Genetic diversity of the forage peanut in the Jequitinhonha, São Francisco, and Paranã River valleys of Brazil

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ABSTRACT. *Arachis pintoi* and *A. repens* are legumes with a high forage value that are used to feed ruminants in consortium systems. Not only do they increase the persistence and quality of pastures, they are also used for ornamental and green cover. The objective of this study was to analyze microsatellite markers in order to access the genetic diversity of 65 forage peanut germplasm accessions in the section *Caulorrhizae* of the genus *Arachis* in the Jequitinhonha, São Francisco and Paranã River valleys of Brazil. Fifty-seven accessions of *A. pintoi*

and eight of *A. repens* were analyzed using 17 microsatellites, and the observed heterozygosity (H_O), expected heterozygosity (H_E), number of alleles per locus, discriminatory power, and polymorphism information content were all estimated. Ten loci (58.8%) were polymorphic, and 125 alleles were found in total. The H_E ranged from 0.30 to 0.94, and H_O values ranged from 0.03 to 0.88. By using Bayesian analysis, the accessions were genetically differentiated into three gene pools. Neither the unweighted pair group method with arithmetic mean nor a neighbor-joining analysis clustered samples into species, origin, or collection area. These results reveal a very weak genetic structure that does not form defined clusters, and that there is a high degree of similarity between the two species.

Key words: *Arachis pintoi*; *Arachis repens*; Forage legume; Molecular marker; SSR

INTRODUCTION

Brazilian livestock production in domestic and international markets has increased in recent years because of a favorable economic environment and market (Valentim and Andrade, 2009; Claudino et al., 2013). According to the Brazilian Ministry of Agriculture, Livestock, and Food Supply (2015), meat production will supply 44.5% of the world market's demand until 2020. However, vigor, pasture quality, and impacts on the environment are some of the factors that limit further increases in productivity.

Among the alternatives to recover degraded pastures and to ensure the sustainability of the soil, grasses intercropped with forage legumes is an important option. In 2013, legume cultivars (*Stylosanthes* spp 'Campo Grande' and *Arachis pintoi* 'Belomonte') occupied two million hectares and generated R$267 million for Brazilian cattle breeders (Valentim and Andrade, 2015).

The advantages of pasture diversification by introducing forage legumes in traditional production systems include a large dry matter yield, high nutritional value, and good biological nitrogen fixation in the soil, which reduce costs for farmers, increase the production of meat and milk, and prevent the opening up of new areas of forest (Carvalho and Pires, 2008; Azevedo et al., 2014).

The forage peanut species *A. pintoi* Krapov. & W.C. Greg. and *Arachis repens* Handro of the taxonomic section *Caulorrhizae* Krapov. & W.C. Greg. have several characteristics related to persistence under grazing and are a food source for animals in the crop-livestock system (Valentim and Andrade, 2015). *A. repens* has potential for forage use; however, it is most commonly used as an ornamental plant due to its good ground cover and fast growth (Azevedo et al., 2011).

The geographical distribution of section *Caulorrhizae* comprises the basins of the rivers Jequitinhonha, São Francisco, and Paranã, which reaches the Atlantic coast and covers parts of the Brazilian states of Goiás, Bahia, and Minas Gerais. Previous research has suggested that the greatest genetic variability in accessions of this section is concentrated in the São Francisco River basin (Gimenes et al., 2000).

Discrimination between original accessions of *A. pintoi* and *A. repens* is based on morphological characteristics, such as leaflet shape and size and bristle density and location (Krapovickas and Gregory, 1994; Monçato, 1995; Menezes et al., 2012). However, with the increase in the number of accessions obtained from germplasm collection expeditions in the

last 20 years, types with intermediate characteristics have been found (Monçato, 1995).

Despite the importance of morphological characteristics in the analysis of diversity, they also have limitations. In plants with a narrow genetic basis, they may not be sufficient for the clear-cut distinction of different genotypes. There is no consensus on the separation of *A. pintoi* and *A. repens* based on molecular studies. Gimenes et al. (2000) reported high similarity between accessions of *A. pintoi* and *A. repens*, whereas Palmieri et al. (2010) did not find any groups that corresponded to the separation of the two species.

