

ORIGINAL ARTICLE

Inferring mating system parameters in forage peanut, *Arachis pintoi*, for Brazilian Amazon conditions

Jônatas Chagas de OLIVEIRA¹ , Polinar Bandeira RUFINO², Hellen Sandra Freires da Silva AZÊVEDO³, Adna Cristina Barbosa de SOUSA⁴, Giselle Mariano Lessa de ASSIS⁵, Lucielio Manoel da SILVA⁵, Alexandre Magno SEBBENN⁶, Tatiana de CAMPOS^{5*}

¹ Universidade Federal do Acre, BR 364, km 4, Distrito Industrial, CEP 69920-900, Rio Branco, Acre, Brasil

² Faculdade Meta, Estrada Alberto Torres, Bairro da Paz, Rio Branco, Acre, Brasil

³ Fundação Oswaldo Cruz, Rua da Beira, BR 364, km 3,5, CEP 76812-245, Porto Velho, Rondônia, Brasil

⁴ Universidade Federal da Paraíba, Cidade Universitária, s/n - Castelo Branco III, CEP 58051-085, João Pessoa, Paraíba, Brasil

⁵ Empresa Brasileira de Pesquisa Agropecuária, Embrapa Acre, Rodovia BR-364, km 14, CEP 69900-970, Rio Branco, Acre, Brasil

⁶ Instituto Florestal de São Paulo, Rua do Horto, 931, CEP 02377-000, São Paulo, SP, Brasil

* Corresponding author: tatiana.campos@embrapa.br;  <https://orcid.org/0000-0001-7421-718X>

ABSTRACT

The search for alternatives to increase productivity and sustainability of livestock production in the Amazon region without increasing deforestation is challenging. Mixed pastures of grasses with forage peanut (*Arachis pintoi*) have shown positive economic impacts. However, gaps in the knowledge of the reproductive biology of *A. pintoi* have limited the development of new cultivars adapted to the environmental variations in the Brazilian Amazon. Pasture consortiums of *Brachiaria humidicola* with forage peanuts (cv. Mandobi) resulted in a 42% increase in weight gain productivity. New cultivars better adapted to the Amazon climate should bring even greater gains. We evaluated the mating system in twenty *A. pintoi* accessions, and approximately 40 offspring per accession genotyped with eight microsatellites (or markers). The parameters of genetic diversity and inbreeding, the outcrossing rate and coancestry were calculated. The observed heterozygosity was significantly higher and the fixation index was significantly lower in adults compared with the offspring. The crossing rate was variable among genotypes (2 to 80%), and the mean outcrossing rate was 36%. These results indicate that pollinator presence in pastures can influence gene flow in *A. pintoi* more than expected. *Arachis pintoi* presented a mixed mating system with a predominance of selfing, and families presented inbreeding and different levels of relatedness. New strategies of genotype conservation are needed to avoid pollinator-mediated crossing between accessions.

KEYWORDS: reproductive system, *Arachis pintoi*, SSR, crossing rate

Inferindo parâmetros do sistema de cruzamento em amendoim forrageiro, *Arachis pintoi*, para as condições da Amazônia brasileira

