Genetic diversity analysis among pigeonpea genotypes adapted to South American regions based on microsatellite markers

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ABSTRACT: The pigeonpea [*Cajanus cajan* (L) Millspaugh] is one of the most important perennial legume crops utilized in the food, fodder, soil conservation, crop-livestock integrated systems, reclaiming of degraded pastures and symbiotic nitrogen fixation. Microsatellite markers were used to estimate the genetic diversity of 77 pigeonpea genotypes selected from the germplasm collections at Embrapa Cattle-Southeast and, to evaluate their transferability to *Phaseolus vulgaris* and *Vigna unguiculata* species. The number of alleles per locus ranged from 2 to12, with an average of 5.1 alleles. The PIC values ranged from 0.11 to 0.80 (average 0.49) and the D values from 0.23 to 0.91 (average 0.58). The averages of observed and expected heterozygosity were 0.25 and 0.47, respectively, showing a deficit in heterozygosity. A model-based Bayesian approach implemented in the software STRUCTURE was used to assign genotypes into clusters. A dendrogram was constructed based on the modified Roger's genetic distances using a neighbor-joining method (NJ). A total of four clusters were assembled by STRUCTURE and a strong tendency of correspondence between the Bayesian clusters in the NJ tree was observed. The genetic distance ranged from 0.09 to 0.62 (average 0.37), showing a low genetic diversity in the pigeonpea genotypes. Transferability of pigeonpea-specific microsatellites revealed a cross-amplification and the presence of polymorphic alleles in *P. vulgaris* and *V. unguiculata*.
Keywords: legumes, transferability, microsatellite, germplasm

Introduction

The pigeonpea [*Cajanus cajan* (L.) Millspaugh] is one of the most important perennial legume crops in the tropic and subtropic regions of the world. Because of its multiple usages in food, fodder, soil conservation, crop-livestock integrated systems, reclaiming of degraded pastures and symbiotic nitrogen fixation, the pigeonpea plays an important role in subsistence agriculture (Reddy et al., 2005).

Because of the potential of the pigeonpea as a forage legume, the Brazilian Agricultural Research Corporation (Embrapa Cattle-Southeast, state of São Paulo-SP) has germplasm collections of selected genotypes with desirable agronomic traits such as high yield, quality of forage and lowest tannin content (Godoy et al., 1995). Over time, the selected genotypes showed phenotypic segregation in subsequent generations. Therefore, these genotypes were self-fertilized and subsequently selected in order to obtain inbred lines (Godoy et al., 1994, 1997). Several studies have been conducted to characterize genotypes and inbred lines of the pigeonpea and provide basic information for breeding. The genetic variability of a partial set of accessions from this collection was assessed using Random Amplification of Polymorphic DNA (RAPD) molecular markers. Results showed low genetic variability and the need to broaden the genetic base for use in crop-livestock integrated systems and reclaiming degraded pastures (Godoy et al., 2003)

The knowledge of the genetic variability is very important in for pigeonpea germplasm collections and pigeonpea breeding programs. Microsatellite markers are quite effective for estimating genetic diversity and genetic relationships and in predicting the genetic value of selected genotypes derived from intraspecific crosses and the performance of their hybrid progenies (Gaitán-Solís et et al., 2002; Varshney et al., 2005). In this study, we used 43 microsatellite markers to evaluate the genetic diversity of 77 pigeonpea selected genotypes from the Embrapa collection. In addition, we studied cross-species amplification in *Phaseolus vulgaris* L. and *Vigna unguiculata* L. Walp.

Materials and Methods

We have selected 43 microsatellite markers described in the literature (Burns et al., 2001; Odeny et al., 2007) to analyze 77 pigeonpea genotypes (Table 1) of the Brazilian Agricultural Research Corporation (Embrapa Cattle-Southeast) germplasm collection, in São Carlos, SP, Brazil. Thirty-nine of them are Brazilian inbred lines, three are commercial cultivars and thirty-five came from the International Crops Research Institute for the Semi-Arid

Table 1 – Characteristics of 43 pigeonpea microsatellite markers.