Microsatellite markers (simple sequence repeats, SSRs) have been successfully used in characterizing plant diversity (Jones et al., 2009), and have detected high variability among the accessions of section *Caulorrhizae* (Palmieri et al., 2002; Gimenes et al., 2007). Microsatellite loci exhibit high indices of polymorphism and transferability within the genus *Arachis*, allowing their use in inter- and intraspecific genetic studies (Palmieri et al., 2002; Moretzsohn et al., 2005; Bravo et al., 2006; Gimenes et al., 2007; Angelici et al., 2008).

A. pintoi and *A. repens* are important genetic resources with potential economic value for agricultural use (Valls, 2005). Therefore, the conservation and use of, and access to, genetic variability are crucial in forage peanut breeding programs (Assis and Valentim, 2009). The objective of this study was to analyze the genetic diversity of 65 accessions of section *Caulorrhizae* collected in the valleys of the Jequitinhonha, São Francisco, and Paranã rivers using microsatellite markers.

MATERIAL AND METHODS

Plant material

We evaluated 57 accessions of *A. pintoi* and eight of *A. repens* obtained from the Active Germplasm Bank of Forage Peanut, Embrapa Acre, Brazil (Table 1). Accession collection started in the early 1980s, and covered the natural geographical distribution of the species along the Jequitinhonha, São Francisco, and Paranã rivers in Brazil (Figure 1).

Table 1. Origin of 65 accessions from the Forage Peanut Active Germplasm Bank (*Arachis pintoi* and *Arachis repens*), Embrapa Acre, Brazil.

Code	Species	Brazilian accession code (BRA)	Collection site**	State*	Code	Species	BRA	Collection site**	State*	Code	Species	BRA	Collection site**	State*
1	*A. pintoi*	014931	J	MG	23	*A. pintoi*	031135	P	GO	45	*A. pintoi*	030325	SF	MG
2	*A. pintoi*	015253	J	MG	24	*A. pintoi*	036544	P	GO	46	*A. pintoi*	030601	SF	MG
3	*A. pintoi*	'Belomonte'	J	BA	25	*A. pintoi*	034355	P	GO	47	*A. pintoi*	030635	SF	MG
4	*A. pintoi*	031526	J	BA	26	*A. pintoi*	030872	P	GO	48	*A. pintoi*	031461	SF	MG
5	*A. pintoi*	'Amarillo'	J	BA	27	*A. pintoi*	030899	P	GO	49	*A. pintoi*	030384	SF	MG
6	*A. pintoi*	NI	J	BA	28	*A. pintoi*	030945	P	GO	50	*A. pintoi*	022683	SF	MG
7	*A. pintoi*	016357	J	MG	29	*A. pintoi*	030929	P	GO	51	*A. pintoi*	032433	SF	MG
8	*A. pintoi*	031895	J	BA+	30	*A. pintoi*	015121	P	GO	52	*A. pintoi*	030392	SF	MG
9	*A. pintoi*	031143	J	MG	31	*A. pintoi*	034347	P	GO	53	*A. pintoi*	030490	SF	MG
10	*A. pintoi*	038900	J	SP	32	*A. pintoi*	NI	P	NI	54	*A. pintoi*	032441	SF	MG
11	*A. pintoi*	038900	J	SP	33	*A. pintoi*	NI	P	NI	55	*A. pintoi*	NI	SF	NI
12	*A. pintoi*	040894	P	GO	34	*A. pintoi*	NI	P	NI	56	*A. pintoi*	038910	SF	MG
13	*A. pintoi*	030333	P	GO	35	*A. pintoi*	NI	P	NI	57	*A. pintoi*	030635	SF	MG
14	*A. pintoi*	034142	P	GO	36	*A. pintoi*	039187	SF	BA	58	*A. repens*	032387	SF	MG
15	*A. pintoi*	031909	P	GO	37	*A. pintoi*	014991	SF	MG	59	*A. repens*	032280	SF	MG
16	*A. pintoi*	031097	P	GO	38	*A. pintoi*	015083	SF	BA	60	*A. repens*	029220	SF	MG
17	*A. pintoi*	031275	P	GO	39	*A. pintoi*	032344	SF	MG	61	*A. repens*	032352	SF	MG
18	*A. pintoi*	034100	P	GO	40	*A. pintoi*	032409	SF	MG	62	*A. repens*	032379	SF	MG
19	*A. pintoi*	039772	P	GO	41	*A. pintoi*	032450	SF	MG	63	*A. repens*	029190	SF	MG
20	*A. pintoi*	031984	P	GO	42	*A. pintoi*	033481	SF	MG	64	*A. repens*	014788	SF	MG
21	*A. pintoi*	015121	P	GO	43	*A. pintoi*	012122	SF	MG	65	*A. repens*	014770	SF	MG
22	*A. pintoi*	034193	P	GO	44	*A. pintoi*	014982	SF	MG					