RESUMO

A busca por alternativas para aumentar a produtividade e a sustentabilidade da pecuária na região Amazônica sem aumentar o desmatamento é desafiadora. Pastagens consorciadas de gramíneas com amendoim forrageiro (*Arachis pintoi*) têm mostrado impactos econômicos positivos. Porém, lacunas no conhecimento da biologia reprodutiva do amendoim forrageiro tem limitado o desenvolvimento de novas cultivares adaptadas as variações ambientais na Amazônia brasileira. Pastos consorciados de *Brachiaria humidicola* com amendoim forrageiro (cv. Mandobi) apresentaram cerca de 42% de aumento na produtividade do ganho de peso. Novas cultivares mais adaptadas ao clima amazônico, poderão trazer ganhos ainda maiores. O objetivo desse estudo foi avaliar o sistema reprodutivo em vinte acessos de *A. pintoi* e aproximadamente 40 progênies por acesso, genotipadas com oito microssatélites (ou marcadores). Os parâmetros de diversidade genética e endogamia, taxa de cruzamento e coancestria foram calculados. A heterozigosidade observada foi significativamente maior e o índice de fixação foi significativamente menor nos adultos comparado às progênies. A taxa de cruzamento foi variável entre os genótipos (2 a 80%) e a média da taxa de cruzamento foi 36%. Esses resultados indicam que a presença de polinizadores em pastagens pode influenciar o fluxo gênico em *A. pintoi* mais do que o esperado. *Arachis pintoi* apresentou um sistema de cruzamento misto com predominância de autofecundação e as famílias apresentaram endogamia e diferentes níveis de parentesco. Novas estratégias de conservação de genótipos são necessárias para evitar polinizadores mediadores de cruzamento entre os acessos.

PALAVRAS-CHAVE: sistema reprodutivo, *Arachis pintoi*, SSR, taxa de cruzamento

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INTRODUCTION

Brazil has the second largest cattle herd in the world, with 39% of the cattle population located in the legal Amazon (the region comprising the Brazilian states of Acre, Amapá, Pará, Amazonas, Rondônia, Roraima, Mato Grosso, Tocantins and Maranhão), which makes livestock farming an important economic activity in the region (Ferraz and Felício 2010; IBGE 2017). However, the growing demand for sustainable livestock production and reduction of deforestation in the Amazon presents a number of challenges (Dias Filho 2011; Mazzetto *et al.* 2015; Zu Ermgassen *et al.* 2018). Among the alternatives for sustainable livestock production, the use of mixed pastures of grasses with forage peanuts (*Arachis pintoi* Krapov and W.C. Greg.) has generated positive results, with an economic impact of R\$ 104 million (US\$ 27 million) in the state of Acre (Embrapa 2018), due to a 42% increase in weight gain per animal compared to grass-only pastures (Urbanski 2016). Mixed pastures also contribute to soil fertility through biological nitrogen fixation, promoting the recovery of degraded areas (Barcellos *et al.* 2008; Garcia *et al.* 2017; Zu Ermgassen *et al.* 2018). Despite these advantages, few forage peanut cultivars are commercially available. Therefore, the development of new cultivars adapted to regional environmental conditions is of great importance (Simeão *et al.* 2017), and requires knowledge on the reproductive biology of forage peanut. The mating system of *A. pintoi* has not been investigated in terms of outcrossing, mating among related individuals and correlated mating. Most wild species of *Arachis* exhibit a floral biology that favors self-fertilization, given that their flowers are hermaphrodites that exhibit self-pollination (Krapovickas and Gregory 2007). However, cross-pollination can occur through the action of pollinator vectors, such as bees of the genus *Centris*, *Trigona*, *Megachile* and *Apis mellifera* (Drumond and Cardoso 2010). Mating system studies in *Arachis* are limited to *A. hypogaea* and are based on morphological markers, revealing a low outcrossing rate that ranged from 1.9 to 8% (Coffelt 1989; Knauff *et al.* 1992). Oliveira and Valls (2003) also used morphological markers to evaluate the reproductive system in five accessions of *A. pintoi* and two of *A. repens*. They detected the presence of autogamy and self-incompatibility in *A. pintoi*, and allogamy in *A. repens* accessions. Mating system studies based on markers, such as microsatellite loci or simple sequence repeats (SSR), allow the determination of the outcrossing rate, mating among relative individuals and correlated mating, as well as the levels of genetic diversity, inbreeding and relatedness within families (Degen and Sebbenn 2014). Therefore, the main aim of this study was to quantify rates of outcrossing, mating among relative individuals and correlated mating in *A. pintoi* using microsatellite markers. We also evaluated the relatedness among plants of an active germplasm bank (AGB) located at Embrapa Acre, which has 139 accesses and is a basis of the forage peanut breeding program.

