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Research Article

Genetic diversity in section *Rhizomatosae* of the genus *Arachis* (Fabaceae) based on microsatellite markers

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Abstract

The genus *Arachis* (Fabaceae) native to South America, contains 80 species divided into nine sections, three of which contain species of special economic importance such as the cultivated peanut (*Arachis hypogaea*), belonging to the section *Arachis*, and some perennial forage species from sections *Caulorrhizae* and *Rhizomatosae*. We used microsatellite markers to assay genetic variability among 77 accessions of four species from section *Rhizomatosae*, the diploid *Arachis burkartii* (2n = 2x = 20) and the tetraploid *Arachis glabrata, Arachis pseudovillosa* and *Arachis nitida* (2n = 4x = 40). A total of 249 alleles were found in the fifteen loci analyzed and a high degree of intra and interspecific polymorphism was detected. The lowest intraspecific variation occurred in *Arachis burkartii*, while the smallest estimated interspecific value was between *A. nitida* and *A. pseudovillosa* and the largest was between *A. burkartii* and *A. nitida*. High observed heterozygosity was detected in *A. glabrata*. The diploid accessions grouped in one cluster and the tetraploid accessions in another. It was possible to distinguish all 77 accessions and the genetic distance between accessions could not be correlated with geographic origin.

Key words: Arachis, genetic diversity, microsatellite, peanut, *Rhizomatosae*.

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Introduction

The genus *Arachis* (Fabaceae Alt. Leguminosae) contains 80 species, all native to South America, and is divided into nine sections (Krapovickas and Gregory, 1994; Valls and Simpson, 2005). This genus contains many species of economic importance, such as the cultivated peanut *Arachis hypogaea* L. in section *Arachis* and perennial forage species in sections *Caulorrhizae* and *Rhizomatosae*, the latter containing four species: the diploid *Arachis burkartii* Handro (2n = 2x = 20) and the tetraploid (2n = 4x = 40) *Arachis glabrata* Benth., *Arachis nitida* Valls & C.E. Simpson and *Arachis pseudovillosa* (Chodat & Hassl.) Krapov. & W.C. Greg. (Fernández and Krapovickas, 1994; Peñaloza and Valls, 2005).

The herbaceous perennial *A. glabrata*, commonly called the rhizoma peanut or perennial forage peanut, is a wild species from which several commercial tropical forage cultivars have been developed (Prine *et al.*, 1981, 1986), including the Florigraze and Arbrook cultivars used in the USA as an alternative to alfalfa because they contain high levels of proteins and have high disease and pest resistance (French *et al.*, 1994). In Australia, *A. glabrata* is valued as high quality forage with the ability to spread through swards of aggressive summer-growing grass species (Bowman *et al.*, 1998). Furthermore, *A. glabrata* also shows multiple disease resistance and has been considered as a potential source of genes to genetic improvement of *A. hypogaea* (Mallikarjuna and Sastri, 2002). However, *A. glabrata* cultivars have a narrow genetic basis because they have been selected from a restricted number of available accessions (Prine, 1972; Cook and Crosthwaite, 1994). There are many *A. glabrata* accessions available in germplasm collections and the evaluation of this material could identify accessions with novel characteristics, such as growth at lower temperatures and in more humid soils, which may result in new cultivars (Prine and French, 1993).

Accessions of *A. glabrata* and all other section *Rhizomatosae* species have been maintained in germplasm banks in Brazil, Colombia, India and the USA, and their ge-

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netic variability has been evaluated using isozymes (Maass and Ocampo, 1995) and random amplification of polymorphic DNA (RAPD) analysis (Nóbile *et al.*, 2004). Although these studies have shown high variability in rhizomatous species and accessions these molecular markers have produced no information on the mating system of the species because of the low polymorphism of isozymes and dominance effects in RAPD analysis. Furthermore, comparison between studies is difficult because isoenzyme and RAPD markers have medium to low reproducibility (Jones *et al.*, 1997). Moreover, RAPD analysis is also sensitive to the reaction conditions and the possible co-migration of RAPD fragments with similar sizes but different sequences cannot be excluded (Tang *et al.*, 2005).

Microsatellites or simple sequence repeats (SSR) are stretches of a short nucleotide sequence that can be repeated many times in tandem (Casacuberta *et al.*, 2000). These molecular markers have been used for the characterization of genetic variability and analysis of the genetic relationships among plant species (Mörchen *et al.*, 1996; Khlestkina *et al.*, 2004). Microsatellite markers have several advantages: they are very abundant and thoroughly distributed in eukaryotic genomes (Tóth *et al.*, 2000); microsatellite primer pairs can be used in related species (Katzir *et al.*, 1996); and microsatellite markers provide a higher incidence of detectable polymorphisms than other molecular markers such as restriction fragment length polymorphism (RFLP) and RAPD markers (Powell *et al.*, 1996).

The objective of the study described in this paper was to use microsatellite markers to evaluate the variability and genetic relationships between the four species of the section *Rhizomatosae*, paying particular attention to the forage species *A. glabrata*.

