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Outcrossing and heterozygosity rates in tropical onion populations

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Abstract

Outcrossing and heterozygosity rates are important to define genetic conservation and breeding strategies, and these estimates are quite rare for onions. This study aimed to estimate cross-pollination and heterozygosity rates in tropical onion populations to guide the development of new varieties. Eight tropical onion pairs were isolated in the field to be open-pollinated by insects. Vernalized parent bulbs were used for pair-crossing in 1:1 alternate rows. Seeds were harvested from the female parents to assess the outcrossing and heterozygosity rates of the progenies based on the color of the red bulb, which is partially dominant over yellow, and four microsatellite loci. The outcrossing estimates based on the morphological marker in the 'BRS Rio Vale' × 'BRS Carrancas', 'Botucatu' × 'BRS Carrancas', 'IPA 11' × 'IPA 10', and 'BRS Alfa São Francisco' × 'IPA10' crosses ranged from 15% to 39%, with an average of 28.2%, whereas with microsatellite loci, in the 'BRS Alfa São Francisco'×'IPA 11', 'Alfa SF RT' × 'BRS A. São Francisco', 'BRS Rio Vale' × 'Botucatu', and 'Cascuda T6' × 'Botucatu' crosses, the values ranged from 33% to 71%, with an average of 42.7%, indicating both cross-pollination and self-pollination within the progenies. The average heterozygosity values in the eight populations ranged from 0.82 to 1.0, highlighting the potential for developing open-pollinated onion varieties highly adapted to biotic and abiotic stresses.

Keywords: *Allium cepa*, cross-pollination, microsatellites, morphological marker

Introduction

Onion (*Allium cepa* L.) is one of the most economically important vegetables worldwide, with a global production of 97.86 million tons in 2017. China and India are the largest producers, accounting for approximately 48% of this total, while Brazil occupies the 12th position with an annual production of 1.72 million tons, representing 1.8% of global production and 40% of the whole production in South America (FAO, 2019). In the Northeast region of Brazil, onion production is concentrated in the states of Bahia and Pernambuco, which together account for almost 21% of Brazilian production (IBGE, 2018). Tropical onion cultivation in Northeastern Brazil is predominantly carried out with short-day cultivars adapted to low latitudes, which require approximately 12 hours of light for bulb formation. Onions are physiologically long-day plants that require about 16 hours of light, characteristic of temperate regions (Currah, 2002).

Onion is an herbaceous species with a biennial cycle whose inflorescences exhibit hermaphrodite flowers and protandrous dichogamy (Currah & Ockendon, 1978). The main pollinators of *A. cepa* are *Apis mellifera* and *Musca domestica* (Currah, 1990). The floral system of *A. cepa* favors outcrossing, although the plants are perfectly capable of self-pollinating (Currah, 2002). Open-pollination (OP) raises the level of heterozygosity and recombination among favorable alleles, generating new and potentially adapted genotypes and increasing the hybrid vigor of the species (Allard, 1999).

Despite the importance of the mating system to define breeding strategies, studies on the pollination rate of onions are scarce. In previous studies, van Der Meer & van Bennekom (1972) and Havey (2018) reported outcrossing rates based on bulbs of different colors or among varieties with yellow and red bulbs,

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using color as a phenotypic marker. Microsatellites have been successfully used to estimate outcrossing rates in other species (Santos et al., 2011; Rajesh et al., 2014; Gebremichael, 2017) but have still not been applied to study onion populations.

For Weir (1996), heterozygosity, or the number of heterozygous individuals, is a measure of the genetic variation of a population. Onion heterozygosity estimates using microsatellites were obtained by Karić et al. (2018), Abdou et al. (2016), and Simó et al. (2013). Heterozygosity estimates in tropical onions were reported by Jayaswall et al. (2019) and Santos et al. (2010). According to Currah (1990), high levels of heterozygosity within onion populations favor greater vigor and, therefore, higher yields in OP populations. Heterozygosity estimates have not yet been reported for tropical onions in Northeastern Brazil.

The present study aimed to estimate the cross-pollination and heterozygosity rates using a morphological marker and microsatellite loci in tropical onion populations to guide breeding programs for this important vegetable.

Material and Methods

Plant material and DNA extraction

Bulbs of eleven onion parents (Table 1) from the germplasm collection of Embrapa Semiárido (Petrolina, PE, Brazil), vernalized in a cold chamber, were used for pair-crossing. Alternate rows (1:1) of parents were adopted at a spacing of 1.0 m × 0.5 m, with one bulb per hole. Each pair of parents was isolated by a minimum distance of 600 m to avoid cross-pollination between different pairs. There was no spraying with insecticides during seed production in order to ensure the presence of pollinating insects. The seeds produced by the female parent plants were stored separately to establish individual progenies.