MATERIAL AND METHODS

Plant material

The study was performed using 20 accessions (V 6727, cv. Alqueire, V 14951, V 13330, V 6741, V 6784, W 34, W 647, V 5895, V 6740, V 13196, V 13198, V 6791wf, cv. Belomonte, Sv 8311, W 1000, cv. Mandobi, cv. Amarillo MG-100, V 13888, and W 34B) of *A. pintoi* from the AGB at Embrapa Acre, Acre state, Brazil. The matrices represent the commercial cultivars available in Brazil and the higher seed producers in the AGB. The 20 matrices were planted by stolon in vessels of 1.0 x 0.9 m in December 2012. The vessels were placed in an open field about 3 m distant from the AGB. Considering the variable maturation period required to obtain sufficient seed numbers per matrix, seeds were collected twice, eight and 15 months after the matrices were planted. To obtain a minimum of 35 offspring per matrix, approximately 60 seeds per matrix were submitted to a breaking dormancy process (Assis *et al.* 2015) and were planted in sterile sand.

DNA extraction and SSR genotyping

DNA extraction, agarose quantification and assessment of eight microsatellites loci (Ah07, Ah11, Ah21, Ag39, Ag140, Ap40, Ap175, and Ap176) (Palmieri *et al.* 2002; Hoshino *et al.* 2006; Gimenes *et al.* 2007) were performed as described by Campos *et al.* (2016). Amplification reaction and genotyping were performed according to Azêvedo *et al.* (2017).

Statistical analysis

The genotypic disequilibrium between a pair of loci was calculated for the matrices. The statistical significance was tested using 1,000 permutations of alleles among individuals and subjected to a Bonferroni correction for multiple tests (95%, $\alpha = 0.05$). For matrices and offspring, genetic diversity was characterized as a mean between all loci using the following indices: total number of alleles per loci (k), allelic richness (R) estimated using rarefaction, observed (H_o) and expected heterozygosity (H_e) under Hardy-Weinberg equilibrium. Inbreeding in the samples was estimated using the population fixation index (F). Statistical significance of F values was tested by 1,000 permutations of alleles among individuals and subjected to a Bonferroni correction for multiple tests (95%, $\alpha = 0.05$). All estimates were calculated using FSTAT software (Goudet 1995). The un-paired t-test was used to evaluate whether the indices of genetic diversity and inbreeding were significantly different between samples. The relatedness between pairwise matrices was estimated using the coancestry coefficient and Spagedi 1.3 software (Hardy and Vekemans 2002). We classified pairwise individuals as related groups, assuming related individuals if pairwise values were at least 0.05.

Mating system indices were estimated at the population and individual levels based on a mixed mating system and correlated mating model using the Expectation-Maximization

(EM) numerical method implemented in MLTR 3.4 software (Ritland 2002). The following indices were calculated at individual and population levels: the fixation index of matrices (F_m), multilocus outcrossing rate (t_m), single-locus outcrossing rate (t_s), rate of mating among relative individuals ($t_m - t_s$), and multilocus paternity correlation ($r_{p(m)}$). At the population level, we also estimated the correlation of t between families (r_f) and correlation of t between loci (r_l). The standard error of the indices was estimated by 1,000 bootstrap resampling within families. We also estimated the effective number of pollen donors ($N_{ep} = 1/r_p$) and the average coancestry coefficient within families: $\Theta = 0.125(1 + F_m)[4s + t_m^2(1 + r_p)]$, where F_m is the inbreeding coefficient in matrices and s is the selfing rate ($s = 1 - t_m$) (Sebbenn 2006). The effective size (N_e) within families was estimated from sample variance in the gene frequency as described by Tambarussi *et al.* (2015) and Wadt *et al.* (2015).

RESULTS

Genetic diversity

Of the 20 matrices, only 12 produced more than the minimum number established of 35 seeds. From 35 to 56 offspring per matrix were obtained for genotyping for a total of 531 individuals (Table 1).