Materials and Methods

Plant material

We investigated 77 accessions from the four species in section *Rhizomatosae* the diploid *Arachis burkartii* (2n = 2x = 20) and the tetraploid (2n = 4x = 40) *Arachis glabrata, Arachis nitida* and *Arachis pseudovillosa* (Table 1), representing natural populations collected in Brazil (all four species) and Paraguay (*A. glabrata* and *A. nitida*). The accessions also include the commercial cultivars Arbrook, a direct release from the PI 262817 accession collected by W. C. Gregory, A. Krapovickas and J.R. Pietrarelli (GKP 9570) near Trinidad (Itapúa Department, Paraguay; the southernmost site sampled by us), and Florigraze, selected from a volunteer plot hybrid in the United States and having

Table 1 - Accessions (n = 77) of the four *Arachis* species in section *Rhizomatosae* analyzed using microsatellite markers.

Species, collection country and accession code (Brazilian gene bank accession number - BRA)*	Collection site, altitude where collected and map reference†	Collectors
A. burkartii (n = 9 accessions)		
Brazil		
016136 (7317)	São Luiz Gonzaga, RS: 230 m; 28°25' S, 54°57' W	Valls J., Moss J., Gerin M., Silva G.
016314 (7320)	S. Antônio das Missões, RS: 150 m; 28°36' S, 55°44' W	Valls J., Moss J., Gerin M., Silva G.
016349 (7334)	Uruguaiana, RS: 45 m; 29°48' S, 56°56' W	Valls J., Simpson C., Moss J., Gerin M., Silva G.
016047 (7363)	Torres, RS: 5 m; 29°21' S, 49°47' W	Valls J., Simpson C., Moss J., Gerin M., Silva G.
030821 (12322)	Dom Pedrito, RS: 130 m; 31°00' S, 54°35' W	Valls J., Zanin A., Moraes C., Oliveira J., Werneck W.
036986 (14239)	Itaqui, RS: 110 m; 29°04' S, 56°15' W	Valls J., Souza-Chies T., Palmieri D.
036994 (14266)	Uruguaiana, RS: 270 m; 29°52' S, 56°52' W	Valls J., Souza-Chies T., Palmieri D.
037117 (14304)	Rosário do Sul, RS: 120 m; 30°14' S, 54°47' W	Valls J., Miotto S. Irgang B., Baptista L., Carneiro A.
037133 (14317)	Caçapava do Sul, RS: 400 m; 30°21' S, 53°27' W	Valls J., Miotto S., Irgang B., Baptista L., Carneiro A.
A. glabrata (n = 58 accessions)		
Brazil		
012076 (5115)	Bom Jesus de Goiás, GO: 600 m; 18°16' S, 49°42' W	Valls J., Sano S., Silva J.
012084 (5153)	Acreúna, GO: 450 m; 17°33' S, 50°34' W	Valls J., Sano S., Silva J.
012157 (5916)	Ituitaba, MG: 500 m; 18°56' S, 49°26' W	Valls J., Werneck W.
012165 (5922)	Goiatuba, GO: 650 m; 18°05' S, 49°17' W	Valls J., Werneck W.
012874 (6436)	Nobres, MT: 300 m; 14°35' S, 56°13' W	Valls J., Simpson C., Gripp A.
016292 (7300)	Uberaba, MG: 490 m; 19°58' S, 47°46' W	Valls J., Moss J., Silva G.
017531 (7642)	Bonito, MS: 460 m; 20°59' S, 56°27' W	Valls J., Rao V., Gerin M., Silva G.
017566 (7647)	Bela Vista, MS: 210 m; 21°53' S, 56°19' W	Valls J., Rao V., Gerin M., Silva G.

Table 1 (cont.)