GenBank Accession no.	Repeat Motif	Primer Sequences (5' - 3')	[3]Ta (°C)	Product Length (bp)	Polymorphic Markers	Monomorphic Markers	Source
CZ445531	$(TA)_{11}$	[1]F: TGAATTGCTGAGAGGACGTTT	56	234-238	+	-	Odeny et al. (2007)
		[2]R: CTGTTCCAATTCCACGGTTT					
CZ445540	$(GGT)_4$	F: ACGCTTCTGATGCTGTGTTG	45	208-210	+	-	Odeny et al. (2007)
		R: CATCAGCATCATCGTTACCC					
CZ445530	$(TTC)_5$	F: CCATTGTGCGTCTTTGTGTT	56	206-208	+	-	Odeny et al. (2007)
		R: GCTTTTCCTCTTCCTTTCTCG					
AJ306901	$(CA)_{10}$	F: AAGGGTTGTATCTCCGCGTG	56	186-202	+	-	Burns et al. (2001)
		R: GCAAAGCAGCAATCATTTCG					
AJ312887	$(CA)_{21}$	F: CCATAATCCAATCCAAATCC	51	160-170	+	-	Burns et al. (2001)
		R: AGAAGGCTTTCATGTAACGC					
AJ312891	$(CA)_6$	F: ACAATGCTAGGGAACACCGC	45.5	180-206	+	-	Burns et al. (2001)
		R: TACCTTAACCCACAATGGCC					
AJ312892	$(CT)_{16}$	F: CAACATTTGGACTAAAAACTG	56	150-158	+	-	Burns et al. (2001)
		R: AGGTATCCAATATCCAACTTG					
AJ312893	$(CT)_{30}$	F: TGCGTTTGTAAGCATTCTTCA	50	126-150	+	-	Burns et al. (2001)
		R: ACTTGAGGCTGAATGGATTTG					
AJ312894	$(CT)_{22}$	F: CACTTGGTTGGCTCAAGAAC	45	152-180	+	-	Burns et al. (2001)
		R: GCCAATGAACTCACATCCTTC					
AJ312895	$(CA)_{15}$	F: CCTTCTTAAGGTGAAATGCAAGC	45	228-242	+	-	Burns et al. (2001)
		R:ATAACAATAAAAGACCTTGAATGC					
CZ681930	$(TC)_8$	F: GCGCTAAGGGAAAACAAAAA	56	164-174	+	-	Odeny et al. (2007)
		R: AACTCCCTTGTTGTCATATGGTG					
CZ681938a	$(ATT)_{21}$	F: TCAGGGGTAAATGCGGTATC	50	236-260	+	-	Odeny et al. (2007)
		R: GAATTGCTTTTTGCTTCCTCA					
CZ681938b	$(ATT)_{21}$	F: TCAGGGGTAAATGCGGTATC	50	212-234	+	-	Odeny et al. (2007)
		R: GAATTGCTTTTTGCTTCCTCA					
CZ682017a	$(AAG)_{13}$	F: TGAAATGAACAAACCTCAATGG	45	200-222	+	-	Odeny et al. (2007)
		R: TGTATTGCACATTGACTTGGCTA					
CZ682017b	$(AAG)_{13}$	F: TGAAATGAACAAACCTCAATGG	45	174-182	+	-	Odeny et al. (2007)
		R: TGTATTGCACATTGACTTGGCTA					
CZ681983	$(TGA)_{11}$	F: GAGGAGGAGGAAGAAGAAGAAGA	45.5	73-79	+	-	Odeny et al. (2007)
		R: TCGTCGCCGTATCACTACAA					
CZ445530	$(TTC)_5$	F: CGGGCTTCCTTTTCTTCTCT	46	200	-	+	Odeny et al. (2007)
		R: AAAACCCCGAAAACACCATT					
CZ445525	$(TTA)_{10}$	F: TTCTGGATCCCTTTCATTTTTC	45	196	-	+	Odeny et al. (2007)
		R: TGACACCCTTCTACCCCATAA					
CZ445522	$(TA)_8$	F: CTTCCCCCAACTAAGATCCA	46	212	-	+	Odeny et al. (2007)
		R: GTTCGTTCTCTTTAATTGACTTGC					

Continue...

Table 1 – Continuation.