The eight analyzed loci were polymorphic. The number of alleles per loci for the total samples of adults and offspring ranged from five to 19, totaling 83 different alleles. After the

Bonferroni correction, no genotypic disequilibrium between pairwise loci was detected for the matrices sampled. According to the t-test, the observed heterozygosity (H_o) was significantly higher in matrices (0.83) as compared with offspring (0.50), whereas the expected heterozygosity (H_e) was not significantly different between matrices (0.78) and offspring (0.73). The fixation index (F) was significantly lower in matrices (-0.07) as compared with offspring (0.31), and the value for offspring was greater than zero, indicating inbreeding.

Relatedness among accessions

Pairwise coancestry coefficients between matrices ranged from -0.162 to 0.211 and were negative in 56% of the 66 estimates, indicating the absence of relatedness in this case (Table 2). This finding confirmed the genetic diversity observed among the genotypes. Comparing pairwise estimates, and assuming related individuals if pairwise values were at least 0.05, four probable groups of related individuals were detected with coancestry ranging from 0.050 to 0.211. The groups were i) genotypes V 13196, V 13198, W 1000 and V 5895 with pairwise coancestry ranging from 0.050 to 0.190; ii) V 6727 and V 6784 with pairwise coancestry of 0.127; iii) V 6740, V 6791wf and Amarillo MG-100 with pairwise coancestry ranging from 0.124 to 0.211; iv) W 34, W 647 and V 13888 with pairwise coancestry ranging from 0.089 to 0.211.

Mating system

Multilocus outcrossing rate (t_m) was highly variable among families, ranging from 0.02 to 0.80 with a general rate of $t_m = 0.36$ (Table 3). The correlation of t among families ($r_f = 0.43$, SE = 0.04) indicated high variation in outcrossing rate among matrices. The correlation of t between the loci ($r_l = 0.52$, SE = 0.04) indicated that inbreeding in offspring originated from approximately 50% self-fertilization and 50% mating among related individuals. The rate of mating among related individuals ($t_m - t_s$) and the paternity correlation ($r_{p(m)}$) were also variable among matrices ($t_m - t_s: -0.24 - 0.37$; $r_{p(m)}: 0.10$

Table 1. Genetic diversity and inbreeding for matrices and offspring of forage peanut, *Arachis pintoi* accessions. P_{value} = probability of un-paired t-test.

Index	Matrices	Offspring	P_{value}
Sample size: n	12	531	-
Total number of alleles: k (range)	61 (4-13)	83 (5-19)	-
Allelic richness for 12 genotypes: R	7.6	6.4	0.153
Observed heterozygosity: H_o	0.83	0.50	< 0.001
Expected heterozygosity: H_e	0.78	0.73	0.312
Fixation index: F	-0.07	0.31	< 0.001

Table 2. Pairwise coancestry coefficient between accessions of forage peanut, *Arachis pintoi*.

	V6727	V6784	W34	W647	V5895	V6740	V13196	V13198	V6791wf	W1000	Amarillo MG-100
V6784	0.127										
W34	0.075	-0.033									
W647	0.036	-0.071	0.127								
V5895	-0.159	-0.016	-0.026	-0.023							
V6740	-0.016	0.002	-0.051	0.036	-0.075						
V13196	0.009	0.068	-0.110	-0.064	0.159	-0.033					
V13198	-0.044	0.057	-0.162	-0.075	0.106	-0.044	0.190				
V6791wf	0.040	0.016	-0.037	0.050	-0.061	0.124	-0.145	-0.071			
W1000	-0.016	-0.040	-0.009	-0.047	0.050	-0.058	0.134	0.165	-0.044		
Amarillo MG-100	0.029	0.005	-0.047	0.040	-0.071	0.197	-0.071	-0.082	0.211	-0.054	
V13888	-0.131	-0.113	0.211	0.089	0.103	0.036	-0.064	-0.033	0.009	-0.047	-0.044

Table 3. Estimates of individual and population mating system indices in forage peanut, *Arachis pintoi*. n = sample size; t_m = multilocus outcrossing rate; t_s = single locus outcrossing rate; $t_m - t_s$ = rate of mating among related individuals; $r_{p(m)}$ = multilocus paternity correlation; N_{ep} = effective number of pollen donors; Θ = coancestry coefficient within family; N_e = effective size; (SE) = standard error.