Species, collection country and accession code (Brazilian gene bank accession number - BRA)*	Collection site, altitude where collected and map reference†	Collectors
017661 (7710)	Rio Brilhante, MS: 290 m; 21°49’ S, 54°32’ W	Valls J., Rao V., Gerin M., Silva G.
020567 (7537)	Uberaba, MG: 920 m; 19°02’ S, 48°01’ W	Valls J., Rao V.
020575 (7516)	Campo Grande, MS: 510m; 20°26’ S, 54°43’ W	Valls J.
029254 (911)	Campo Grande, MS: other data unavailable	Sousa Costa N.
029262 (912)	Campo Grande, MS: other data unavailable	Sousa Costa N.
029386 (11922)	Douradina, MS: 450 m; 21°55’ S, 54°30’ W	Valls J., Quarin C., França Dantas M., Silva G.
030864 (13273)	Conc. das Alagoas, MG: 450 m; 20°01’ S, 48°13’ W	Valls J., Moss J., Pizarro E., Werneck W.
033359 (1134)	Santa Albertina, SP: 410 m; 20°05’ S, 50°45’ W	Ferreira F., Werneck W.
033561 (13652)	Goiatuba, GO: 850 m; 18°02’ S, 49°14’ W	Valls J., Werneck W.
034053 (unavailable)	São Manuel, SP: 700 m; 22°43’ S, 48°35’ W	Bertozo M.
034061 (2091)	Indiara or Mineiros, GO: other data unavailable	Vieira R., Vinicius M., Werneck W.
034371 (13936)	Mineiros, GO: 700 m; 17°34’ S, 53°37’ W	Valls J., Peñaloza A, Werneck W.
034495 (13964)	Camapuã, MS: 430 m; 19°31’ S, 54°02’ W	Valls J., Moss J., Pizarro E., Werneck W.
034533 (13971)	DoisIrmãos, MS: 230 m; 20°28’ S, 55°16’ W	Valls J., Moss J., Pizarro E., Werneck W.
034541 (13976)	Miranda, MS: 120 m; 20°14’ S, 56°22’ W	Valls J., Moss J., Pizarro E., Werneck W.
034550 (13977)	Miranda, MS: 120 m; 20°14’ S, 56°22’ W	Valls J., Moss J., Pizarro E., Werneck W.
034576 (13979)	Miranda, MS: 140 m; 20°11’ S, 56°30’ W	Valls J., Moss J., Pizarro E., Werneck W.
034592 (13983)	Miranda, MS: 130 m; 20°08’ S, 56°39’ W	Valls J., Moss J., Pizarro E., Werneck W.
034703 (14013)	Porto Murtinho, MS: 250 m; 21°04’ S, 56°54’ W	Valls J., Moss J., Pizarro E., Werneck W.
034754 (14019)	Bonito, MS: 300 m; 21°12’ S, 56°27’ W	Valls J., Moss J., Pizarro E., Werneck W.
034924 (14061)	Bela Vista, MS: 220 m; 22°04’ S, 56°30’ W	Valls J., Moss J., Pizarro E., Werneck W.
037061 (3037)	Aparec. do Taboado, MS: 395 m; 20°02’ S, 51°06’ W	Sousa Costa N., Karia C.
037141 (3100)	Antônio João-B.Vista, MS: 610 m; 22°03’ S, 56°22’ W	Sousa Costa N., L. Zago Machado
037176 (3116)	Santa Vitória, MG: 330 m; 19°01’ S, 50°27’ W	Sousa Costa N., Andrade R.
037184 (3118)	Campina Verde, MG: 600 m; 19°16’ 49°43’ W	Sousa Costa N., Andrade R.
037192 (3120)	Prata, MG: 630 m; 19°13’ S, 48°48’	Sousa Costa N., Andrade R.
037729 (14498)	Itarumã, GO: 500 m; 18°45’ S, 51°20’ W	Valls J., Moss J., Pizarro E., Werneck W.
037737 (14505)	Paranaíba, MS: 430 m; 19°39’ S, 51°21’ W	Valls J., Moss J., Pizarro E., Werneck W.
037745 (14513a)	Inocência, MS, 400 m; 19°57’ S, 52°12’ W	Valls J., Moss J., Pizarro E., Werneck W.
037745 (14513b)	Inocência, MS: 400 m; 19°57’ S, 52°12’ W	Valls J., Moss J., Pizarro E., Werneck W.
037761 (14516)	Ribas do Rio Pardo, MS: 400 m; 20°26’ S, 53°45’ W	Valls J., Oliveira R., Moraes M., Silva G.
037826 (14565)	Nioaque, MS: 350 m; 21°18’ S, 56°00’ W	Valls J., Moss J., Pizarro E., Werneck W.
037834 (14571)	Ponta Porá, MS: 600 m; 22°30’ S, 55°27’ W	Valls J., Moss J., Pizarro E., Werneck W.
037842 (14572)	Ponta Porá, MS: 600 m; 22°30’ S, 55°27’ W	Valls J., Moss J., Pizarro E., Werneck W.
037885 (14601)	Coronel Sapucaia, MS: 500 m; 23°21’ S, 55°31’ W	Valls J., Moss J., Pizarro E., Werneck W.
037893 (14617)	Dourados, MS: 35 m; 22°16’ S, 54°48’ W	Valls J., Moss J., Pizarro E., Werneck W.
Paraguay		
011878 (Cv Arbrook)	Itapúa Department: 80 m; 27°10’ S, 55°40’ W	(Standard cultivar, ex Florida, USA)
035645 (3778)	Concepción Dept.: 190 m; 22°17’ S, 57°06’ W	Silva G., Pizarro E., Heyn R.
035793 (3798)	Concepción Dept.: 260 m; 22°15’ S, 56°23’ W	Silva G., Pizarro E., Heyn R.
035823 (3801)	Concepción Dept.: 150 m; 23°26’ S, 57°11’ W	Silva G., Pizarro E., Heyn R.
035831 (3802)	Concepción Dept.: 70 m; 22°52’ S, 57°24’ W	Silva G., Pizarro E., Heyn R.
035840 (3803)	Concepción Dept.: 80 m; 22°53’ S, 57°24’ W	Silva G., Pizarro E., Heyn R.
035858 (3805)	Concepción Dept.: 90 m; 22°53’ S, 57°22’ W	Silva G., Pizarro E., Heyn R.

Table 1 (cont.)

