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Are there differences in heterozygosity of strains obtained from intercrossed and self-fertilized onion plants?

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ABSTRACT

The commercial use of onion hybrids is preferred by producers. In contrast, the production of hybrid onion seeds is extremely inefficient. This is due to the use of lineages obtained by successive self-fertilizations assuming the effect of inbreeding depression per se. Therefore, it is necessary to understand new alternatives to reduce the effect of inbreeding depression in the strains. The objective of this study was to evaluate possible differences in polymorphism and levels of heterozygosity of strains obtained from intercrossed and self-fertilized plants. Twelve onion populations belonging to Bayer's breeding program (Granex, IPA-1, IPA-2 and IPA-3) were used, obtained by self-fertilization of one plant, intercrossing of two plants or intercrossing of three plants. Three individuals from each strain were used in the analyzes. The amplifications were performed using 8 microsatellite primers with greater polymorphism, according to germplasm characterization studies carried out by CITA. Heterozygosity generally decreases with self-fertilization and increases as more plants are used in the cross. The SSR markers used in the present study were efficient in detecting variability in different genetic backgrounds. With the results obtained, it is suggested to carry out the obtaining of hybrids between the different combinations and to analyze the performance *per se* of the different modalities of obtaining strains proposed in the present research.

Keywords: *Allium cepa*, hybrid, onion polymorphism, genotyping, primers.

RESUMO

Há diferenças de heterozigosidade de linhagens obtidas de plantas intercruzadas e autofecundadas de cebola?

O uso comercial de híbridos de cebola é preferido pelos produtores. Em contrapartida, a produção de sementes híbridas de cebola é extremamente ineficiente. Isso se deve ao fato do uso de linhagens obtidas por sucessivas autofecundações assumindo o efeito de depressão por endogamia *per se*. Diante disso, faz-se necessário buscar novas alternativas capazes de reduzir o efeito de depressão por endogamia nas linhagens. Objetivou-se neste trabalho avaliar possíveis diferenças de polimorfismo e níveis de heterozigosidade de linhagens obtidas de plantas intercruzadas e autofecundadas. Foram utilizadas 12 populações de cebola pertencentes ao programa de melhoramento da Bayer (Granex, IPA-1, IPA-2 e IPA-3), obtidas por autofecundação de uma planta, entrecruzamento de duas plantas ou intercruzamento de três plantas. Três indivíduos de cada linhagem foram utilizados nas análises. As amplificações foram realizadas a partir do uso de 8 microsatélite primers com maior polimorfismo, de acordo com estudos de caracterização de germoplasma realizados pela CITA-Espanhola. A heterozigosidade geralmente diminui com a autofertilização e aumenta à medida que mais plantas são usadas no cruzamento. Os marcadores SSRs usados no presente estudo foram eficientes para detectar variabilidade nos diferentes backgrounds genéticos. Com os resultados obtidos, sugere-se realizar a obtenção de híbridos entre as diferentes combinações e analisar o desempenho *per se* das diferentes modalidades de obtenção de linhagens propostas na presente pesquisa.

Palavras-chave: *Allium cepa*, híbridos, polimorfismo de cebola, genotipagem, primers.

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Onion cultivation represents an important activity for the economic and social sector (Comin *et al*., 2018; Nile *et al*., 2018; Ouyang *et al*., 2018). In 2017, 57 thousand hectares of onion were grown in Brazil (Agrianual, 2017).

The use of hybrid onion seeds has promoted greater productivity (Santos *et al*., 2018; Maciel *et al*., 2019). Despite all the agronomic potential expressed in hybrids, there is a major obstacle in seed production. For the production of seeds of hybrid onion cultivars, two agricultural years are necessary, which results in greater need for vigor from the parents (Leite, 2014). Due to the complexity involved in the production of hybrid onion seeds, in some agricultural years, the seed stock has run out (ABCSEM, 2013). There are indications that, because it is a species highly influenced by inbreeding depression, successive self-fertilization results in highly susceptible parents, low vigor, low bulb productivity and consequently lower seed productivity in the field (Machado *et al*., 2019).

Therefore, it is necessary to search for new alternatives capable of reducing the effect of inbreeding depression in the strains and enabling the production of hybrid seeds.