Matrix	n	t_m (SE)	t_s (SE)	$t_m - t_s$ (SE)	$r_{p(m)}$ (SE)	N_{ep}	Θ	N_e
V6727	42	0.70 (0.07)	0.33 (0.02)	0.37 (0.05)	0.86 (0.02)	1.2	0.264	1.86
V6784	41	0.62 (0.08)	0.52 (0.06)	0.10 (0.05)	0.19 (0.05)	5.2	0.249	1.96
W34	44	0.17 (0.05)	0.22 (0.03)	-0.05 (0.03)	0.18 (0.03)	5.6	0.419	1.19
W647	40	0.78 (0.06)	0.76 (0.05)	0.02 (0.04)	0.13 (0.03)	7.6	0.196	2.46
V5895	55	0.17 (0.05)	0.13 (0.01)	0.03 (0.06)	0.21 (0.05)	4.7	0.421	1.18
V6740	38	0.80 (0.07)	0.53 (0.04)	0.27 (0.05)	0.44 (0.06)	2.3	0.215	2.25
V13196	56	0.12 (0.04)	0.11 (0.01)	0.02 (0.03)	0.25 (0.06)	4.0	0.442	1.13
V13198	35	0.46 (0.08)	0.32 (0.02)	0.14 (0.06)	0.67 (0.03)	1.5	0.313	1.57
V6791wf	50	0.31 (0.07)	0.39 (0.04)	-0.08 (0.04)	0.15 (0.02)	6.6	0.360	1.38
W1000	46	0.26 (0.06)	0.33 (0.04)	-0.07 (0.04)	0.17 (0.03)	6.0	0.379	1.31
Amarillo MG-100	42	0.02 (0.00)	0.26 (0.00)	-0.24 (0.00)	0.10 (0.00)	9.9	0.492	1.02
V13888	42	0.27 (0.07)	0.32 (0.04)	-0.05 (0.04)	0.19 (0.05)	5.2	0.376	1.32
A. pintoi	531	0.36 (0.08)	0.18 (0.01)	0.18 (0.08)	0.83 (0.04)	1.2	0.372	1.33

- 0.86). The effective number of pollen donors (N_{ep}) ranged from 1.2 to 9.9 among matrices with a general rate of 1.2.

The mean population and family coancestry coefficients (Θ) were higher (mean: 0.372; range: 0.196 - 0.492), and the effective size (N_e) was lower (mean: 1.33; range: 1.02 - 2.46) than what would be expected in panmictic populations ($\Theta = 0.125$; $N_e = 4$).

DISCUSSION

The high frequency of heterozygous genotypes of *A. pintoi* was higher than expected for an autogamous species. Observed heterozygosity was increased compared with expected heterozygosity, confirming the presence of high genetic diversity in matrices. The same unexpected heterozygous profile was observed for *A. glabrata* (Angelici *et al.* 2008). These authors already considered that cross-pollination could be significant. The high heterozygosity in our accessions resulted from the combination of different alleles that were conserved through vegetative propagation (Palmieri *et al.* 2010).

Our estimated outcrossing rate was greater than expected because *Arachis* was once generally accepted as autogamous by analogy to *A. hypogaea*. Our results corroborate previous reports of heterozygous profiles (Angelici *et al.* 2008; Palmieri *et al.* 2010). Our large sample size of 531 progenies allowed to infer more precisely the crossing rate.