Species, collection country and accession code (Brazilian gene bank accession number - BRA)*	Collection site, altitude where collected and map reference†	Collectors
035947 (3814)	Concepción Dept.: 100 m; 23°01' S, 57°01' W	Silva G., Pizarro E., Heyn R.
036331 (3822)	Concepción Dept.: 120 m; 23°20' S, 57°19' W	Silva G., Pizarro E., Heyn R.
036366 (3829)	Concepción Dept.: 180 m; 22°29' S, 57°34' W	Silva G., Pizarro E., Heyn R.
036374 (3831)	Concepción Dept.: 250 m; 22°40' S, 57°31' W	Silva G., Pizarro E., Heyn R.
036382 (3832)	Concepción Dept.: 200 m; 22°51' S, 57°24' W	Silva G., Pizarro E., Heyn R.
036404 (3834)	Concepción Dept.: 230 m; 22°53' S, 56°51' W	Silva G., Pizarro E., Heyn R.
USA		
011819 (Cv Florigraze)	Gainesville, FL: Cv. PI 421707: other data unavailable	[Standard cultivar]
A. nitida (n = 7 accessions)		
Brazil		
034983 (14040)	Porto Murtinho, MS: 200 m; 21°52' S, 57°34' W	Valls J., Moss J., Pizarro E., Werneck W.
Paraguay		
035581 (3771)	Concepción Dept.: 180 m; 22°19' S, 57°14' W	Silva G., Pizarro E., Heyn R.
035602 (3774)	Concepción Dept.: 180 m; 22°19' S, 57°11' W	Silva G., Pizarro E., Heyn R.
035688 (3782)	Amambay Dept.: 200 m; 22°23' S, 56°38' W	Silva G., Pizarro E., Heyn R.
035696 (3783)	Amambay Dept.: 200 m; 22°23' S, 56°38' W	Silva G., Pizarro E., Heyn R.
036340 (3823)	Concepción Dept:. 100 m; 23°19' S, 57°18' W	Silva G., Pizarro E., Heyn R.
036421 (3836)	Concepción Dept.: 230 m; 22°53' S, 56°51' W	Silva G., Pizarro E., Heyn R.
A. pseudovillosa (n = 3 accessions)		
Brazil		
017701 (7695)	Aral Moreira, MS: 450 m; 22°51' S, 55°17' W	Valls J., Rao V., Gerin M., Silva G.
032883 (13593)	Antônio João, MS: 660 m; 22°10' S, 55°56' W	Valls J., Oliveira R., Silva G.
037869 (14590)	Coronel Sapucaia, MS: 520 m; 23°19' S, 55°33' W	Valls J., Oliveira R., Moraes M., Silva G.

*Brazilian gene bank sited at Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil.
†GO, Goiás; MG, Minas Gerais; MS, Mato Grosso do Sul; MT, Mato Grosso; RS, Rio Grande do Sul; SP, São Paulo.

an old accession (PI 118457) collected in the Brazilian town of Campo Grande in 1936 by W. Archer as the probable female parent. All samples were made available by Embrapa Recursos Genéticos e Biotecnologia (Cenargen, Brasília, DF, Brazil) where plants have been maintained in pots and vegetatively propagated since no seed production has been observed in the greenhouse.

DNA extraction and amplification

For each accession, we extracted total DNA from the leaves of a single plant using the procedure described by Doyle and Doyle (1987) and the minor modifications given by Ferreira and Grattapaglia (1996).

The 15 microsatellite primer pairs used in this study (Table 2) were isolated from three different species from three *Arachis* sections: six from *A. hypogaea* (Ah) in section *Arachis*, five from *Arachis pintoi* (Ap) in section *Caulorrhizae* and four from *A. glabrata* (Ag) in section *Rhizomatosae*. The isolation and characterization of microsatellites Ap40 and Ap176 have been described by Palmieri *et al.* (2002) and Ap33 by Palmieri *et al.* (2005). Microsatellites Ah3, Ah7, Ah11, Ah21, Ah126 and Ah282 were isolated by Gimenes *et al.* (2007) while microsatellites Ap32, Ap38 Ag39, Ag151, Ag167 and Ag171 were described by Moretzsohn *et al.* (2005). For each plant, individual amplification was carried out in a reaction mixture containing 15 ng of total DNA, 0.3 mM of each primer, 0.375 µM of each dNTP, 0.5 units of *Taq* DNA polymerase (GE Healthcare, USA), 1 X amplification buffer and appropriate concentration of $MgCl_2$ (1.5 mM for Ah3, Ah7, Ah21, Ah126, Ap176; 2.0 mM for Ap32, Ap33, Ap38, Ap40; and 2.5 mM for Ah11, Ag39 Ag151, Ag167, Ag171, Ah282) and made up to 10 µL with deionized water. All amplifications were performed in a PTC100 thermal cycler (MJ Research, United Kingdom). The amplification parameters used consisted of 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 45 s at 50 °C, and 1 min at 72 °C. The PCR products were denatured by adding 7 µL of a loading buffer (95% formamide, 5% NaOH 0.2 M, 0.25% bromophenol blue) and heating for 5 min at 90 °C, separated on 4% (w/v)

Table 2 - Results obtained for analysis of 77 accessions from the four *Arachis* species in section *Rhizomatosae*. The table shows the locus, primer sequence, number of observed alleles (A_o), number of different band patterns observed (NBP), mean observed heterozygosity for each species and locus (H_o) and the overall mean observed heterozygosity per locus (H_o*). The source species categories and the loci are ordered by decreasing overall H_o value, with zero indicating no heterozygosity.