Improvements in onion yield and quality are normally achieved by increasing the value of desirable characters using molecular markers (Sudha *et al*., 2019).

SSRs (Simple Sequence Repeats), also known as microsatellites, are very informative molecular markers that have consequently become tools of great benefit for plant breeding programs. SSRs are abundant, co-dominant, multi-allelic, highly polymorphic markers and they are spread through the whole genome, allowing differentiation between homozygotes (Xanthopoulou *et al*., 2014; Song *et al*., 2016; Luo *et al*., 2018).

Microsatellites are small sequences that are repeated in tandem and range from 1 to 6 base pairs. These markers are distinguished by great variation in the number of repetitions that results from dynamic and complex mutagenic events such as unequal crossing-over, retrotransposition and, mainly, DNA polymerase slippage (Bhargava & Sharma, 2013; Qiu *et al*., 2016).

A taxonomic study by Fischer & Bachmann (2000) was the first to develop specific SSR markers for onions. The authors used 30 microsatellite primers to try to discriminate among interspecific accessions of *Allium*. In recent years, SSR markers have been used in onion for genome mapping studies (Baldwin *et al*., 2012), cultivar discrimination (Khar *et al*., 2011; Kisha & Cramer, 2011) and to assess the genetic diversity in local varieties from Spain (Mallor *et al*., 2014).

Despite advances in obtaining onion-specific SSR markers, there are no studies using these to investigate the effects of inbreeding. Therefore, the current study was proposed to molecularly characterize onions (*Allium cepa*) from different backgrounds and levels of inbreeding and that had been bred by cross-fertilization ('sib') of two and three plants and by self-fertilization.

The objective of this study was to evaluate possible differences in polymorphism and levels of heterozygosity of strains obtained from intercrossed and self-fertilized plants.

MATERIAL AND METHODS

Molecular analyses were carried out from March to July 2017 at the Center for Agrifood Research and Technology of Aragon (CITA) in Spain (41°39'N).

The onion lines used in the present study belong to Bayer's onion breeding program located in Uberlandia, Brazil (18°44'6"S, 48°24'3"W, 872 m altitude) and were analyzed from 2014 to 2015. The study evaluated 12 onion lines from four different genetic backgrounds (Granex, IPA-1, IPA-2 and IPA-3) derived from the self-fertilization of one plant, crossbreeding with two plants and crossbreeding with three plants (Table 1).

The plants were grown in a hoop houses (7 x 21 m and 4 m high) with a top covering of UV-filtering polyethylene film (150 micron) and white, anti-aphid side screens.

The study was carried out with microsatellite molecular markers and using the CTAB DNA extraction protocol (Doyle & Doyle, 1990) and quantified using a Multiscan GO (Thermo Fisher Scientific, USA) and stored in 100 µL aliquots in 1XTE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0).

The amplifications were performed using a set of primers (Table 2) selected for their high polymorphism (according to previous germplasm characterization studies performed by CITA researchers) and ability to represent the onion genome (one for each of the eight *A. cepa* chromosomes).

PCR amplifications were performed in 20 µL solutions containing 10 ng of genomic DNA, 1x PCR buffer {75 mM Tris-HCl,, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$}, 2 mM $MgCl_2$, 0.20 mM of each dNTP (Invitrogen), 0.25 µL DMSO (≥99.9% Sigma), 0.075 U Taq DNA polymerase (Biotools, Madrid, Spain), 0.125 µM of forward-M13 primer and 0.500 µM of the reverse primer and the M13 tail (5'-CACGACGTTGTAAAACGAC-3') tagged at the 5' end with a fluorescent dye: 6-FAM or HEX (Schuelke, 2000). The PCR reactions were carried out using an Applied Biosystems thermal cycler (model GeneAmp® PCR System 9700, Perkin-Elmer Corp., Norwalk, CT, USA) programmed with a touch-down profile: included two phases, starting with an initial denaturation phase at 95°C for 5 min. The first phase was a touchdown (TD) PCR profile with 20 cycles, starting with 95°C denaturation for 45 s, where the annealing temperature was reduced by -0.7°C per cycle for 45 s, followed by the extension stage at 72°C for 1 min. The second PCR phase with 15 cycles in total, included 95°C denaturation for 45 s, the annealing temperature for each primer according Fischer & Bachmann (2000), extension at 72°C for 1 min. The amplified fragments were separated by capillary electrophoresis in an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Madrid, Spain) using the internal size standard GeneScan™-LIZ500 (Applied Biosystems). Raw data and genotypes were obtained using GeneMarker® software version 2.7.0 (Softgenetics, LLC, State College, PA, USA). Eight microsatellite markers (Table 2) were compared based on the size of the amplified fragments that were identified by observing the different fluorochromes used in each microsatellite and chromosomal location. Potential relationships between each locus (identified by primer) in onions was achieved by referencing GenBank (NCBI – National Center for Biotechnology), ENA (European Nucleotide Archive).