NI, no information. *Brazilian states: BA, Bahia; MG, Minas Gerais; GO, Goiás. + [BRA-031895: autotriploid accession (2n = 30) naturally appearing in GK 12787 field plot at San José, Costa Rica, and identified as distinct by Pedro Argel/CIAT]. **Collection sites: J, Jequitinhonha; P, Paranã; SF, São Francisco.

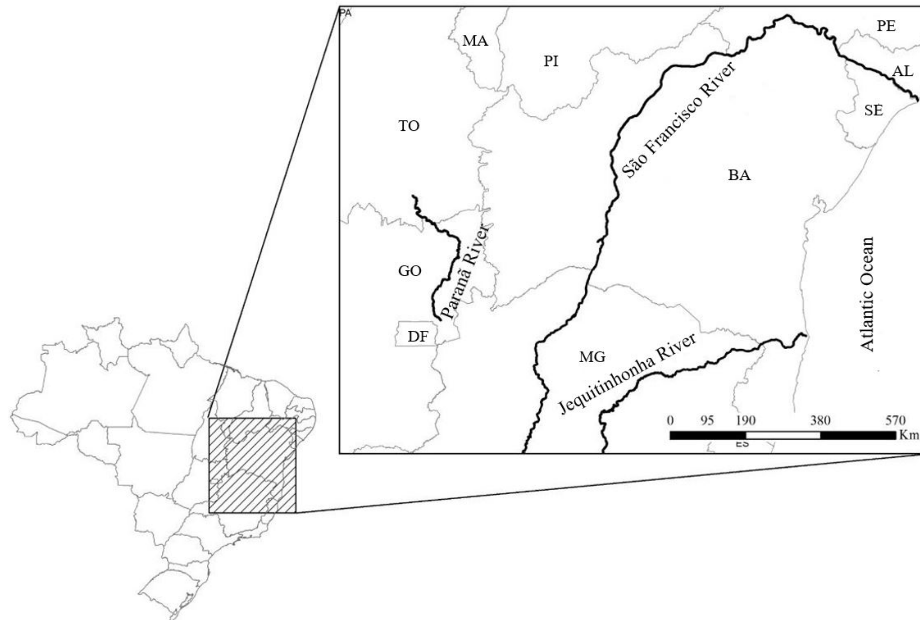

Figure 1. Geographical locations of the Jequitinhonha, São Francisco, and Paranã River valleys in Brazil.

DNA extraction and microsatellite loci

Molecular analyses were performed in the Morphogenesis and Molecular Biology Laboratory (LabMol) at Embrapa Acre. Total genomic DNA was extracted from fresh young leaves using the protocol described by Hoisington et al. (1994), and was diluted and quantified by comparison with standard DNA using agarose gel electrophoresis (1%).

Seventeen SSR markers that are described in the literature were tested and optimized for the annealing temperature. Of these, four loci were developed based on specific sequences for *A. hypogaea* L. (Ah) (Gimenes et al., 2007), 10 were developed for *A. pintoi* (Ap) (Palmieri et al., 2002), and three were developed for *Arachis glabrata* Benth. (Ag) (Hoshino et al., 2006). *A. hypogaea* and *A. glabrata* belong to the sections *Arachis* and *Rhizomatosae* Krapov. & W.C. Greg., respectively.

Statistical analysis

The following genetic diversity parameters were estimated: the observed heterozygosity (H_O), expected heterozygosity (H_E), number of alleles (N) per locus, and the polymorphism information content (PIC) using the Tools For Population Genetic Analyses (TFPGA) software (http://www.marksgeneticsoftware.net/tfpga.htm). The PIC was calculated according to the equation of Botstein et al. (1980):

$$Pic = 1 - \sum_{i=1}^{n} f_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 fi^2 fj^2$$

where f_i is the frequency of the ith allele and f_j is the frequency of the jth allele, summing over alleles.