Locus and source species	Forward and reverse primer sequences	A_o	*Arachis* species in section *Rhizomatosae*								H_o*
			A. burkartii		*A. glabrata*		*A. nitida*		*A. pseudovillosa*		
			NBP	H_o	NBP	H_o	NBP	H_o	NBP	H_o	
From *Arachis hypogaea*											
Ah126	5' CCCTGCCACTCTCACTCACT3' 5' CGTACAAGTCAGGGGGTGAC3'	23	9	0.888	48	0.880	6	1	2	1	0.9420
Ah21	5' CAGCCTAGAGCCGAATTCAC3' 5' ATAGGGAGAAGGCAGGGAGA3'	26	8	0.777	50	0.793	7	1	3	0.666	0.8090
Ah282	5' GCAAACACACCACATTTCA3' 5' GGCTCCAATCCCAAACACTA3'	18	8	0.555	41	0.690	6	0.857	3	1	0.7755
Ah3	5' TCGGAGAACCAAGCACACATC3' 5' TTGCGCTCTTTCTCACACTC3'	25	9	0.777	42	0.638	6	1	3	0.666	0.7703
Ah7	5' CAGAGTCGTGATTTGTGCACTG3' 5' ACAGAGTCGGCCGTCAAGTA3'	19	6	0.555	41	0.552	7	0.857	3	0.666	0.6575
Ah11	5' AAATAATGGCATACTTGTGAACAATC3' 5' TTCCACCAAGGCAAGACTATG3'	15	5	0.333	25	0.534	2	0.143	2	1	0.5025
From *Arachis glabrata*											
Ag171	5' TGACCGTTGGGGTTTTTG3' 5' CAAACCCAAACACACGTCAC3'	25	9	0.555	48	0.862	7	0.714	3	0.666	0.6993
Ag151	5' GCATCAGCAGAATCAGTGGA3' 5' GAGTGGGACGAGCTAAGGTG'	22	9	0.555	46	0.862	7	0.714	3	0.666	0.6993
Ag39	5' TGTAGTCAGCTGCTCCAAAA3' 5' ATGAAAGTTCACTTGAGCAAA3'	25	4	0	40	0.534	6	0.714	3	1	0.5620
Ag167	5' CTCACCTTCAAAGCCCTTGT3' 5' AGAGGGGACAACGACAACC3'	24	1	0	43	0.552	7	0.571	3	0.666	0.4473
From *Arachis pintoi*											
Ap40	5' CTGTTTGATCGCCGCTATG3' 5' GTCAAGTGCTTCCTCCGATG3'	14	4	0.333	29	0.759	3	0.286	3	0.338	0.4290
Ap176	5' CCAACACAGGGCTTACCAAG3' 5' TCACCGATCCCACTTTTCC3'	10	3	0	6	0.517	3	0.429	1	0	0.2365
Ap32	5' GATCATGCTCATCATCAACACC3' 5' ATAGGGAGAAGGCAGGGAGA3'	1	1	0	1	0	1	0	1	0	0.0000
Ap33	5' CATCGTGTGGAGACGAAGGT3' 5' AACAGAGAGCGGATGAAAGC3'	1	1	0	1	0	1	0	1	0	0.0000
Ap38	5' CAGCCTAGAGCCGAATTCAC3' 5' GCGAACAAAGGAGGAAGAGA3'	1	1	0	1	0	1	0	1	0	0.0000

polyacrylamide denaturing gels at 30 W and 1100-1500 V for about 2.5 h and stained with silver according to a commercial protocol (Promega, Madison, USA).

Data analysis

For each sample the amplified fragments were scored as present or absent. A genetic distance matrix was generated using the Nei and Li Genetic Distance Coefficient (Nei and Li, 1979). Genetic relationships between the 77 accessions were viewed in a neighbor-joining tree (Saitou and Nei, 1987). All analyses were computed with the TREECON 1.3 software (Van der Peer and Watcher, 1994). The mean genetic distance coefficient for each species was estimated by calculating the mean genetic distance for each pairwise comparison in each species.

Results

Band patterns formed by one, two, three and four fragments were observed in the samples. Patterns formed by one or two fragments were observed for *A. burkartii* and from one to four fragments in *A. glabrata*, *A. nitida* and *A. pseudovillosa* (data not shown). The number of fragments observed in the patterns was in line with the expected pattern for a locus in species with the corresponding ploidy levels. The data indicated that *A. burkartii* is diploid, with a maximum of two alleles, while the others are tetraploid with up to four putative alleles. Therefore, accessions presenting a single amplified fragment were considered homozygote while those presenting more than one fragment were considered heterozygote for the locus in question.