CZ445538	$(TTA)_4$	F: CCAAGAAAAGGTGCTCCAAGT	45.5	155	-	+	Odeny et al. (2007)
		R: TTGCTTCTTTTCTCGCTTGC					
CZ445539	$(CAT)_4$	F: TGATAGGGACCACAACGACA	56	200	-	+	Odeny et al. (2007)
		R: AGCGTTGACTCCTCCCTCTT					
CZ445519	(CT)6TT $(CT)_2$	F: GACTCTTCACCTCACACTCATCAC	46	190	-	+	Odeny et al. (2007)
		R: ACCTCATACAACAACCCTAAGCAC					
CZ445544	$(TTAT)_4$	F: TACAGCAGCCACATCAAAGC	45.5	290	-	+	Odeny et al. (2007)
		R: TGAACCGTGAAAGTGGGATT					
CZ445553	$(TTA)_4$	F: ACCCATTATTGATTTGGGTA	45.5	200	-	+	Odeny et al. (2007)
		R: CCAAATTTCACCCAAGAAA					
CZ445545	$(AAT)_4$	F: TCTTCCATTGCATGGTGTT	56	202	-	+	Odeny et al. (2007)
		R: GCATGATATGAGATGATGACGA					
CZ445554	$(AAC)_4$	F: ATAGGCCCATCTCCAGGTTC	47	158	-	+	Odeny et al. (2007)
		R: TTAATGCCCAGCCAATTCTT					
CZ445553	$(TTA)_4$	F: ACCCATTATTGATTTGGGTA	45.5	200	-	+	Odeny et al. (2007)
		R: CCAAATTTCACCCAAGAAA					
CZ445545	$(AAT)_4$	F: TCTTCCATTGCATGGTGTT	56	202	-	+	Odeny et al. (2007)
		R: GCATGATATGAGATGATGACGA					
CZ445554	$(AAC)_4$	F: ATAGGCCCATCTCCAGGTTC	47	158	-	+	Odeny et al. (2007)
		R: TTAATGCCCAGCCAATTCTT					
CZ445521	$(TA)_4$ $(AT)_4$ $(AT)_4$	F: CTACAATCCCAGGGAAAAGG	46	210	-	+	Odeny et al. (2007)
		R: ACAAACGTAATCTGTGTTGATCTC					
CZ681935	$(TC)_8$	F: CATTTATTTCTCTCTGGCATTCAC	56	158	-	+	Odeny et al. (2007)
		R: CGAGCTGCAAGCATAAACG					
CZ681923	$(AAG)_5$	F: CATCGCCTACAATCATACAAAGA	54	106	-	+	Odeny et al. (2007)
		R: TCTTGTCCTTTTTCAGTCATCGT					
CZ681927	$(GAA)_{16}$	F: CTCTTGCTTACGCGTGGACT.	45.5	206	-	+	Odeny et al. (2007)
		R: CTTTTGCTTTTGCGTGCTT					
CZ681929	$(AGA)_5$	F: TCACAGAGGACCACACGAAG	50	200	-	+	Odeny et al. (2007)
		R: TGGACTAGACATTGCGTGAAG					
CZ681933	$(AGA)_4$	F: AGAGGGAAAGGGAAGAGAAGA	54	200	-	+	Odeny et al. (2007)
		R: TCAAGCAACTCCAAGAAATTCA					
CZ681946	$(CTT)_4$	F: TAATCCCATTCCGTTGTCGT	45	256	-	+	Odeny et al. (2007)
		R: CCCAGGAAGAGATGAGACCA					
CZ681968	$(ATT)_4$	F: CAGGATTTTAATGGATTCTGCAA	45.5	280	-	+	Odeny et al. (2007)
		R: GGGTGAATACTATTTAAAAGGATA					
CZ681969	$(ACT)_4$	F: ATCCCAGACTTCATAGGGAGATAG	57.5	200	-	+	Odeny et al. (2007)
		R: GTCTAGTCCCAGGTACAAAGAGGT					
CZ681961	$(AGA)_{10}$	F: ATGGGCATGGTAGAGGAGGT	47	198	-	+	Odeny et al. (2007)
		R: CGCTCATCATCGTCATCAAA					

Continue...

Table 1 – Continuation.

CZ681943	$(GAT)_5$ $(GAT)_4$ $(GAT)_4$	F: TGGGCATGGTAGAGGAAGTT	46	186	-	+	Odeny et al. (2007)
		R: CGTCATGAAGCAACAGGAGA					
CZ681977	$(CA)_7$	F: ACCTTGCTTGTTTCGCTTTT	46	148	-	+	Odeny et al. (2007)
		R: AAGGGAGGTGGACTACAAGGA					
CZ681979	$(GT)_7$	F: GTGAGTGAGAGTGAGTGTATTTGT	60	200	-	+	Odeny et al. (2007)
		R: GCTCTGATGCCAAATGTTGA					
CZ681998	$(TC)_6$	F: ACAAATCCGGTGACCCATAA	60	206	-	+	Odeny et al. (2007)
		R: CCGAGAACAAAAACATTGAACA					
CZ682005	$(AC)_6$	F: TGTATGTTCGTTTAGAGGCTTCC	56	200	-	+	Odeny et al. (2007)
		R: GCCCCTTTTCACTTTTCTCA					
CZ682009	$(TG)_7$	F: AGCCACTTAATAACCAAGCCTTTT	60	258	-	+	Odeny et al. (2007)
		R: GTGTATGCTTTACTTGCTTTCCTTT					
CZ682011	$(GT)_7$	F: AAATTCACCACCATGATCCAA	45	196	-	+	Odeny et al. (2007)
		R: TCTTCACTTCCGAGACACAACT					

Tropics (ICRISAT) (Table 2). The inbred lines have distinct morphological characteristics such as color of the stem, flowers, seeds and pods. These inbred lines were obtained from selfing of genotypes introduced from ICRISAT and have been incorporated to the breeding programs at Embrapa. In addition, cross-amplification evaluations were made using two other legume species: *Phaseolus vulgaris* (CAL-143, IAC-UNA, BAT-93 and JALO-EEP558 varieties) and *Vigna unguiculata* ("Fradinho" cultivar), both from the germplasm collection of the Agronomic Institute of Campinas (IAC) (Campinas, SP, Brazil).

Genomic DNA was extracted from freeze-dried leaf samples using the cetyltrimethyl ammonium bromide (CTAB) method with modifications (Faleiro et al., 2003). DNA samples were quantified by comparison with known quantities of λ phage DNA on a 1% agarose gel.

The PCR was carried out in a total reaction volume of 25 μL containing 0.5 ng of DNA template, 0.8 μM of each forward and reverse primers, 100 μM of each dNTP (MBI Fermentas), 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl and 0.5 U Taq DNA Polymerase (Invitrogen). All PCR amplifications were performed in a PTC–200 thermal cycler (MJ Research, Waltham, MA/USA) using the following conditions: 94°C for 1 min followed by 30 cycles of 94°C for 1 min, specific annealing temperature for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min. Amplification products were genotyped by electrophoresis on 6% denaturing polyacrylamide gels in 1X TBE buffer using a 10 bp ladder (Invitrogen) as a standard size. The DNA fragments were visualized by silver staining according to Creste et al. (2001).