To compare the efficiency of the markers to identify varieties, the power of discrimination (*D*) was estimated for each site based on the following equation described by Tessier et al. (1999):

$$D_k = 1 - \sum_{j-1}^{l} p_j \frac{Np_j - 1}{N - 1}$$

Genetic distances among the accessions were calculated using the modified Roger's distance method in TFPGA, and a dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) clustering criterion in NTSYSpc (http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html).

Clustering consistency was tested by resampling using 10,000 bootstraps in the program BOOD (Coelho, 2002). The consistency of the dendrogram was evaluated by a cophenetic correlation between the distances represented by the original dendrogram and the genetic distances between pairs of accessions. The significance of this correlation was tested using the Mantel test with 10,000 random permutations. The cophenetic correlation and its significance were calculated using NTSYSpc.

The Bayesian method implemented in the STRUCTURE software was used to investigate the genetic organization of the accessions. This analysis considers the separation of the total number of individuals analyzed in clusters, giving them a K value that represents the number of different gene pools, assuming Hardy-Weinberg equilibrium and a lack of linkage disequilibrium among the loci. Consequently, clusters of individuals sharing the same gene pool are inferred, with no need for prior information on its origin.

Analysis was performed on all of the accessions, and K ranged from 1 to 12. Five independent simulations were conducted for each K using the admixture model, independent allele frequencies, a burn-in of 10,000, and 10,000 Markov chain Monte Carlo iterations. The most probable K number was determined with delta K values according to Evanno et al. (2005) using STRUCTURE HARVESTER (Earl and vonHoldt, 2012). The accessions were allocated to clusters according to the probability of each individual belonging to each cluster. Cluster analysis was performed using the neighbor-joining (NJ) method in DARwin (http://darwin.cirad.fr/) using the same matrix as Roger's modified distance that was obtained from TFPGA.

RESULTS AND DISCUSSION

Seventeen microsatellite markers were tested. Only 10 loci (58.8%; Table 2) were polymorphic and produced definite bands for genotyping. The other loci were monomorphic or nonspecific (41.2%; Ap33, Ah2, Ah26, Ah30, Ap40, Ah51, and Ah126). A total of 125 alleles were found, with fragments ranging from 97 bp (Ah7) to 322 bp (Ap152) in length. *N* varied from four (Ap175) to 23 (Ap175), with an average of 12.5 alleles per locus. Palmieri et al. (2010) used 26 microsatellite markers and found 20 polymorphic loci, detecting a total of 196 alleles in 33 accessions of *A. pintoi* and 10 of *A. repens* with an average of 9.8 alleles per locus, a smaller number than reported here.

Table 2. Characterization of loci and annealing temperature (AT), allele length (bp), number of alleles per locus (*N*), expected heterozygosity (H_E), observed heterozygosity (H_O), polymorphism information content (PIC), and discriminatory power (*D*) for 65 accessions of forage peanuts (*Arachis pintoi* and *Arachis repens*) from the Active Germplasm Bank in Embrapa Acre, Brazil.

Locus	AT (°C)	Allele length (bp)	*N*	H_E	H_O	PIC	*D*
Ap152*	58.5	259-322	11	0.88	0.30	0.88	0.98
Ap175*	55.4	174-230	23	0.89	0.46	0.88	0.98
Ap176*	50.0	194-246	15	0.89	0.30	0.89	0.97
Ag39**	52.1	150-190	22	0.94	0.23	0.93	0.99
Ag140**	57.3	164-191	4	0.39	0.03	0.38	0.73
Ag171**	48.2	164-196	4	0.32	0.06	0.31	0.72
Ah6-125***	48.2	170-194	11	0.30	0.26	0.30	0.77
Ah7***	52.1	97-122	11	0.85	0.26	0.84	0.92
Ah21***	57.3	100-135	13	0.76	0.21	0.75	0.96
Ah282***	55.4	173-202	11	0.75	0.88	0.74	0.91
Total			125				
Mean			12.5	0.70	0.30	0.69	0.89

*Palmieri et al. (2002); **Hoshino et al. (2006); ***Gimenes et al. (2007). Specific sequences for *Arachis pintoi* (Ap), *A. glabrata* (Ag), and *A. hypogaea* (Ah).

