0.4615	0.3475
GA029	0.7231	0.4005	0.4923	0.3203
GA035	0.7538	0.3711	0.3692	0.3023
GA037	0.8077	0.3107	0.3231	0.2624
GA055	0.7000	0.4200	0.5692	0.3318
GA127	0.7154	0.4072	0.3231	0.3243
GA138	0.7077	0.4137	0.4615	0.3281
GA139	0.6385	0.4617	0.6615	0.3551
Mean	0.6909	0.4201	0.5035	0.3308

EST-SSR markers used (Cubry et al*.,* 2008). The total reaction volume and volume for each reactant including the volume of Taq DNA Polymerase was determined following the most promising PCR reaction mixture that used in article (Cubry et al*.,* 2008).

Gel electrophoresis

Gel electrophoresis was used to analyze the presence and absence of genomic DNA and PCR product. Simple sequence repeat were analyzed using polyacrylamid gel electrophoresis, to compute the size and number of alleles (Wang et al*.,* 2009). The accuracy of the PCR experiment was checked and yield of the PCR product was properly quantified in 1.2% agarose gel. 40% of (29:1, acrylamid: bis acrylamid solution) Polyacrylamid gel was used to count alleles and see patterns of SSR in the genome of the entire varieties using vertical electrophoresis apparatus (Cleaver, CS500 volt).

Scoring and data analysis

The clear and visible amplified PCR products were scored for presence (1) and absence (0) and the data were subjected to analysis using diverse tools of statistics and bioinformatics softwares. Dendrogram was constructed using Darwin 6.0 software (Perrier et al., 2003; 2006). A dendrogram was constructed using neighbour joining (NJ) as implemented in the same software. The number of alleles, gene diversity and polymorphism information content (PIC) were calculated using Power Marker V3.25 (Liu and Muse, 2005), Observed heterozygosity was calculated using Arlequin V3.1, and the number of private alleles were calculated using GenAlEx 6.41.

RESULTS

SSR amplified product and PIC statistics

In this study, the genetic relationships of 65 *Niger* germplasm were analyzed using 11 Expressed Sequence Tagged derived Simple Sequence Repeats (EST-SSRs) markers. 19 SSR markers were obtained from published articles (Dempewolf et al*.,* 2010a), and screened to select 11 of them which were polymorphic with understandable and solid band for statistics on 65 germplasm, and produced 66 total numbers of alleles with an average of 9 alleles per locus. Locus GA037 showed greater allele frequency, gene diversity and PIC. Whereas GA003 showed lower allele frequency, gene diversity, heterozygosity and PIC (see Table 3).

Dendrogram constructed using EST-SSR markers

Based on the dissimilarity matrix the entire accessions were grouped in to three major clusters and six sub clusters. The minimum and maximum dissimilarity values for the analyzed accessions were 0.056 and 0.75 respectively. The phylogenetic tree constructed using Jaccard coefficients (Figure 1). The dendrogram was shown with three different colours (Red= for accessions collected from Tigray regions, Green= from Amhara and Black= from oromia region) which is used to indicate the location of major *Niger* growing area in Ethiopia.

DISCUSSION

All the 11 EST-SSR markers used in this study are polymorphic (100%). Gupta et al. (2003) identified only 55% of 20 EST-SSR markers used were polymorphic among 52 wheat accessions. Eujayl et al. (2002) reported a lower level of polymorphism (25%) when 42 EST-SSR markers screened against 64 durum wheat germplasm lines. This clearly indicates that the percentage of polymorphism depends on number and nature of the material used under analysis.

In the present study, data analysis was carried out with 11 EST-SSR markers by following SMM model which assumes that each mutation (insertion and deletion) creates a new allele. A similar pattern of allele scoring was observed with genomic SSR markers in Folkertsma et al. (2005).