The polymorphism information content (PIC) values were calculated for estimates of marker informativeness according to the equation of Botstein et al. (1980),

$$PIC = 1 - \sum_{i=1}^{n} f_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 f_i^2 f_j^2$$

where f_i is the frequency of the ith allele, f_j is the frequency of the jth allele and the summation extends over n alleles. In order to compare marker efficiencies in varietal identification, the discriminating power (D) was estimated for each primer based on the formula,

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

where N is the number of individuals and p^j is the frequency of the jth pattern (Tessier et al., 1999).

The observed heterozygosity (H_O) and the expected heterozygosity (H_E) were analyzed using the GDA software (Lewis and Zaykin, 2002). Genetic distance was calculated from microsatellite marker data using modified Roger's genetic distances. A genetic distance matrix was estimated using tools for genetic population analysis (TFPGA v 1.3) (Miller, 1997). Cluster analysis was performed using the neighbor-joining (NJ) method with the DARwin v. 5.0.157 software (Perried and Jacquemound-Collet, 2006). The reliability of the generated dendrogram was also tested by bootstrap analysis using the BooD program with 1000 iterations (Coelho, 2002). The software STRUCTURE version 2.2 (Pritchard et al., 2000) was used to generate a Bayesian inference of the structure of the populations. By this method, a model of *K* populations is assumed and samples are grouped in order to minimize linkage disequilibrium and to maximize conformity to Hardy-Weinberg equilibrium across all analyzed loci. As a preliminary step, analysis was performed a single time for each *K* value ranging from 2 to 20. Each run was performed using the admixture model and

Table 2 – Information of *Cajanus cajan*, *Phaseolus vulgaris* and *Vigna unguiculata* genotypes evaluated with microsatellite markers.

Sample Code	[1]Genotype ID	Genetic Origin	Sample Code	Genotype ID	Genetic Origin	Sample Code	Genotype ID	Genetic Origin
1	G1m-95	[2]ECS	29	G106	India	57	G151	India
2	G3	Brazilian Cultivar	30	G108-99	ECS	58	G154	Bangladesh
3	G05-94	ECS	31	G109	ECS	59	G154-95	ECS
4	G06-95	ECS	32	G112	India	60	G158	India
5	G8-95	ECS	33	G114	India	61	G165	India
6	G9m	ECS	34	G115	India	62	G166	India
7	G10-94	ECS	35	G116	India	63	G167-97	ECS
8	G17c-94	ECS	36	G118	ECS	64	G168	India
9	G18-95	ECS	37	G119	ECS	65	G168-99	ECS
10	G19m-95	ECS	38	G120	India	66	G169	India
11	G21-99	ECS	39	G121-99	ECS	67	G171	India
12	G27	India	40	G123	ECS	68	G174	India
13	G27-94	ECS	41	G124	India	69	G176	n.a.
14	G29b-94	ECS	42	G124-95	ECS	70	G184-97	ECS
15	G29m-94	ECS	43	G126	India	71	G186-98	ECS
16	G30	India	44	G127	ECS	72	G197	India
17	G39-94	ECS	45	G128	India	73	G198	India
18	G40-95	ECS	46	G131	India	74	N0 314	[3]Brazilian - IZ
19	G47-94	ECS	47	G135	India	75	INPA	Amazônia
20	G48-95	ECS	48	G137	India	76	FAVA LARGA	Brazilian Cultivar
21	G57-95	ECS	49	G137-99	ECS	77	ANÃO	Brazilian Cultivar
22	G58	ECS	50	G138	ECS	78	CAL-143	[4]*P. vulgaris* - IAC
23	G59-95	ECS	51	G141	India	79	IAC-UNA	*P. vulgaris* - IAC
24	G66-95	ECS	52	G142	India	80	BAT-93	*P. vulgaris* - IAC
25	G100	Bangladesh	53	G142-95	ECS	81	JALO-EEP558	*P. vulgaris* - IAC
26	G101	India	54	G148	India	82	FRADINHO	*V. unguiculata* - IAC
27	G101-97	ECS	55	G149	India			
28	G104	India	56	G149-99	ECS			

[1]Genotype ID: Unidade de Execução de Pesquisa de Âmbito Estadual (UEPAE). [2]ECS: Embrapa Cattle-Southeast. [3]IZ: Institute of Animal Husbandry. [4]IAC: Agronomic Institute of Campinas.

1000 replicates for burn-in and 10,000 replicates during analysis. The most probable number of K was calculated based on Evanno et al. (2005) using an *ad hoc* statistic ΔK, which represents the rate of change in log probability of the data between successive K values rather than the log probability of the data.