PIC values ranged from 0.30 to 0.93, with a mean value of 0.69 (Table 2). Of the 10 loci analyzed, seven had PIC values greater than 0.5, which is considered highly informative for genetic diversity studies (Botstein et al., 1980).

Microsatellites are present both in coding and non-coding regions, and can be found in nuclear and organellar genomes (Kalia et al., 2011). The Ap176 locus was highly informative (PIC = 0.89), and had 92% identity and 90% similarity to an mRNA sequence (GW937987.1) isolated from a cDNA library of roots of *Arachis duranensis* Krapov. & W.C. Greg. Palmieri et al. (2010) found that the locus Ap176 is similar to a lipoxygenase enzyme (41% identity and 47% similarity). Therefore, the locus is closely related to the expression of a gene and can be classified as a functional marker.

Among the polymorphic loci found, locus Ag39 had the highest PIC value (0.93). The sequence from which this marker was derived originated from *A. glabrata*, and had 100% similarity and 94% identity with a sequence from *A. hypogaea* (DQ099178.1).

The conservation of microsatellite sites among species and even among genera makes it possible to use the same markers in related species. This characteristic of transferability is limited by the homology of DNA sequences between related species. It was found in the present study that regardless of the species (*A. hypogaea*, *A. glabrata*, or *A. pintoi*) from which the locus was developed, all of the loci used were successfully amplified in both species.

The *D* values ranged from 0.73 to 0.99, with a mean of 0.89 (Table 2). *D* values for all of the loci were high, demonstrating the high discriminatory power of these loci and their potential in fingerprinting studies.

The H_E was high for most loci, and varied from 0.30 to 0.94, with a mean of 0.70. The H_O values ranged from 0.03 to 0.88, with a mean value of 0.30. The low value obtained for the average H_O was expected, because species in the *Arachis* genus are autogamous (Krapovickas and Gregory, 1994). Similar results were obtained in another study with species of the genus *Arachis* (Hoshino et al., 2006), confirming the reproductive system of the species studied.

Only locus Ah282 had an observed heterozygosity value that was higher than expected, indicating that plants often have heterozygotic regions and do not solely exhibit autogamous homozygosis. This may have been a result of crossings between different accessions in the natural habitat before collection, where heterozygosity was maintained due to vegetative

propagation in the conservation of accessions. This genomic trait supports the classification of the reproductive system of *A. pintoi* as mixed, in which a cross-fertilization rate of 36.7% has been detected (Oliveira, 2015). Values of H_O higher than expected have also been obtained in genetic diversity studies in section *Caulorrhizae* (Palmieri et al., 2010).

A total of 65 private alleles distributed between the two species were found. Thirteen alleles (20.3%) were exclusive to *A. pintoi*, two (3.1%) were only found in accessions of *A. repens*, and the majority (50 alleles, 78.1%) was shared between the two species; *A. repens* and *A. pintoi* have a very close genetic relationship. Palmieri et al. (2010) obtained 99 (49%) private alleles in *A. pintoi*, 21 (10.7%) exclusive to *A. repens* and 79 (40.3%) shared between the two species. It is noteworthy that these authors analyzed different microsatellite loci that were developed from specific sequences of *A. pintoi*. As in the present study, seven loci were used for other species, and the alleles detected were highly conserved between *A. pintoi* and *A. repens*.

The Bayesian analysis revealed that the largest delta K value was for K = 3 (Figures 2 and 3), indicating the existence of three distinct gene pools.

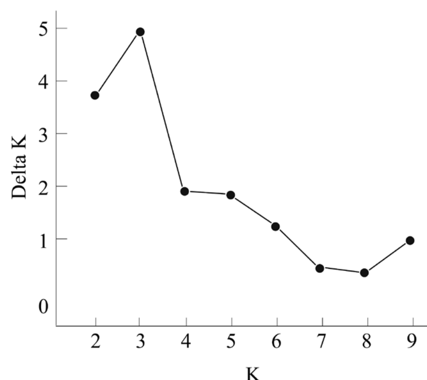

Figure 2. Delta graph (K) with K = 3, obtained from STRUCTURE analysis using the model from Evanno et al. (2005) in 65 forage peanut accessions collected in the valleys of the São Francisco, Paranã, and Jequitinhonha rivers, belonging to the Active Germplasm Bank of Embrapa Acre, Brazil.