We detected 249 observed alleles (A_o) in the 15 loci amplified, of which three loci (20%) were monomorphic (Ap32, Ap33 and Ap38) for all the species while locus Ag167 was monomorphic only for *A. burkartii* and Ap176 for *A. pseudovillosa* only (Table 2). In the 12 polymorphic loci (Ag151, Ag167, Ag171, Ag39, Ah11, Ah126, Ah21, Ah282, Ah3, Ah7, Ap176 and Ap40) the observed number of alleles ranged from 10 for Ap176 to 26 for Ah21, with the mean number of observed alleles per locus being 20.5 while the overall mean observed heterozygosity per locus (H_o*) ranged from 0.2365 for Ap176 to 0.9420 for Ah126 (Table 2). The six primer pairs developed for *A. hypogaea* (section *Arachis*) detected polymorphism for all four species of section *Rhizomatosae*. Three of the five primer pairs developed for *A. pintoi* (section *Caulorrhizae*) detected only monomorphic loci in the species from section *Rhizomatosae*. We also found that the Ap40 locus was polymorphic in the four species studied, while locus Ap176 was polymorphic for *A. glabrata*, *A. nitida* and *A. burkartii* and monomorphic only for *A. pseudovillosa*. Three primer pairs (Ag39, Ag151 and Ag171) of the four pairs developed for *A. glabrata* were polymorphic for the other three species of section *Rhizomatosae*, and the Ag167 locus was monomorphic only for *A. burkartii* and polymorphic for the three tetraploid species (*A. glabrata*, *A. nitida* and *A. pseudovillosa*).

The number of observed alleles found in each species varied widely, with A_D = 209 in *A. glabrata*, 109 in *A. nitida*, 72 in *A. burkartii* and 53 in *A. pseudovillosa* (Table 3). Furthermore, the number of specific alleles (A_s) for each species was also different between the species analyzed, with *A. glabrata* having 88 (88/209 = 42%), *A. burkartii* 25 (25/72 = 35%), *A. nitida* 10 (10/109 = 9%) and *A. pseudovillosa* only one (1/53 = 2%) (Table 3).

The general mean observed heterozygosity per species (H_o') for the polymorphic loci analyzed was highest (H_o' = 0.6947) for *A. pseudovillosa* and lowest (H_o' = 0.4444) for *A. burkartii* (Table 3). In addition, the mean number of putative alleles (A_p) per locus (A_p/A_D) ranged from 4.81 for *A. pseudovillosa* to 15.92 for *A. glabrata*, while the percentage of different band patterns per total of accession analyzed (NBP/N_A) ranged from 65.95% for *A. glabrata* to 89.00% for *A. pseudovillosa* (Table 3).

The mean genetic distances within accessions of each species and between the species were high (Table 4). Diploid *A. burkartii* showed the lowest intraspecific variation of all the species analyzed. The smallest estimated interspecific value (0.7106) was between *A. nitida* and *A. pseudovillosa* and the largest (0.8279) was between *A. burkartii* and *A. nitida*.

The band presence and absence data matrix was used to produce a dendrogram, which showed that no pair of accessions presented 100% similarity and that the accessions were distributed in two large clusters (Figure 1). One cluster was formed by the accessions of the diploid species

Table 3 - Statistical analysis of the data for 77 accessions of the four *Arachis* species in section *Rhizomatosae*, showing the number of accessions (N_A), total alleles detected (A_D), number of specific alleles (A_s), ratio of putative alleles (A_p) to total alleles detected (A_p/A_D), general mean observed heterozygosity per species (H_o'), mean number of different band patterns (NBP') and percentage of different band patterns per number of accessions (NBP' /N_A, %).

Species	N_A	A_D	A_s	A_p/A_D	H_o'	NBP'	NBP' /N_A (%)
A. burkartii	9	72	25	6.55	0.4444	6.25	69.44
A. glabrata	58	209	88	15.92	0.6810	38.25	65.95
A. nitida	7	109	10	9.08	0.6904	5.58	79.71
A. pseudovillosa	3	53	1	4.81	0.6947	2.67	89.00

Table 4 - The mean genetic distance estimates (Nei and Li, 1979) for the four *Arachis* species in section *Rhizomatosae*.

	A. glabrata	*A. nitida*	*A. burkartii*	*A. pseudovillosa*
A. glabrata	0.7108	0.7823	0.8166	0.7563
A. nitida	-	0.6105	0.8279	0.7106
A. burkartii	-	-	0.5661	0.7832
A. pseudovillosa	-	-	-	0.6123

A. burkartii while the accessions of the tetraploid species *A. glabrata*, *A. nitida* and *A. pseudovillosa* formed another cluster made up of two sub-clusters, the first, subdivided into several small clusters, consisted of 55 of the 58 *A. glabrata* accessions and the second contained the tetraploid species (*A. nitida* and *A. pseudovillosa)* and the three remaining *A. glabrata* accessions (Figure 1).

The relationship between the number of randomly sampled accessions from the *A. glabrata* and the number of alleles detected is presented in Figure 2. The number of alleles increased to 178 when 27 accessions were sampled and from then onwards the number of alleles tended to stabilize and about two alleles were added with each new accession.

Discussion

All the accessions studied could be differentiated with the primer pairs used, indicating that microsatellite markers are highly polymorphic and discriminative in *Arachis* species of the section *Rhizomatosae*. These same characteristics of microsatellite primers have previously been detected in many plant species, including the apricot *Prunus armeniaca* L. (Hormaza, 2002), the tetraploid potato *Solanum tuberosum* L. (McGregor *et al.*, 2000) and a wild and cultivated wheat collection (Medini *et al.*, 2005). Furthermore, studies comparing molecular markers such as Amplified Fragment Length Polymorphisms (AFLP), RAPD, RFLP and microsatellites have already indicated that AFLP and microsatellite markers are highly reproducible within and between laboratories (Rafalski and Tingey, 1993; Cordeiro *et al.*, 2000). Apart from reproducibility, we also found that a further advantage of the microsatellites used in our study was the cross-species transferability of these markers between *Arachis* species belonging to different sections (*Arachis*, *Caulorrhizae* and *Rhizomatosae*).