Results and Discussion

Of the 43 microsatellite markers, 16 were polymorphic (Table 3). A total of 83 putative alleles were obtained from the 16 microsatellite markers. The number of alleles ranged from 2 to12, with an average of 5.1 alleles per locus (Table 3). Screening of 77 pigeonpea genotypes with these 16 markers indicated low polymorphism information content. The PIC values ranged from 0.11 to 0.80 with an average of 0.49. The D values ranged from 0.23 to 0.91 with an average of 0.58. The highest PIC and D values ware found in locus CZ681938a which contains 8 alleles. The observed (H_O) and expected heterozygosity (H_E) values ranged from 0.01 to 0.53 (average 0.25) and 0.01 to 0.82 (average 0.47), respectively, indicating high heterozygote deficiency. The low variability in these collections may be due to a narrow genetic base of the original germplasm collection or pre-selection of these genotypes based on agronomic characteristics, mainly related to the production of dry matter (Godoy et al., 2004).

All polymorphic markers were tested for cross-amplification in *P. vulgaris* (CAL-143, IAC-UNA, BAT-93 and JALO-EEP558) and *V. unguiculata* (Fradinho) (Table 4). Thirteen microsatellite markers (CZ445540, CZ445530, AJ306901, AJ312887, AJ312891, AJ312892, AJ312893, AJ312894, AJ312895, CZ681930, CZ681938a, CZ681938b and CZ681983) amplified in at least one bean species. Six markers (CZ445530, CZ681983, AJ312891, AJ312893, AJ312895

Table 3 – Characteristics of pigeonpea microsatellite *loci*, including number of alleles, PIC, D, H_O and H_E values.

GenBank Accession no.	(1)Alelle number	(2)PIC	(3)D	(4)H_O	(5)H_E
CZ445531	2	0.29	0.36	0.01	0.01
CZ445540	2	0.29	0.35	0.33	0.27
CZ445530	2	0.11	0.23	0.01	0.10
AJ306901	2	0.32	0.41	0.19	0.30
AJ312887	4	0.51	0.62	0.20	0.52
AJ312891	4	0.32	0.43	0.29	0.25
AJ312892	5	0.49	0.56	0.28	0.51
AJ312893	12	0.61	0.72	0.42	0.55
AJ312894	7	0.69	0.73	0.36	0.72
AJ312895	5	0.60	0.75	0.29	0.66
CZ681930	6	0.66	0.78	0.29	0.70
CZ681938a	8	0.80	0.91	0.53	0.82
CZ681938b	8	0.68	0.78	0.32	0.71
CZ682017a	8	0.74	0.82	0.21	0.77
CZ682017b	2	0.26	0.35	0.01	0.01
CZ681983	4	0.48	0.57	0.23	0.54
Average	5.1	0.49	0.58	0.25	0.47

[1]Number of alleles in pigeonpea. [2]PIC - Polymorphism information content. [3]D - Discriminating power. [4]H_O - Observed heterozygosity. [5]H_E - Expected heterozygosity.

Table 4 – Characteristics of pigeonpea-specific microsatellite markers transferable to *Phaseolus vulgaris* and *Vigna unguiculata*.

GenBank Accession no.	*Vigna unguiculata*	*Phaseolus vulgaris*			
	FRADINHO	CAL-143	IAC-UNA	BAT-93	JALO-EEP558
CZ445531	-	-	-	-	-
CZ445540	208/208	208/208	208/208	208/208	208/208
CZ445530	208/208	208/208	208/208	208/208	208/208
AJ306901	200/200	198/198	186/200	200/200	200/200
AJ312887	160/160	-	160/160	160/160	160/160
AJ312891	180/206	206/206	206/206	206/206	206/206
AJ312892	-	154/154	154/156	154/156	-
AJ312893	130/130	128/128	128/146	128/146	130/130
AJ312894	166/166	-	166/166	166/166	166/168
AJ312895	242/242	242/242	242/242	242/242	242/242
CZ681930	164/164	166/166	166/166	172/172	164/164
CZ681938a	252/252	260/260	252/252	252/252	-
CZ681938b	220/228	228/228	228/228	224/228	-
CZ682017a	-	-	-	-	-
CZ682017b	-	-	-	-	-
CZ681983	73/73	73/73	75/75	75/75	73/73

Alleles observed for each locus are displayed in base pairs (bp). (-) No amplification.

and CZ681930) were successfully amplified in *P. vulgaris* and *V. unguiculata*, indicating very good transferability. Non-specific amplification of the loci CZ445531, CZ682017a and CZ682017b was observed between species. Eight markers (AJ306901, AJ312891, AJ312892, AJ312893, AJ312894, CZ681930, CZ681938a and CZ681938b) revealed polymorphism between the *Phaseolus* and *Vigna* genotypes. These results suggest considerable sequence conservation within the primer regions flanking microsatellite loci. The high level of cross-species amplification and the observed polymorphic alleles suggest that they can be used for inter- and intraspecific studies. This level of amplification efficiency is similar to that

observed by Gepts et al. (2008) and Gupta et al. (2008), where chickpea and Azuki bean microsatellite markers were used to amplify DNA from other related legume species such as *Vigna* and *Phaseolus*, respectively.

