Genetic relationships of Arachis (Fabaceae) accessions based on microsatellite markers

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Research Article

Abstract

The genus *Arachis* is endemic to South America and contains 83 described species assembled into nine taxonomical sections. The section *Arachis* is of particular interest because it includes the cultivated peanut (*A. hypogaea*) and its closely related wild species. The knowledge of the genetic relationships among species and accessions is necessary for a more efficient management of germplasm collections and use of wild species for crop improvement. In this study, we used 26 microsatellite markers to analyze the genetic variability and relationships of some recently collected accessions of species in the *Arachis* section, with emphasis on the B genome species. The analyses showed a generally high level of intraspecific genetic variability, but usually grouped the accessions according to their genome types and species. However, accessions of some species did not group as expected, and these results suggest the need of further taxonomic revision of a few taxa, especially some accessions of *A. gregoryi*, *A. magna* and *A. kuhlmannii* and the circumscriptions of sections *Erectoides* and *Procumbentes*.

Introduction

The genus *Arachis* contains 83 described species, assembled into nine taxonomic sections according to their morphology, cross-compatibility relationships and geographical distribution in South America (Krapovickas and Gregory 1994; Valls and Simpson 2005; Valls et al. 2013; Valls and Simpson 2017; Seijo et al. 2021). Brazil is the largest holder of wild *Arachis*, as 65 species of all nine sections occur in its territory and 46 are exclusive to the country. Most of the *Arachis* species are diploid with 2n = 2x = 20, five are tetraploid (2n = 4x = 40), and four are aneuploid or dysploid, with 2n = 2x = 18 chromosomes (Fernández and Krapovickas 1994; Krapovickas and Gregory 1994; Lavia 1998; Peñaloza and Valls 2005; Ortiz et al. 2017; Silvestri et al. 2017).

The section *Arachis* is the most important because it contains the cultivated *A. hypogaea*, and its wild progenitors: *A. duranensis* and *A. ipaënsis* (Kochert et al. 1996; Seijo et al. 2004; Fávero et al. 2006; Grabiele et al. 2012; Moretzsohn et al. 2013; Bertioli et al. 2016). Within section *Arachis*, six genome types (A, B, D, F, G, and K) have been described, based on classical and molecular cytogenetic studies (Husted 1933; Stalker 1991; Fernández and Krapovickas 1994; Robledo and Seijo 2008; Robledo and Seijo 2010; Silvestri et al. 2015). Most species in the *Arachis* section have an A genome type, characterized by the presence of a so-called A chromosome pair, which has a reduced size (Husted 1936) and a lower level of euchromatin condensation in comparison to the other chromosomes (Seijo et al. 2004). Genome A species were arranged into three subgroups (Chiquitano, La Plata River Basin, and Pantanal) based on the variability observed in the heterochromatin and 18S−26S rRNA loci (Robledo et al. 2009). The remaining diploid species with x = 10 do not have the "A chromosome" pair and have been divided into four genome types: (1) B *stricto sensu*, without centromeric bands and similar to the B genome of *A. hypogaea*; (2) K, showing large centromeric bands and similarities with the Chiquitano A genome subgroup; (3) F, with reduced but uniform amount of centromeric heterochromatin, and (4) the D genome, characterized by an asymmetric karyotype with several subtelocentric or submetacentric chromosomes, only found in *A. glandulifera* (Stalker 1991; Robledo and Seijo 2008; Robledo and Seijo, 2010). Finally, the

G genome was described for three species of section *Arachis* with reduced basic chromosome number (x = 9), and uniform DAPI bands (Silvestri et al. 2015).

In the last decades, some 1250 accessions of 63 described native wild *Arachis* species have been collected with germplasm in Brazil (http://alelobag.cenargen.embrapa.br/AleloConsultas). Wild species are highly promising sources of genes for desirable agronomic traits, such as earliness, drought tolerance, resistance to foliar diseases, viruses, and nematodes (Stalker 2017). The characterization of these new accessions is important for their conservation and use for the improvement of cultivated peanut.

Microsatellite or SSR (Simple sequence repeat) markers have been widely used to analyze the genetic variability in plant species, since they are multiallelic, polymorphic, randomly distributed through plant genomes, and typically codominant markers. In addition, microsatellites have proven to be highly transferable between species of *Arachis* (Moretzsohn et al. 2004; Hoshino et al. 2006; Gimenes et al. 2007; Koppolu et al. 2010; Moretzsohn et al. 2013).