Table 1. Identification and bulb color of the genotypes used to form onion populations.

Genotypes	Bulb color
'BRS Alfa São Francisco'	Yellow
'IPA 11'	Yellow
'Alfa SF RT'	Yellow
'BRS Carrancas'	Dark red
'BRS Rio Vale'	Yellow
'Botucatu'	Yellow
'Cascuda T6'	Yellow
'Cascuda T7'	Yellow
'IPA 10'	Dark red
'IPA 11'	Yellow
'BRS Alfa São Francisco'	Yellow

The seeds harvested from the female parents were sown in polystyrene trays containing black substrate, where they grew until reaching the appropriate stage for transplantation. The trays were placed in a nursery and irrigated daily with micro-sprinklers. Transplantation occurred after 35 days to 1.2 m wide soil beds at a spacing of 0.12 m x 0.08 m. Irrigation and crop management were the same as those usually adopted for onion production in the region (Ferreira et al., 2017).

Young leaves were collected from the parents during the growth period for seed production and from the progenies during growth to produce the bulbs. The leaves were stored at -80°C for later DNA extraction.

Leaf samples from seven plants of each progeny and parent were collected 30-40 days after sowing to constitute a bulk for total DNA extraction. The protocol for DNA extraction was the 2x CTAB method modified to 10,500 and 12,500 rpm in the first and second centrifugations, respectively, with 2% beta-mercaptoethanol incubation at 60°C for 30 minutes for all samples (Ferreira & Santos, 2018). The DNA concentration extracted from each sample was determined by direct reading with a Multiskan Spectrophotometer (Thermo Scientific) at absorbance ratios of 260/280 nm. DNA quality was verified on 0.8% agarose gel followed by genomic DNA dilution to 30 ng μL^{-1}.

PCR and microsatellite analysis

Parental genotyping was used to detect polymorphic loci using 50 microsatellites, 30 of which were published by Fischer & Bachmann (2000) and 20 by Jakse et al. (2005). Of the 50 microsatellite loci, eight showed polymorphism, and four that had at least one exclusive allele in the male parent were selected.

The polymerase chain reaction (PCR) described by Fischer & Bachmann (2000) was carried out in a final volume of 20 µL, which contained 30 ng of genomic DNA, 1x of PCR buffer, 2.0 mM $MgCl_2$, 0.22 mM of each dNTP, 0.53 µM of each primer, and 0.4 units of the Taq DNA Polymerase enzyme. The thermocycler program for the amplifications consisted of initial denaturation at 94°C for 2 minutes, 94°C for 40 seconds, 58°C for 45 seconds, 72°C for 60 seconds, followed by 34 cycles and a final extension step at 72°C for 5 minutes. The PCR amplification reaction described by Jakse et al. (2005) was carried out in a final volume of 15 µL, which contained 30 ng of genomic DNA, 1x of PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.4 units of the Taq DNA Polymerase enzyme. PCR programming was as follows: initial denaturation at 95°C for 4 minutes, 95°C for 30 seconds, 58°C for 45 seconds, 72°C for 60 seconds, followed by 28 cycles and a final extension step at 72°C for 8 minutes.

After amplification, the PCR reactions were

heated for 3 minutes at 94ºC in denaturing formamide buffer and then immediately placed on ice before application to the polyacrylamide gel. The gels were stained with silver nitrate, as described by Ferreira & Santos (2018).

Statistical analysis

The outcrossing rate was obtained based on the method described by Jain (1979) as the ratio of the number of heterozygotes (a) to the number of heterozygous individuals (a) plus the number of recessive homozygotes (b). The formula is expressed as T (%) = (a / a + b) * 100.

Microsatellite loci were used to analyze the progenies of the following yellow × yellow bulb crosses: 'BRS Rio Vale' × 'Botucatu', 'BRS Alfa São Francisco' × 'IPA 11', 'Cascuda T6' × 'Botucatu', and 'Alfa SF RT' × 'BRS Alfa São Francisco', while the red bulb morphological marker was applied to the following progenies: 'BRS Rio Vale' × 'BRS Carrancas', 'Botucatu' × 'BRS Carrancas', 'IPA 11' × 'IPA 10', and 'BRS Alfa São Francisco' × 'IPA10' (Table 1).