The high frequency of *A. glabrata* accessions with several heterozygous loci indicated that this is a characteristic commonly present in this species and is not due to any other factor, such as a long period of cultivation in greenhouses, outside the area of natural distribution. High heterozygosity is not an expected characteristic among *Arachis* species, since they are generally considered autogamous, although this is not necessarily the case for species in the least investigated taxonomic sections of this genus. For example, the rhizomatous species *A. glabrata* has some pecu-

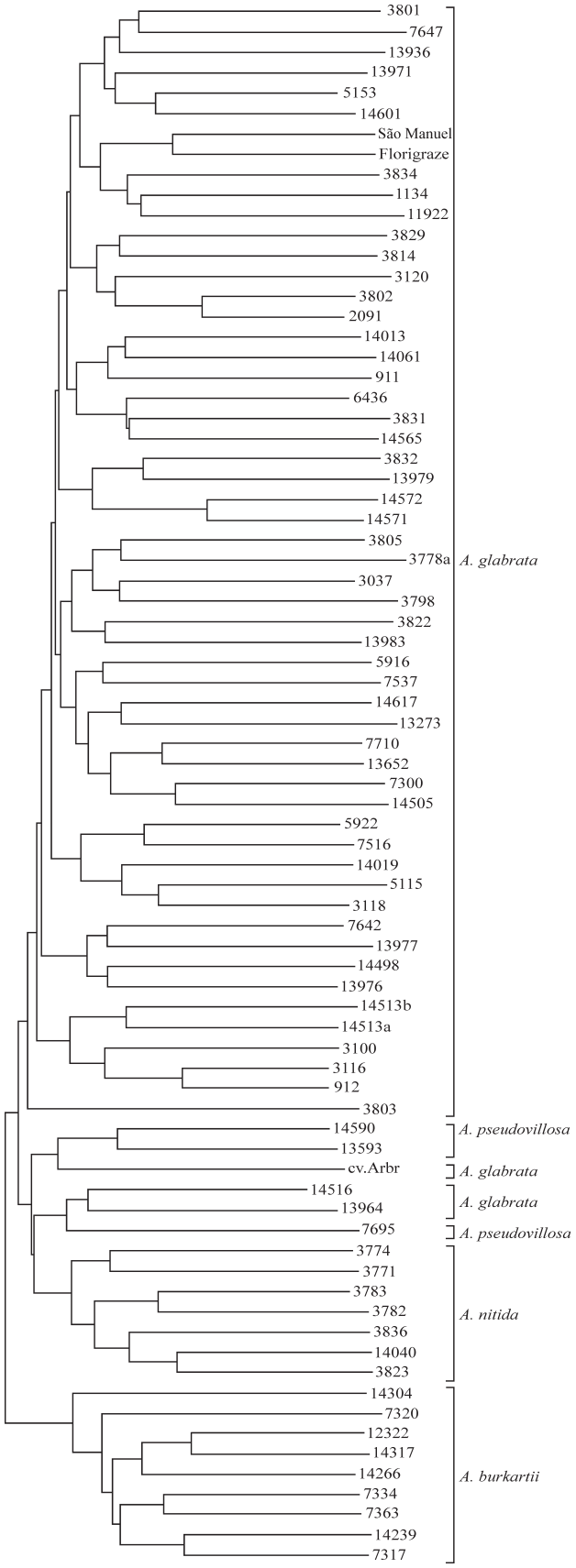

Figure 1 - Neighbor joining tree showing the relationships between accessions of the four *Arachis* species in section *Rhizomatosae*. The accession numbers are those shown in Table 1.

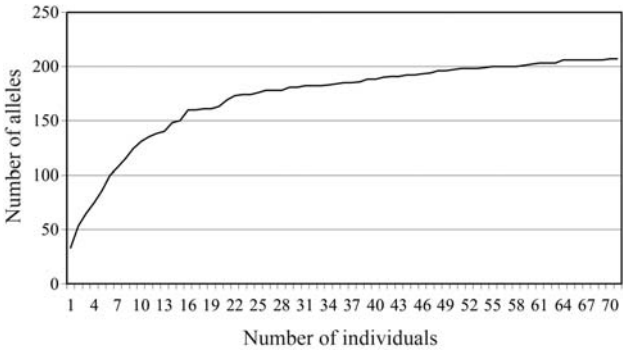

Figure 2 - The relationship between the number of randomly sampled *Arachis glabrata* accessions and the number of alleles detected.

liarities, scattered wild populations occasionally produce seeds while only a small quantity of seed, if any, is sporadically found when this plant is cultivated in greenhouses and nurseries (Valls, 1996), making vegetative propagation by cuttings the normal method of artificial multiplication of this species. The absence of seeds in plants maintained in germplasm banks may be related to incompatibility systems (Cook and Crosthwaite, 1994; French *et al.*, 1994), since *A. glabrata* pollen viability appears to be high and is not in itself seen to be an obstacle to seed production (Niles and Quesenberry, 1992). It thus appears that there is a high frequency of cross-pollination in the original area of *A. glabrata* distribution, resulting in high heterozygosity among *A. glabrata* accessions which is retained during vegetative propagation.