STRUCTURE analysis coupled with computation of Evanno ΔK statistics suggested a primary partition of pigeonpea and genotypes of the *P. vulgaris* and *V. unguiculata* into four clusters (K = 4). This analysis can help to identify clusters of genetically similar genotypes. Thus, the subpopulations from the STRUCTURE analysis were grouped into four clusters (C): C1, C2, C3, and C4 (Figure 1a). Cluster C1 is comprised of the 28 pigeonpea genotypes, which were collected in India (G141, G142, G148, G149, G151, G165, G171, G174, G198, G176, NO 314, G168, G149, G166, G197, G158, G137, G126, G119 and G118), Bangladesh (G154) and the Brazilian inbred lines (G154-95, G142-95, G149-99, G184-97, G168-99, G167-97 and G108-99). Cluster C2 includes 18 pigeonpea Brazilian inbred lines obtained from the breeding program of Embrapa Cattle-Southeast (G1m-95, G05-94, G06-95, G8-95, G9m, G10-94, G17c-94, G19m-95, G27-94, G29b-94, G47-94, G48-95, G39-94, G18-95, G40-94, G57-95 and G58) the cultivar G3-Guandu Mandarim and 2 pigeonpea genotypes from India (G30 and G27). These Brazilian inbred lines were selected for use in crop-livestock integrated systems and reclaiming degraded pastures. The field data confirmed that these genotypes are closely related. Cluster C3 consisted of 26 pigeonpeas, which were collected in India (104, G114, G120, G124, G116, G135, G112, G115, G101, G106, G131, G169 and G128), Bangladesh (G100) and the Brazil inbred lines (G124-95, G121-99, G101-97, G21-99, G137-99, G109, G123, G127, G138, G59-95, G66-95 and G29m-94). Clusters C1 and C3 had mixed origins (India, Bangladesh and Brazil inbred lines). These results indicate the presence of