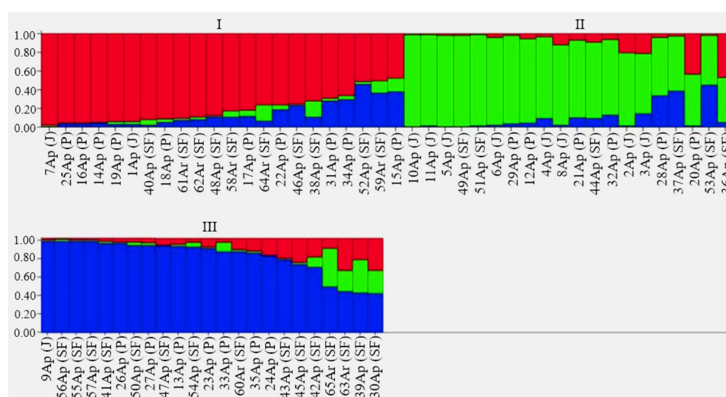

Figure 3. Analysis of gene pools in 65 accessions of forage peanut according to a Bayesian analysis using STRUCTURE. Each individual is represented by a column. The evaluated accessions were divided into three gene pools (K = 3): red, gene pool I; green, gene pool II; blue, gene pool III.

Gene pool I contained 22 accessions, 17 accessions of *A. pintoi* and 5 of *A. repens*. Ten accessions (41.7%) collected from the Paranã valley, 10 (33.3%) from the São Francisco River valley, and two (18.2%) from the Jequitinhonha valley were grouped into gene pool I.

Gene pool II included 20 accessions, all of which were accessions of *A. pintoi*. In this pool, six (25.0%) accessions were from the Paranã valley, six (20.0%) from the São Francisco valley, and eight (72.3%) from the Jequitinhonha valley. Gene pool III included 23 accessions, 20 accessions of *A. pintoi* and three of *A. repens*. In this gene pool, seven (29.2%) accessions were from the valley of the Paranã River, 15 (50.0%) were from the São Francisco valley, and one (9.1%) was from the Jequitinhonha valley.

According to these results, no clustering pattern was found that supported the separation of *A. pintoi* and *A. repens*, or the geographical subdivision of the area of occurrence over the valleys. However, although there was a formation of groups with both species, most (62.5%) of the *A. repens* accessions were gathered in one gene pool (I). The other *A. repens* accessions were grouped in gene pool III. Accession 60Ar exhibited a high degree of ancestry from gene pool III. This result corroborates those obtained in studies by Gimenes et al. (2000) and Menezes et al. (2012), who found that accession 60Ar was grouped with *A. pintoi* accessions, confirming the proximity of this *A. repens* accession to *A. pintoi*.

Molecular phylogeny has confirmed the monophyletic nature of *A. pintoi* and *A. repens* accessions of the section *Caulorrhizae* (Friend et al., 2010). Therefore, the accessions should have very similar genomes, because they share a recent common ancestor and the same original gene pool. In addition, the existence of fertile interspecific hybrids between the two species has not been proven (Pucciariello et al., 2013).

The dendrogram obtained by the UPGMA (Figure 4) and NJ analyses (Figure 5) revealed that there was no clustering of accessions according to their naturally occurring sites or species designations, showing a lack of genetic structure that allows the formation of defined groups. The cophenetic correlation coefficient between the dendrogram and genetic distance matrix was high ($r = 0.80$), demonstrating that the dendrogram revealed consistency in the genetic relationship expressed by the distances obtained from the dataset.

Despite the lack of structure in the formation of the clusters, the extreme values of genetic distance found were consistent with the distances between the collection sites. The shortest genetic distance (0.22) was observed between two accessions of *A. repens* (Ar62 and Ar61) collected in the valley of the São Francisco River. The greatest genetic distance (0.94) was observed between Ap27 and Ap10, two *A. pintoi* accessions that originated in the valleys of the Paranã and Jequitinhonha rivers, respectively.