The correlation of t among families indicates high variation in outcrossing rate among matrices, which exhibited a predominance of mixed mating systems. However, some matrices were even close to allogamy ratios (V 6727, W 647 and V 6740). This variation directly interferes with the conservation of the germplasm collection, as the presence of pollinators may allow considerable gene flow and lead to loss of accession identity. As only matrices with sufficient seed production were

genotyped for this study, our estimated outcrossing ratio for *A. pintoi* is not representative of all accessions in the AGB of Embrapa Acre. The 12 matrices represent 8.6% of the AGB. Seed production is not a uniform trait among genotypes (Oliveira and Valls 2003). It also has to be considered that our estimated t_m value reflects the reproductive performance of the accessions under the experimental conditions used in this study, including the presence of a set of undetermined pollinators and the specific climatic conditions in 2012/2013. It will thus be important to repeatedly evaluate the mating systems of the accessions in different environmental conditions and geographic locations. Characteristics of the pollen grain, as pore number, also modulate the outcrossing rate and will be analyzed in future studies.

Studies using molecular markers to estimate the mating system in wild species of *Arachis* have not been published. Outcrossing rates using morphological markers were estimated at 1.9% and 8% in *A. hypogaea* cultivars as described by Coffelt (1989) and Knauft *et al.* (1992). Our estimates obtained from molecular markers have detected higher values compared with morphological traits. Similarly, another leguminosae forage, *Stylosanthes* was described as autogamous based on morphological marker analyses (Bray and Hutton 1976). However, studies using microsatellites reported more accurate estimates (Santos-Garcia *et al.* 2011; Sousa *et al.* 2012; Ramos *et al.* 2019), as the analyses are based on many loci, detect high polymorphism in any growth stage, and can be applied in many progeny samples. Thus, our results support molecular genotyping as a more precise method to evaluate mating systems.

The values of t_s , which were generally lower than those of t_m , confirm the occurrence of crosses between relatives, which leads to biparental endogamy. These values were also influenced by the self-fertilization rate. The high value for paternity correlation ($r_{p(m)}$) indicates that most offspring

originated from outcrossing are full-sibs due to the low effective number of pollen donors (1.2) that fertilized the mother plants. The receptivity of the stigma and the pollen viability in *A. pintoi* may vary throughout the day (Flores *et al.* 2015), which, in addition to the late dehiscence of the anthers in some accessions (Knauff *et al.* 1992), may cause the low number of effective pollen donors.

The high coancestry coefficients (Θ) and low effective size (N_e) were caused by the high selfing, the individual variation in the outcrossing rate, and the correlation of paternity. Frequency of identification, according to the descent alleles within progenies, and relatedness are increased by selfing and correlated with paternity (Sebbenn 2006; Spoladore *et al.* 2017).

Among the six forage-peanut cultivars included in the National Registry of Cultivars of the Brazilian Ministry of Agriculture, Livestock, and Food Supply (*Registro Nacional de Cultivares do Ministério da Agricultura, Pecuária e Abastecimento* – RNC-MAPA), Amarillo MG-100 is the most used commercially. Our t_m estimate for this cultivar was 2%, but only maternal alleles were identified in progeny genotyping. This very low crossing rate is very important to conserve the genotype identity. The features of floral biology, such as stigma surface and floral morphology, should be further investigated. Those traits could promote specific crosses between genotypes and generate genotypes with low crosses, which is desirable in an open field with pollinators present, as is the case in pastures.

CONCLUSIONS

Our analysis of the mating system in twenty accessions of forage peanut, *Arachis pintoi*, in an active germplasm bank in the southwestern Brazilian Amazon using genotyping with eight microsatellites, indicated the occurrence of a mixed mating system with a predominance of selfing, and that pollinators can influence *A. pintoi* gene flow more than expected. We detected inbreeding and different levels of relatedness within families. Variable outcrossing rates among genotypes and the presence of pollinators could produce crosses and recombinant seeds inside parcels of the bank, so that further studies are required to assure the adequate conservation of accession identity.

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