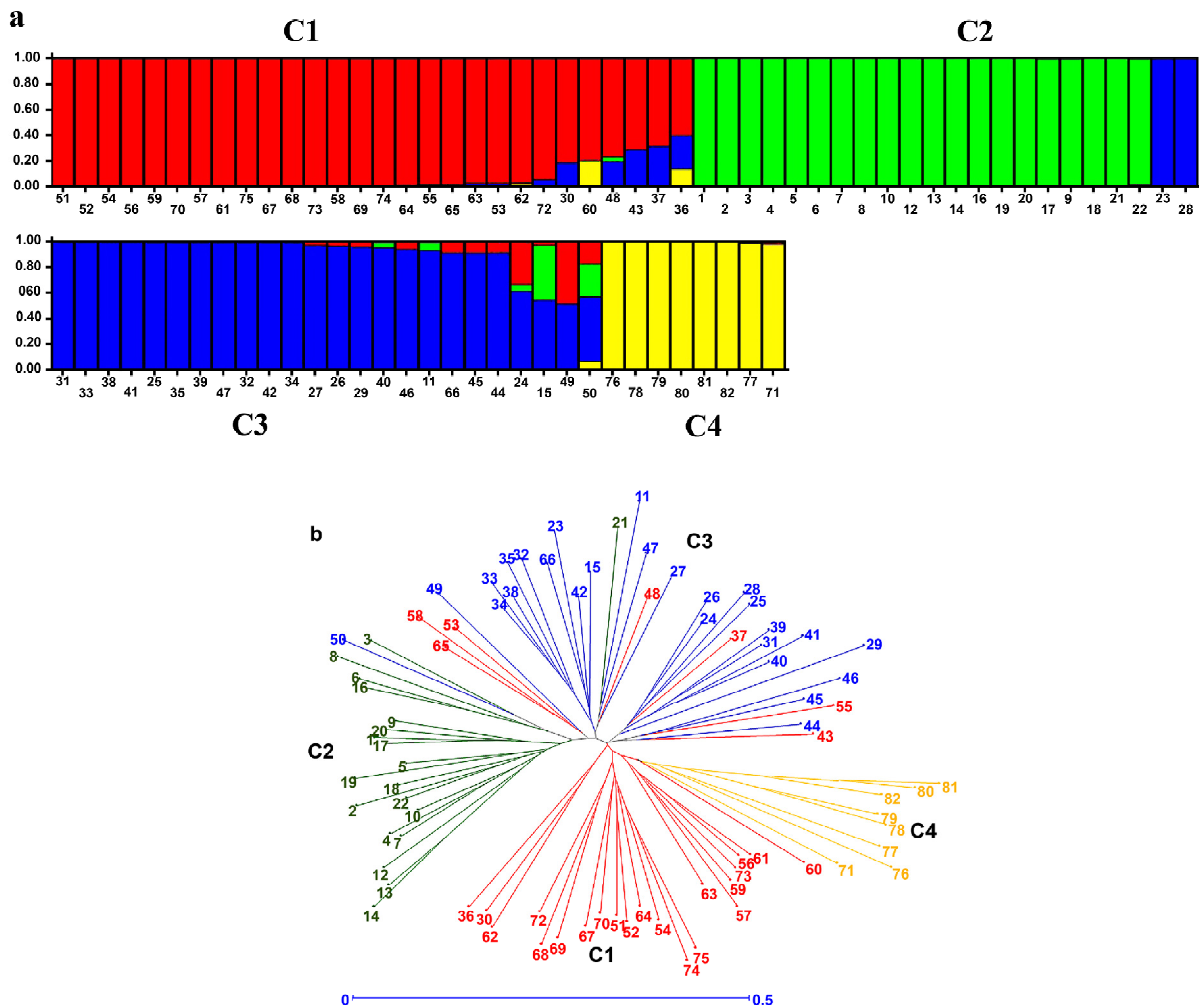

Figure 1 – Genetic diversity of pigeonpea genotypes and cross-species amplification between *Phaseolus vulgaris* and *Vigna unguiculata*. **a** Population structure analysis. Each genotype is represented by a thin vertical segment, which can be partitioned into K colored segments that represent the individual estimated membership to the K cluster. Membership coefficients obtained at the optimal K value (K = 4 clusters). **b** Neighbor-joining tree analysis. The numbers at the tip of tree branches indicate the accession number. The colors of the bar and the tree branch indicate the 4 groups identified through the STRUCTURE program (C1 = red, C2 = green, C3 = blue and C4 = yellow).

different gene pools among these clusters. Cluster C4 contained two pigeonpea cultivars (Fava Larga and Anão), the inbred line G186-98 and the four varieties of *P. vulgaris* (CAL-143, IAC-UNA, BAT-93 and JALO-EEP558) and one *V. unguiculata* cultivar (Fradinho).

The phylogenetic NJ tree, which was constructed based on the modified Roger's genetic distance matrix, was colored according to STRUCTURE results (Figure 1b). Furthermore, a strong tendency of correspondence between the Bayesian clusters in the NJ tree was observed. Clusters C1 and C3 comprised the pigeonpea genotypes from India, Bangladesh and some Brazilian inbred lines. Cluster C1 include 18 Brazilian inbred lines. Cluster C4 includes the 2 cultivars, the G186-98 Brazilian inbred line of the pigeonpea and all genotypes of *Phaseolus* and *Vigna*. Genotypes of pigeonpea which were self-fertilized (by controlled pollination, and subsequently selected in order to obtain inbred lines) grouped together (G27 and G27-94, G168 and G168-99, G154 and G154-95, G149 and G149-99, G142 and G142-95, G124 and G124-95, G101 and G101-97), except G137 and G137-99. The pigeonpea genotype G137-99 presented a heterozygote profile. Although pigeonpea is considered an autogamous species, in the presence of pollinators, the cross-pollination can occur, ranging from 3% to 26%. Consequently, a percentage of heterozygous strains can be observed, even if it is a low percentage (Reddy et al., 2004). The bootstrap value of the center point of the group (82.0%) indicates the robustness of the genetic relationship depicted by the dendrogram (Figure 1b). The genetic distances among the 77 genotypes of pigeonpea ranged from 0.09 to 0.62 with an average of 0.39. The lower genetic distances were found among the genotypes of cluster C2, such as G06-95 and G9m (0.09). These two genotypes have similar stem color (green) and thickness (10mm) (Godoy et al., 2004; Provazi et al., 2007). The largest genetic distances were found among the genotypes G1m-95 (cluster 2) and G158 (0.62) (cluster 1). Genetic distances between clusters C1 and C3 were higher than the C2 cluster revealing moderate diversity among these genotypes. The cultivars Fava Larga and Anão were grouped in a distinct cluster (C4) with 0.42 genetic distance between them. The cluster analysis based on modified Roger's genetic distances shows the narrowing of the genetic basis among genotypes.

Knowledge of the genetic diversity in germplasm collections is fundamental for further breeding programs to fully exploit existing diversity by genotypes selection. As evident from the clustering of genotypes, it is clear that these microsatellite markers are efficacious. The pigeonpea is an important crop of the Phaseoleae tribe, which has limited genomic resources. As microsatellite markers are highly polymorphic, reproducible, co-dominant in nature and distributed throughout the genome, they have become the ideal marker system for genetic analysis and breeding applications.

Conclusions

The microsatellite markers revealed low genetic diversity among genotypes of pigeonpea, especially between the Brazilian inbred lines selected for use in crop-livestock integrated systems and reclaiming degraded pastures. The modified Roger's genetic distances revealed the presence of genetically close genotypes.

Pigeonpea-specific microsatellite markers were transferable to *P. vulgaris* and *V. unguiculata*. The transferable loci exhibited polymorphism among some genotypes. Transferability studies of microsatellite loci from other cultures can be highly advantageous.

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References

Burns, M.J.; Edwards, K.J.; Newbury, H.J.; Ford-Lloyd, B.V.; Baggott, C.D. 2001. Development of simple sequence repeat (SSR) markers for the assessment of gene flow and genetic diversity in pigeonpea (*Cajanus cajan*). Molecular Ecology Notes 1: 283-285.

Botstein, D.; White, R.L.; Skolnick, M.; Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American Journal of Human Genetics 32: 314-331.

Coelho, A.S.G. 2002. BooD: assessment of errors associated with estimates of distances/genetic similarities across the bootstrap procedure with variable number of markers. UFG, Goiânia, GO, Brazil (in Portuguese). (CD-ROM).

Creste, S.; Tulmann Neto, A.; Figueira, A. 2001. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Molecular Biology Reporter 19: 299-306.

Evanno, G.; Regnaut, S.; Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 18: 2611-2620.