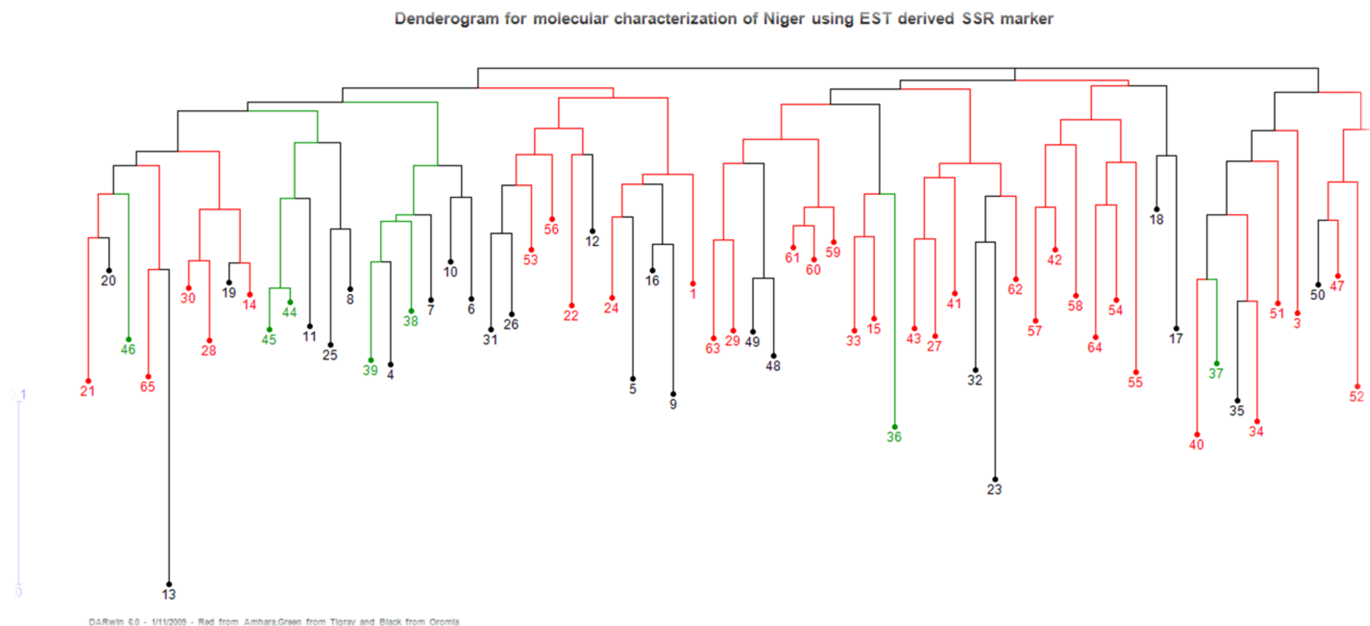

Figure 1. Dendrogram showing the genetic diversity of 65 *Niger* germplasm using SSR markers.

In total, 11 EST-SSR markers produced 66 alleles with an average of 9 alleles per locus (Table 3). This is the maximum number of alleles per markers reported using EST-SSR markers in any cereals to date. In case of tall fescue grass, an average of 2.78 alleles/marker were reported (Saha et al., 2004), while 1.8 alleles/marker in bread wheat (Gupta et al., 2003) with 20 EST-SSRs, 4.5 alleles/markers in durum wheat with 42 EST-SSRs (Eujayl et al., 2002), 3 alleles/markers in 54 barley accessions using 38 EST-SSR markers (Thiel et al., 2003), and 4.6 alleles/marker in *Crotalaria* species (Wang et al., 2006). Compared to results obtained with neutral genomic SSRs, the average number of alleles per marker detected in this study is comparable to that found in limited size core collection (Caniato et al., 2007), or on geographically limited studies (e.g. Barnaud et al., 2007; Deu et al., 2008), but is lowest than found on the same material (Billot et al., 2013).

The PIC values of markers can provide an estimate of discrimination power in a set of accessions by taking not only the number of alleles, but also the relative frequencies of each allele (Smith et al., 2000). The average PIC value of EST-SSR markers (0.3308) was a bit higher in this references set of *Niger* in comparison with previous studies using EST-SSR markers for genetic diversity analysis in other crops, e.g., 0.443 in bread wheat (Gupta et al., 2003), 0.45 in barley (Thiel et al., 2003). However, the average PIC value was lower compared to PIC values of genomic SSR markers in sorghum [0.62 in both studies of Agrama and Tuinsta (2003), and Caniato et al. (2007)]. However, this is higher than PIC value reported by Folkertsma et al. (2005) using 100 guinea race accessions and 21 genomic SSR markers and Ali et al. (2008) using 72 sorghum accessions with 41 SSR markers. This is on par with PIC (0.54) reported by Wang et al. (2009) in a study involving 96 sweet sorghum lines and 95 SSRs. High PIC values and large number of alleles per markers can also be attributed to the nature of the *Niger* materials that is studied. SSR markers containing dinucleotide repeats produced more alleles and hence, greater PIC values (Table 3). These results were in harmony with previous studies by Smith et al. (2000), Agrama and Tuinstra (2003), Casa et al. (2005), and Deu et al. (2008).

Conclusion

Genetic diversity analysis with proper genotyping using EST-SSR marker will help the breeders to mine trait-specific alleles and facilitate an effective way of identifying the gene for different agronomic traits. The present study showed the presence of considerable variations among *Niger* genotypes. The presence of this considerable variation among *Niger* genotypes has great promise as parents to obtain promising heterotic expression in F1's and may create considerable variability in the segregating populations.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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