The similarity between *A. pintoi* and *A. repens* accessions and the lack of a clustering pattern based on species assignment have already been observed in morphological, agronomic, bromatological, and molecular characterization studies. Assis et al. (2009) characterized the bristle of the stigmatic surface and found four clusters, three of which clustered accessions of both species. Menezes et al. (2012) studied the agronomic and bromatological characterization of the section *Caulorrhizae* and found six clusters, four of which consisted of *A. pintoi* and *A. repens* genotypes.

The taxonomic classification of *A. pintoi* and *A. repens* by Krapovickas and Gregory (1994) was based on the only two accessions known before the 1980s, which were GKP 10538 (*A. repens*) and GK 12787 (*A. pintoi*). These accessions have divergent and contrasting morphological characteristics.

However, as the number of collected accessions increases, it is possible to see the

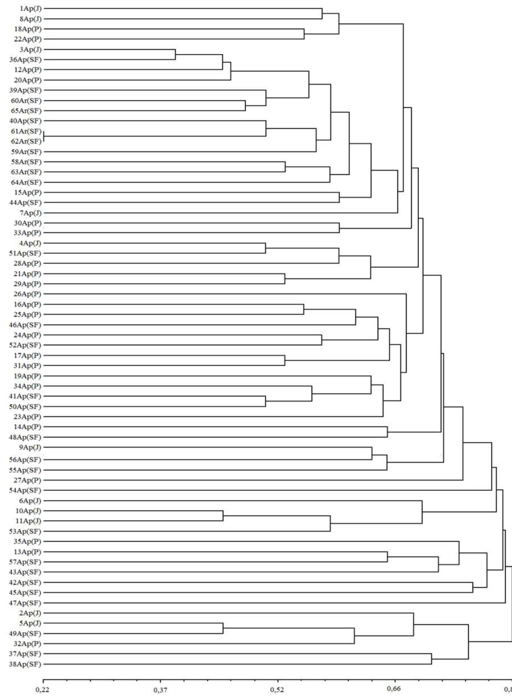

Figure 4. Dendrogram of 65 forage peanut accessions constructed using the unweighted pair group method with arithmetic mean with Roger's modified distance and 10 microsatellite loci.

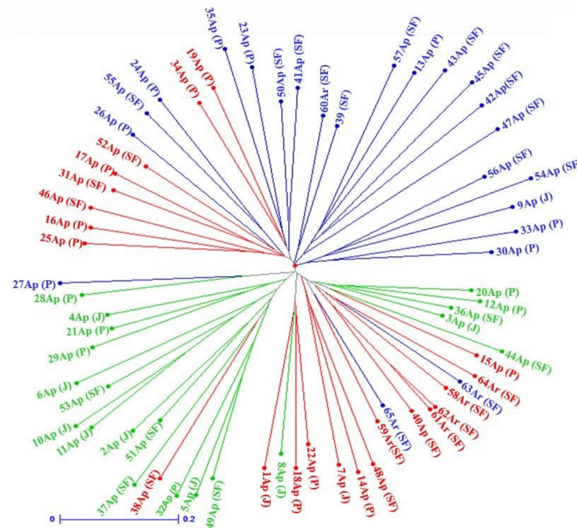

Figure 5. Neighbor-joining tree representing the genetic relationships among 65 accessions of forage peanut. Dots follow clustering formation generated by the STRUCTURE software with K = 3: red, gene pool I; green, gene pool II; blue, gene pool III.

appearance of materials with intermediate morphological characteristics among the original classification used by the above-mentioned authors. As reported by Monçato (1995), there are accessions with intermediate characters between *A. pintoi* and *A. repens* that may have appeared by natural hybridization. As described above, 78.1% of private alleles were shared between the species. Despite the similarity between *A. pintoi* and *A. repens* and the use of markers with high discriminatory power, no duplicates were found in the accessions analyzed. Therefore, the samples in the Active Germplasm Bank have been diversified. The close proximity between *A. pintoi* and *A. repens* has been demonstrated once more and highlights the complexity among related species. Knowledge of genetic diversity and the correct identification of forage peanut accessions are also important for proper conservation planning and the use of crosses in breeding programs.

Conflicts of interest

The authors declare no conflict of interest.

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