Besides analyzing primer amplifications, the mean number of alleles per locus (Na) was also obtained (the ratio of the total number of alleles to the total number of loci). Diversity between segregating lines was calculated using allele frequency (pi) and observed heterozygosity using the diversity software.

RESULTS AND DISCUSSION

Two out of the eight microsatellite loci (25%) did not amplify while six (85%) were polymorphic. These

values confirm the high level of genetic information expected from microsatellite markers, as also observed in similar studies on *Allium cepa* by Callum *et al.* (2008) and Santos *et al.* (2010). However, the polymorphic amplifications were difficult to interpret, and the stutter peaks produced during genotyping made the peaks difficult to read. Several authors (e.g. Jakse *et al*., 2005 and Lee *et al*., 2013) have also reported on the challenge of interpreting SSR markers in onion genotyping, mainly because of the complexity of PCR reactions and the great size of the onion genome.

For these reasons, the onion genome has not yet been completely sequenced. Once completed, studies like this one will greatly benefit from it. However, other recent studies, such as the published by Shukla *et al.* (2016) and the Sequon (Onion Genome Sequencing project), state that complete sequencing of the onion genome is only a matter of time and propose that all data on EST-SSR functional markers and RNA sequencing for *Allium cepa* should be combined with markers that have already been registered in GenBank. Another objective of these authors is the prediction of mRNA from respective sequenced genes, which could be used to identify the transfer process of DNA coding.

The ACM235 microsatellite showed an interesting degree of polymorphism, in relation to the other lines. It is suggested that it may be a possible marker in new research. Callum *et al.* (2006) carried out genetic mapping to identify which gene had the greatest effect on the sugar content of the onion bulb. The study identified that the ACM235 marker was the most polymorphic and had the greatest relative significance for the levels of fructan bulb.

The eight loci evaluated in the present study showed that the 12 segregating lines were genetically diverse, with the number of alleles in the self-fertilized progenies (49) was lower than that of the two-plant sib, suggesting allele migration due to greater gene segregation. These results only suggest that the effect of inbreeding was lower when two or three plants were crossbred than when one plant was self-fertilized.

The mean number of alleles in each locus (R) ranged from 1.71 to 4.43. Another molecular characterization study of onion cultivars using microsatellite markers, detected 40 alleles in 13 polymorphic SSRs, with an average number of alleles per locus ranging from two to seven, and a mean of three SSR alleles in 44 onion cultivars (Santos *et al*., 2010).

Table 1. Description of genetic backgrounds, generations and inbreeding levels of twelve onion lines. Uberlândia, UFU, 2018.

Background	Generations*	Code	Average inbreeding level
GRxGR	F_2S_3	A_1	Moderate
GRxGR	F_2S_2 SIB 2 plants	B_2	
GRxGR	F_2S_2 SIB 3 plants	C_3	
IP-1	S_4	A_1	
IP-1	S_3 SIB 2 plants	B_2	
IP-1	S_3 SIB 3 plants	C_3	
GRxIP-1	F_2 S1	A_1	Low
GRxIP-1	F_2 SIB 2 plants	B_2	
GRxIP-1	F_2 SIB 3 plants	C_3	
IP-2xIP-3	F_2 S1	A_1	
IP-2xIP-3	F_2 SIB 2 plants	B_2	
IP-2xIP-3	F_2 SIB 3 plants	C_3	

* S/ indicates the number of self-fertilizations A_1= self-fertilized; B_2= crossbred from two plants; C_3= crossbred from three plants. GR= Granex, IP-1 = IPA-1, IP-2 = IPA-2 and IP-3 = IPA-3.