We found that the tetraploid species of section *Rhizomatosae* presented higher heterozygosity levels than the diploid species *A. burkartii*. However, this was not observed when the same species were assessed using RAPD markers (Nóbile *et al.*, 2004), this difference between studies probably being due to the greater ability of microsatellite markers to detect polymorphism and amplified polymorphic loci in tetraploid species which appeared null when assessed with RAPD markers.

The genetic distance estimates calculated by us were high (Table 4), indicating a large amount of genetic variability in section *Rhizomatosae* species, as expected in wild *Arachis* species. High variability has also been detected in the germplasm of wild *Arachis* species in other studies using isozyme (Maass and Ocampo, 1995), RAPD (Nóbile *et al.*, 2004), RFLP (Gimenes *et al.*, 2002) and microsatellite (Moretzsohn *et al.*, 2004; Hoshino *et al.*, 2006; Gimenes *et al.*, 2007) markers. The high genetic variability which we found in *A. glabrata* supports the work by Maass and Ocampo (1995), who analyzed four isoenzyme systems (alpha-esterase, acid phosphatase, glutamate oxaloacetate transaminase and diaphorase) in 12 *A. glabrata* accessions from the International Tropical Agriculture Center germplasm bank (CIAT, Colombia). In addition, Nóbile *et al.* (2004) used RAPD markers to assess 57 *A. glabrata* accessions, 11 *A. burkartii* accessions, 11 *A. nitida* accessions and 1 *A. pseudovillosa* accession from the CENARGEN *Arachis* Germplasm Bank and also detected high variability in the species.

In our study, it is interesting to note that the number of general and specific alleles detected were similar to the number of accessions analyzed of each of the four rhizomatous species, reflecting not only the different extent of the geographic distribution of the different species but also the fact that not only is *A. glabrata* germplasm preferentially collected because it is a potential forage but it has a much greater ability to survive for many years under greenhouse or nursery conditions than the other three section *Rhizomatosae* species.

As in the study of the genetic integrity of the Gatersleben *Triticum aestivum* L. germplasm bank by Börner *et al.* (2000), we found that the accessions of the species already represented in the CENARGEN *Arachis* germplasm bank and the new accessions collected may be efficiently monitored by microsatellite markers for the maintenance and preservation of the genetic variability of this material.

Our study showed that the species investigated shared 124 (50.2%) alleles, a relatively large number. Differentiation between the species is, therefore, due not only to the specific alleles but also to the differences in the frequencies of the shared alleles in each species (Table 3). This was the case for *A. pseudovillosa* and *A. nitida*, which presented a very small number of specific alleles (Table 3). In the dendrogram (Figure 1) the first cluster contains tetraploid species which were separated from the diploid species in the second cluster, confirming the results of RAPD analysis (Nóbile *et al.*, 2004). The larger subcluster formed in the first cluster included 55 accessions that belong exclusively to *A. glabrata* while the second smaller subcluster did not distinguish the species perfectly. Although *A. nitida* was clearly separated from the other accessions, the *A. pseudovillosa* and *A. glabrata* accessions in this subcluster did not separate perfectly. These results support those of Krapovickas and Gregory (1994), who reported natural hybrids of these two species, and the results of the RAPD analysis carried out by Nóbile *et al.* (2004). Valls (1996) pointed out that no hybrid exists between the diploid and tetraploid species of section *Rhizomatosae*, which seems to indicate that different genomes are present in the tetraploid and diploid species (Stalker and Moss, 1987). However, many of the accessions used in these crossing attempts were not good seed producers, and unsuccessful hybridization may have other causes that do not involve genetic or genomic incompatibility (Valls, 1996).

As Figure 2 shows, *A. glabrata* harbors a large number of alleles and the allele content in a random sample can be monitored using microsatellites loci that would help the formation of a core collection of this species. However, it is necessary to point out that, although extensive, the present sampling did not include representatives of wild popula-

tions from Argentina (*A. burkartii* and *A. glabrata*), Uruguay (*A. burkartii*), and southern Paraguay (*A. glabrata*, except for the `Arbrook' cultivar). Southern Paraguay and the adjacent Province of Corrientes in Argentina harbor the morphologically distinct *A. glabrata* var. *hagenbeckii,* with narrow leaflets, in sympatry with local types of *A. glabrata* var. *glabrata* (Krapovickas and Gregory, 1994). Much more scarce, *A. pseudovillosa* occurs also in the Department of Amambay, Paraguay, near the Brazilian border, but only a few Brazilian accessions are available and this species is poorly represented in gene banks. Further efforts to cover such gaps in our knowledge of the genetic diversity of section *Rhizomatosae* using the microsatellite primers described in the present paper are obviously needed.

In conclusion, the microsatellite markers used allowed us to distinguish the species and accessions of section *Rhizomatosae*, detected high variation among accessions and can be very useful for monitoring the genetic variability in germplasm banks when establishing core collections.

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