Faleiro, F.G.; Faleiro, A.S.G.; Cordeiro, M.C.R. Karia, T.K. 2003. DNA extraction methodology from leaves of native central Brazilian region species to operate molecular analysis. Boletim Técnico da Embrapa Cerrados, Brasília, DF, Brazil 92: 1-5. (in Portuguese).

Gaitàn-Solìs, E.; Duque, M.C.; Edwards, K.J.; Tohme, J. 2002. Microsatellite repeats in common bean (*Phaseolus vulgaris*): isolation, characterization, and cross-species amplication in *Phaseolus* ssp. Crop Science 42: 2128-2136.

Gepts, P.; Francisco, J.L.A.; Everaldo, B.; Matthew, W.B.; Rosana, B.; William, B.; Incoronata, G.; Gina, H.; James, K.; Patricia, L.; Phillip, M.; Maeli, M.; Peter, P.; Andrea, P.H.; Timothy, P.; Federico, S.; Francesa, S.; Kangfu, Y. 2008. Genomics of *Phaseolus* beans, a major source of dietary protein and micro-nutrients in the tropics. p. 113-143. In: Moore, P.H.; Ming, R., eds. Genomics of tropical crop plants. Springer, New York, NY, USA.

Gupta, S.K.; Souframanien, J.; Gopakrishna, T. 2008. Construction of a genetic linkage map of black gram, *Vigna mungo* (L.) Hepper, based on molecular markers and comparative studies. Genome 51: 628-637.

Godoy, R.; Batista, L.A.R.; Souza, F.H.D.; Primavesi, A.C. 2003. Description of selected Pigeon-pea (*Cajanus cajan* (L.) Millsp) pure lines. Revista Brasileira de Zootecnia 32: 546-555. (in Portuguese, with abstract in English).

Godoy, R.; Fushita, A.T.; Dübbern De Souza, F.H. 2004. Description of eleven pigeonpea pure lines selected in São Carlos, SP. Revista Brasileira de Zootecnia 33: 2206-2213. (in Portuguese, with abstract in English).

Godoy, R.; Batista, L.A.R.; Negreiros, G.F. 1994. Agronomic evaluation and selection of germplasm of pigeonpea forage (*Cajanus cajan* (L.) Millsp). Revista Brasileira de Zootecnia 23: 742-749. (in Portuguese, with abstract in English).

Godoy, R.; Batista, L.A.R.; Negreiros, G.F. 1995. Agronomic evaluation of pigeonpea forage (*Cajanus cajan* (L.) Millsp). Revista Brasileira de Zootecnia 23: 730-742. (in Portuguese, with abstract in English).
Godoy, R.; Batista, L.A.R.; Negreiros, G.F. 1997. Agronomic evaluation and selection of germplasm of pigeonpea forage (*Cajanus cajan* (L.) Millsp from the India. Revista Brasileira de Zootecnia 26: 447-453. (in Portuguese, with abstract in English).
Lewis, P.; Zaykin, D. 2002. Genetic Data Analysis (GDA): computer program for the analysis of allelic data: version 1.1 (d12). Available at: http://alleyn.eeb.uconn.edu/gda/. [Accessed Sep. 22, 2009].
Miller, M.P. 1997. Tools for Population Genetic Analysis (TFPGA) 1.3: A windows program for the analysis of allozyme and molecular population genetic data. (Computer software distributed by author).
Odeny, D.A.; Ayashree, B.; Ferguson, M.; Hoisington, D.; Crouch, J.; Gebhardt. 2007. Development, characterization and utilization of microsatellite markers in pigeonpea. Plant Breeding 126: 130-136.
Perrier, X.; Jacquemound-Collet, J.P. DARwin software. Available at: http://www.darwin.cirad.fr/darwin. 2006. [Accessed Jul. 12, 2008].
Pritchard, J.K.; Stephens, M.; Donnelly, P. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945-959.
Provazi, M.; Camargo, L.H.G.; Santos, P.M.; Godoy, R. 2007. Botanical description of selected pigeonpea pure lines. Revista Brasileira de Zootecnia 36: 328-334. (in Portuguese, with abstract in English).
Reddy, L.J.; Chandra, S.; Pooni, H.; Branmel, P.J. 2004. Rate of outcrossing in pigeonpea under intercropped conditions. p. 324-502. In: Bramel, P.J., ed. Assessing the risk of losses in biodiversity in traditional cropping systems: a case study of pigeonpea in Andhra Pradesh. International Crops Research Institute for the Semi-Arid Tropics. Patancheru, Andhra Pradesh, India.
Reddy, L.J.; Upadhyaya, H.D.; Gorda, C.L.L.; Sube, S. 2005. Development of core collection in pigeonpea [*Cajanus cajan* (L.) Millspaugh] using geographic and qualitative morphological descriptors. Genetic Resources and Crop Evolution 52: 1049-1056.
Tessier, C.; David, J.; Boursiquot, P.; Charrieir, A.J.M. 1999. Optimizations of the choice of molecular markers for varietal identification in *Vitis vinifera* L. Theoretical and Applied Genetics 98: 171-177.
Varshney, R.K.; Graner, A.; Sorrells, M.E. 2005. Genic microsatellite markers in plants: features and applications. Trends Biotechnology 23: 48-55.

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