Table 2. Microsatellite primers of *Allium cepa* used in analyses. Locus name, Forward (F) and Reverse (R) primer sequence and GenBank accession number (https://www.ncbi.nlm.nih.gov/genbank/). Uberlândia, UFU, 2018.

Locus	Primer sequence (5'-3')	GenBank accession number
ACM101	F-M13CCTTTGCTAACCAAATCCGA R-CTTGTTGAGAAGGAGGACGC	CF443425
ACM138	F-M13ACGGTTTGATGCACAAGATG R-CCAACCAACAGTTGATACTGC	CF451850
ACM134	F-M13ACACACACAAGAGGGAAGGG R-CACACACCCACACACATCAA	CF449417
ACM146	F-M13ATGTCCCAATTCGACCAGAG R-CGTTACGGCTGAGAACTTCC	CF446333
ACM240	F-M13GTGCAACTCCAAGAGAAGGG R-AATATAAAGGCGTTGGCCTG	CF444554
ACM255	F-M13AAATTCCCAAAACGAAACCC R-GGGTTTCAGGAACAGTCAGC	CF449065
ACM322	F-M13TTCTTCTCCTATCCAGCTATCG R-GTGATTTGGGAGGGGATTTT	ES449660
ACM235	F-M13ACGCATTTTCAAATGAAGGC R-TGAGTCGGCACTCACCTATG	CF441946

†M13: 5'-CACGACGTTGTAAAACGAC-3'

Allelic variations for the ACM146 and ACM134 primers were the highest while allelic variation for ACM240 was the lowest. Rivera *et al.* (2016) evaluated genetic diversity in onion varieties (*Allium cepa*) in northeastern Spain and found that ACM146 was also one of the primers that provided the greatest allelic variation, which demonstrates the potential of this SSR for characterizing variation among populations. The average number of alleles did not vary substantially among the three methods.

The mean heterozygosity (H) of the six amplified SSR loci was 0.21 with ACM146 and ACM134 having the highest heterozygote frequencies (Table 3). Heterozygote frequency indicates variability since each diploid individual, as in the case of onion, can have up to

Table 3. Baseline allelic pattern estimated for twelve onion (2n) strains with three individuals in each of three different genetic generations, genotyped with eight SSR markers. Uberlândia, UFU, 2018.

Crossings	ACM101	ACM146	ACM240	ACM255	ACM138	ACM322	ACM134	H	
GR x GR (A_1)	235/235	231/231	N/N	N/N	241/241	268/268	205/205		
	235/235	231/231	209/209	N/N	241/241	268/268	205/205	0	
	235/235	231/231	209/209	N/N	N/N	268/268	205/205		
GR x GR (B_2)	235/235	231/231	N/N	N/N	241/241	268/268	203/205		
	235/235	231/231	209/209	N/N	241/241	268/268	N/N		
	235/235	231/234	209/209	N/N	241/241	268/268	203/205		
GR x GR (C_3)	235/235	231/231	209/209	176/176	N/N	268/268	203/205		
	N/N	231/231	209/209	N/N	241/241	268/268	203/205	0.19	
	234/234	231/234	209/209	N/N	241/241	268/268	N/N		
IP-1 (A_1)	235/235	231/231	N/N	N/N	241/241	268/268	205/205		
	234/235	231/231	N/N	N/N	241/241	268/268	205/205	0.06	
	235/235	231/231	N/N	N/N	241/241	268/268	205/205		
IP-1 (B_2)	235/235	232/232	N/N	N/N	241/241	268/268	203/203		
	235/235	232/232	N/N	N/N	241/241	268/268	203/203	0	
	235/235	232/232	N/N	176/176	241/241	268/268	N/N		
IP-1 (C_3)	235/235	231/231	N/N	N/N	241/241	268/268	203/205		
	230/235	231/231	N/N	N/N	241/241	268/268	203/203	0.20	
	235/235	231/231	N/N	N/N	241/241	268/268	203/203		
GR x IP-1 (A_1)	232/232	231/235	N/N	N/N	241/241	268/268	203/203		
	232/232	231/235	N/N	N/N	241/241	268/268	203/203	0.20	
	232/232	231/235	N/N	N/N	241/241	268/268	203/203		
GR x IP-1 (B_2)	230/235	231/235	N/N	N/N	241/241	268/268	N/N		
	230/235	231/235	N/N	N/N	N/N	N/N	N/N	0.22	
	235/235	231/235	N/N	N/N	241/241	N/N	N/N		
GRxIP-1 (C_3)	235/235	231/235	N/N	N/N	241/241	N/N	203/203		
	230/235	231/235	N/N	N/N	241/241	N/N	203/203	0.33	
	235/235	231/235	N/N	N/N	241/241	268/268	N/N		
IP-2xIP-3 (A_1)	235/235	231/231	N/N	N/N	241/241	268/268	203/205		
	N/N	231/231	N/N	N/N	241/241	268/268	203/205	0.15	
	N/N	231/231	N/N	N/N	241/241	268/268	203/203		
IP-2xIP-3 (B_2)	235/235	231/231	205/209	N/N	241/241	268/269	203/205		
	N/N	231/231	205/209	N/N	241/241	268/269	203/205	0.27	
	235/235	231/231	N/N	N/N	241/241	268/268	N/N		
IP-2xIP-3 (C_3)	235/235	231/231	N/N	N/N	241/245	N/N	N/N		
	N/N	231/231	N/N	N/N	241/245	N/N	N/N	0.43	
	N/N	231/231	N/N	N/N	241/245	N/N	N/N		
Average (H):	A_1= 0. 08	B_2 = 0. 14	C_3 = 0. 23						