Hybrid plants were identified by 1) counting the male parent allele in microsatellite loci among the progenies of yellow × yellow bulb crosses, as applied by Santos and Lima Neto (2011) in *Mangifera indica*, and by 2) the presence of red bulbs among the progenies of yellow × red bulb crosses, as discussed by Jain (1979). According to Kim et al. (2004), F_1 derived from crosses between doubled haploid yellow and red onion parents were always red, though the intensity of the red color was lighter than in red parents, indicating that the red phenotype is partially dominant over yellow.

Heterozygosity was estimated as described by Weir (1996), according to whom heterozygosity represents the frequency of heterozygotes observed in a population or the frequency of heterozygous alleles over all alleles. The software PowerMarker was used for this analysis (Liu & Muse, 2005).

Results

Population sizes were greater in the four progenies analyzed morphologically according to bulb color, with 242 plants on average, than in the four progenies analyzed by microsatellite loci, with 52 plants on average (Table 2). The small number in the second situation was due to limiting conditions during electrophoresis, and it did not interfere with the results since the traits analyzed are single inherited, following a simple Mendelian proportion.

Table 2. Estimation of cross-pollination rates (CPR) in onion populations based on microsatellite loci and bulb color.

Marker	Cross	Loci	TNP	TNH	CPR (%)
Microsatellite	'BRS Alfa São Francisco'×'IPA 11'	4^B	54	22	40
Microsatellite	'Alfa SF RT' × 'BRS A. São Francisco'	91^B	55	18	33
Microsatellite	'BRS Rio Vale' × 'Botucatu'	3^A, 4^B, 6^B	55	39	71
Microsatellite	'Cascuda T6' × 'Botucatu'	6^B	52	14	27
Bulb color	'BRS Rio Vale' × 'BRS Carrancas'	-	267	40	15
Bulb color	'Botucatu' × 'BRS Carrancas'	-	399	155	39
Bulb color	'IPA 11' × 'IPA 10'	-	101	38	37
Bulb color	'BRS Alfa São Francisco' × 'IPA10'	-	199	44	22

[A]=AMS loci. [B]=ACM loci. TNP=Total number of plants. TNH=Total number of hybrids.

No lack of flowering synchronization was observed in the crossing phase between plants of different pairs, thus not interfering with the cross-pollination estimates. The paired management of the genotypes in the absence of insecticides also contributed to the satisfactory presence of pollinator insects, especially *A. mellifera*, which is common in the experimental areas.

Cross-pollination rates based on bulb color and microsatellite loci

The progenies of four crosses were identified by directly counting the number of red bulbs among the bulbs that originated from crosses involving a yellow bulb maternal plant. A total of 40, 155, 38, and 44 red bulb plants resulted from the 'BRS Rio Vale' × 'BRS Carrancas, 'Botucatu' × 'BRS Carrancas', 'IPA 11' × 'IPA 10', and 'BRS Alfa São Francisco'×'IPA 10' crosses, respectively, resulting in cross-pollination estimates of 15%, 39%, 37%, and 22% (Table 2), with an average of 28.25%. The highest rates were observed in the 'Botucatu' × 'BRS Carrancas' and 'IPA 11' × 'IPA 10' crosses, with 39% and 37%, respectively.

The progenies of the other four crosses were identified by the presence of exclusive alleles from the male parent. A total of 22, 18, 39, and 14 hybrid plants were identified in the 'BRS Alfa São Francisco' × 'IPA 11', 'Alfa SF RT' × 'BRS Alfa São Francisco', 'BRS Rio Vale' × 'Botucatu', and 'Cascuda T6' × 'Botucatu' crosses, resulting in cross-pollination estimates of 40%, 33%, 71%, and 27% (Table 2), with an average of 42.75%.

The highest cross-pollination rate was observed in the 'BRS Rio Vale' × 'Botucatu' progeny, evaluated based on three microsatellite loci, including AMS 03, showing homozygosis in both parents and heterozygosis in the

progeny, while the other three progenies were evaluated based on only one microsatellite locus (Table 2).

Microsatellite loci, onion populations, and progeny heterozygosity estimates

Of the 50 microsatellite loci used to evaluate eight parents, 28 had 'amplicons' easily identified in the gels. The average heterozygosity value (H) of the 28 microsatellite loci was 0.87, ranging from 0.37 to 1.0, with the lowest values being observed in the ACM 138 and ACM 152 loci. The ACM 169, ACM 078, ACM 134, ACM 045, AMS 03, and ACM 068 loci showed H<1, while the other loci had H=1 (Table 3). The eight onion populations showed heterozygosity rates ranging from 0.82 to 1.0, with the highest value observed in 'BRS Alfa São Francisco' and 'Alfa SF RT' populations, while the lowest value was observed in 'Cascuda T7' (Table 3).