Moderate degree of endogamy

Low degree of endogamy

*H= Heterozygosity N/N= Not conclusive for the two alleles. GR= Granex, IP-1 = IPA-1, IP-2 = IPA-2 and IP-3 = IPA-3.

two alleles per locus (Brown & Weir, 1983). Thus, greater variability is related to greater heterozygote frequency.

In general, heterozygote frequencies were low in the progenies derived from the various breeding methods, ranging from 0.06 to 0.43. Trifonova *et al.* (2017) worked with microsatellites to analyze genetic diversity in endemic species of *Allium regelianum* and found heterozygote frequencies that varied from 0.14 to 0.29, similar to those of the current study. Mallor *et al.* (2014) worked with different *Allium* species and found higher values that reached 0.92. The greater proportion of homozygotes in this study is most likely due to the narrow genetic base of the onion lines, which naturally increases the effects of inbreeding.

The lowest heterozygosity values (H = 0, 0.19, 0.19, 0.06, 0.20) (Table 3) were found in the 'GR x GR' and 'IP-1' backgrounds (all with moderate levels of inbreeding). Conversely and as expected, the 'GR x IP-1' and 'IP-2 x IP-3' backgrounds (low-level inbreeding) yielded the highest heterozygosity values (H = 0.20, 0.22, 0.33, 0.15, 0.27 and 0.43), demonstrating that initial self-fertilizations (two and three) made in the first group of backgrounds ('GR x GR' and 'IP-1') helped reduce genetic variability, regardless of generation (A_1, B_2 and C_3). These results also allow comparisons between SSR markers and their capacity for predicting genetic variability in onions.

The mean heterozygosity (H) values for all four generations of A_1, B_2 and C_3 (0.08, 0.14 and 0.23, respectively, Table 3) show that overall, heterozygosity was lowest in self-fertilized progeny and increased as more plants were added to the crosses.

The findings of the present study, regarding allele pattern and base pair estimates of the alleles in six SSR loci, are an initial step in using SSR markers in research on inbreeding and may encourage future breeding efforts and research on hybrid onion production in Brazil.

The present study is the first on onions that discusses the impact of inbreeding on the breeding of onion lineages and subsequent hybrids. Investigating the crossbreeding of a few plants, as an alternative to the traditional method of successive self-fertilizations, was useful because it yielded initial findings and raised questions about the underlying genetics. The phenotypic results of this study identified differences among breeding methods and showed that crossbreeding a few plants can be used to breed onion lines.

It is suggested to carry out the obtaining of hybrids between the different combinations and to analyze the performance *per se* of the different modalities of obtaining strains proposed in the present research.

In function of the results, heterozygosity generally decreases with self-fertilization and increases as more plants are used in the cross, and the SSR markers used in the present study were efficient in detecting variability in different genetic backgrounds.

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