Table 3. Allelic pattern and heterozygosity rates (HR) and populations[1 to 8] of tropical onions genotyped with 28 microsatellite loci.

Loci	1	2	3	4	5	6	7	8	HR
AMS03	537/524	524/487	524/487	524/524	524/524	537/524	537/524	537/524	0.7
AMS04	544/530	544/530	544/530	544/530	544/530	544/530	544/530	544/530	1.0
AMS08	460/450	460/450	460/450	460/450	460/450	460/450	460/450	460/450	1.0
AMS10	231/223	231/223	231/223	231/223	231/223	231/223	231/223	231/223	1.0
AMS12	737/707	737/707	737/707	737/707	737/707	737/707	737/707	737/707	1.0
AMS14	364/349	364/349	364/349	364/349	364/349	364/349	364/349	364/349	1.0
AMS17	507/495	507/495	507/495	507/495	507/495	507/495	507/495	507/495	1.0
AMS20	611/597	611/597	611/597	611/597	611/597	611/597	611/597	611/597	1.0
AMS21	611/597	611/597	611/597	611/597	611/597	611/597	611/597	611/597	1.0
AMS22	358/350	358/350	358/350	358/350	358/350	358/350	358/350	358/350	1.0
ACM04	606/589	651/632	606/589	632/589	606/589	632/606	632/606	632/606	1.0
ACM006	539/525	576/553	539/525	576/553	576/553	553/539	539/525	539/525	1.0
ACM024	286/273	273/261	273/261	286/273	273/261	286/273	286/273	286/273	1.0
ACM045	797/772	797/772	797/772	797/772	797/772	797/797	797/797	797/797	0.6
ACM068	916/883	916/883	916/883	916/883	883/883	916/883	916/883	916/883	0.9
ACM071	328/321	328/321	328/321	335/328	335/328	328/321	328/321	328/321	1.0
ACM078	952/952	952/952	952/952	952/899	952/952	952/899	952/899	952/899	0.5
ACM091	433/412	433/412	412/394	433/412	412/394	433/412	433/412	433/412	1.0
ACM101	586/570	586/570	586/570	570/563	570/563	570/563	570/563	570/563	1.0
ACM102	314/311	314/311	314/311	314/311	314/311	314/311	314/311	314/311	1.0
ACM119	648/639	695/685	648/639	648/639	648/639	648/639	648/639	648/639	1.0
ACM124	555/541	555/541	555/541	555/541	555/541	555/541	555/541	555/541	1.0
ACM132	506/485	506/485	506/485	506/485	527/485	506/485	506/485	506/485	1.0
ACM134	390/384	422/384	405/384	405/384	422/415	405/405	405/405	405/405	0.6
ACM138	616/543	543/543	568/543	543/543	568/543	543/543	543/543	543/543	0.4
ACM152	596/577	577/577	596/577	596/577	577/577	577/577	577/577	577/577	0.4
ACM169	692/671	692/692	692/671	739/692	739/739	692/692	739/692	692/692	0.5
ACM187	547/532	547/532	547/532	547/532	547/532	547/532	547/532	547/532	1.0
HR	0.96	0.86	0.96	0.93	0.82	0.82	0.86	0.82	0.87 / 0.88

1='BRS Alfa São Francisco'; 2='IPA 11'; 3='Alfa SF RT', 4='BRS Rio Vale'; 5='BRS Carrancas'; 6='Botucatu'; 7='Cascuda T6'; 8='Cascuda T7'.

Complete heterozygosity was observed in the progenies of the 'BRS Alfa São Francisco' × 'IPA 11', 'BRS Rio Vale' × 'Botucatu', and 'Cascuda T6' × 'Botucatu' crosses, while the 'Alfa SF RT' × 'BRS Alfa São Francisco' progeny showed a heterozygosity value of only 0.50 (Table 4).

Table 4. Heterozygosity rates (H) in four onion populations based on microsatellite loci.

Population	Loci	H
'BRS Alfa São Francisco' × Vale Ouro 'IPA 11'	AMS 03	1.00
'Alfa SF RT' × 'BRS Alfa São Francisco'	ACM 004	0.50
'Cascuda T6' × 'Botucatu'	ACM 006	1.00
'BRS Rio Vale' × 'Botucatu'	AMS 03	0.93
'BRS Rio Vale' × 'Botucatu'	ACM 004	1.00
'BRS Rio Vale' × 'Botucatu'	ACM006	1.00

Discussion

Coss-pollination estimates based on bulb color ranged from 15% to 39%, with an average of 28.2%, while the estimates based on microsatellite loci ranged from 33% to 71%, with an average of 42.7%, indicating both cross-pollination and self-pollination within eight tropical onion populations. These values are close to the ones obtained by van Der Meer & van Bennekom (1972), whose results ranged from 23 to 56% in experiments conducted in an open field in the Netherlands using red bulb color as a phenotypic marker in the Rijnsburger yellow bulb onion population. Our estimates are also close to the 66% outcrossing rate reported by Havey (2018) when caging together male-fertile plants of different bulb colors and

introducing house flies as pollinators. According to Currah & Ockendon (1978), low cross-pollination rates may be associated with the lack of flowering synchrony or the absence of pollinating insects.

The average value of the estimates obtained using microsatellite loci, 42.7%, was higher than the average value using the bulb color phenotypic marker, 28.2%, with the former being impacted by the high pollination rate estimated in the 'BRS Rio Vale' × 'Botucatu' progeny, evaluated based on three microsatellite loci. Both the morphological marker in four populations and the microsatellite loci in three populations allow unilocus estimates of the crossing rate, which are less accurate than multilocus estimates. For Allard (1999), multilocus estimates are more accurate than estimates obtained using a single molecular marker.

It should be emphasized that, prior to the application of isoenzymatic markers to estimate crossing rates, studies were carried out with phenotypic markers such as flower color, bulb color, and stem color, among others (Jain, 1979), all of which are of simple inheritance or unilocus. The parents used in the present study were true lines for red or yellow bulbs since bulbs of different colors were not identified during seed multiplication.

Allium cepa is well known as a cross-pollinated species (Jo et al., 2017). However, it also has hermaphrodite and male flowers in the same inflorescence. According to Currah (1990), the anthers of individual flowers ripen and lose their pollen before the stigmas are fully receptive, a phenomenon known as protandry. However, for Currah (2002), these plants are perfectly capable of self-pollinating. Thus, it can be deduced that the low rates estimated in the present study agree with the floral characteristics of the species, favoring self-pollination.

The low number of microsatellites used to estimate cross-pollination in this study is associated with the low allelic diversity of parents and the high heterozygosity rates of both parents. The absence of rare alleles or homozygosity in one of the parents also contributed to the unexpected number of loci to estimate multilocus outcrossing rates. Santos & Lima Neto (2011) adopted this strategy to estimate multilocus cross-pollination rates in mango.

Heterozygosity was high among the progenies, ranging from 0.93 to 1.0, except for the 'Alfa SF RT' × 'BRS Alfa São Francisco' progeny, 0.50. The parents of the 'BRS Alfa São Francisco' × 'IPA 11', 'Cascuda T6' × 'Botucatu', and 'BRS Rio Vale' × 'Botucatu' progenies are of different origins and breeding programs, explaining their high heterozygosity. The 'Alfa SF RT' population was originated from recurrent selection within the 'BRS Alfa São Francisco' population, which may explain the low heterozygosity of the 'Alfa SF RT' × 'BRS Alfa São Francisco' progeny, 0.50.

The heterozygosity values reported by Simó et al. (2013) suggest that heterozygosity around 50% occurs in predominantly allogamous populations. Simó et al. (2013) reported heterozygosity ranging from 0.15 to 0.66 in Spanish onion populations, below the values estimated in the present study. Still according to Simó et al. (2013), high heterozygosity could be applied to develop hybrids, exploiting the vigor of heterosis. Another impact of high heterozygosity is related to the development of OP populations since a high frequency of intra-population heterozygotes can act as a buffer to biotic and abiotic adversities, highlighting the potential of the regional OP population.

The AMS 03, ACM 004, ACM 006, and ACM091 microsatellite loci are recommended for the identification of hybrids resulting from natural or artificial pollination in the 'BRS Alfa São Francisco' × 'IPA 11', 'Alfa SF RT' × 'BRS Alfa São Francisco', 'BRS Rio Vale' × 'Botucatu', and 'Cascuda T6' × 'Botucatu' crosses. This pioneering study with four tropical onion populations presents cross-pollination and heterozygosity estimates based on the direct counting of microsatellite alleles. This information will contribute to guiding onion improvement studies, such as the development of hybrids, synthetic varieties, and OPs of this vegetable of great importance for Northeastern Brazil.

Conclusions

1. The outcrossing estimates based on the phenotypic marker of bulb color ranged from 15% to 39%, with an average value of 28.2%, while the estimates based on microsatellite loci ranged from 33% to 71%, with an average value of 42.7%, indicating both cross-pollination and self-pollination within eight tropical onion populations;

2. The heterozygosity estimates in eight onion populations ranged from 0.82 to 1.0, suggesting the development of open pollination (OP) populations with high productive potential.

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