The objective of this study was to analyze the genetic variability and relationships of some recently collected accessions of species in the *Arachis* section, highlighting the five previously described species determined to have a B genome *stricto sensu* (Robledo and Seijo 2010), that is, not yet considering the recently described *A. inflata* (Seijo et al. 2021), and compare them with accessions of the *Erectoides* and *Procumbentes* sections, contributing to a more efficient conservation and use of this germplasm. We also took the opportunity to examine some small doubts regarding the identity of a few accessions, as described below, which can become a burden in the management of germplasm.

Material And Methods

Plant material

Plants were obtained from the Genebank of Wild Species of *Arachis*, maintained at Embrapa Genetic Resources and Biotechnology (Cenargen), Brasília-DF, Brazil (http://alelobag.cenargen.embrapa.br/AleloConsultas, Williams 2022). With the exception of the shy seed producer *A. pflugeae*, transplanted directly from its natural site, all plants were grown from seeds and maintained in greenhouses. A total of 93 accessions belonging to three taxonomic sections were included. These accessions represent the six varieties of *A. hypogaea* (Krapovickas and Gregory 1994), three landraces of *A. hypogaea* from the Xingu Indigenous Park (Freitas et al. 2007) and 30 wild species, being 24 of section *Arachis*, four of section *Procumbentes* and two of section *Erectoides* (Table 1).

Table 1

Species and accessions of *Arachis* analyzed with SSR markers and their genome types, collectors and origins.

Arachis species	Genome	Collectors & numbers[a]	Origin[b]	Lat (S)	Long (W)	Alt (m)
Section Arachis						
A. batizocoi Krapov. & W.C.Greg.	K	K 9484	BOL, SC, Parapetí	20°05'	63°14'	700
	K	K 9484-mut	BOL, SC, Parapetí	20°05'	63°14'	700
A. benensis Krapov., W.C.Greg. & C.E.Simpson	F	KGSPSc 35005	BOL, BE, Trinidad	14°47'	64°55'	156
A. cardenasii Krapov. & W.C.Greg.	A	GKP 10017	BOL, SC, Roboré	18°20'	59°46'	260
A. cruziana Krapov., W.C.Greg. & C.E.Simpson	K	WiSVg 1302-2	BOL, SC, San José Chiquitos	18°50'	60°53'	285
	K	WiSVg 1302-3	BOL, SC, San José Chiquitos	18°50'	60°53'	285
A. decora Krapov., W.C.Greg. & Valls	G	VSPmWiPzSv 13307	BRA, GO, Iaciara	14°03'	46°50'	583
	G	VSPmWiPzSv 13290	BRA, GO, Monte Alegre de Goiás	13°18'	46°42'	548
	G	VSW 9955	BRA, GO, Campos Belos	12°59'	46°36'	536
A. diogoi Hoehne	A	Vp 5000	BRA, MS, Corumbá	17°50'	57°33'	102
A. glandulifera Stalker	D	VSPmSv 13738	BRA, MT, Porto Esperidião	16°13'	59°07'	159
	D	VOfSv 14730	BRA, MT, Vila Bela da Santíssima Trindade	15°24'	60°12'	231
A. gregoryi C.E.Simpson, Krapov. & Valls	B	VSGr 6389	BRA, MT, Vila Bela da Santíssima Trindade	15°22'	60°14'	224
	B	VOfSv 14728	BRA, MT, Vila Bela da Santíssima Trindade	15°24'	60°12'	251

Arachis species	Genome	Collectors & numbers[a]	Origin[b]	Lat (S)	Long (W)	Alt (m)
	B	VOfSv 14735	BRA, MT, Vila Bela da Santíssima Trindade	15°23'	60°11'	243
	B	VOfSv 14739	BRA, MT, Vila Bela da Santíssima Trindade	15°24'	60°13'	225
	B	VOfSv 14740	BRA, MT, Vila Bela da Santíssima Trindade	15°24'	60°13'	226
	B	VOfSv 14743	BRA, MT, Vila Bela da Santíssima Trindade	15°27'	60°10'	219
	B	VOfSv 14753	BRA, MT, Pontes e Lacerda	15°59'	59°33'	275
	B	VOfSv 14760	BRA, MT, Vila Bela da Santíssima Trindade	16°08'	59°47'	262
	B	VOfSv 14765	BRA, MT, Vila Bela da Santíssima Trindade	16°05'	59°57'	241
	B	VOfSv 14767	BRA, MT, Vila Bela da Santíssima Trindade	16°05'	59°58'	247
	B	VS 14957	BRA, MT, Vila Bela da Santíssima Trindade	15°22'	60°14'	224
	B	VS 14960	BRA, MT, Vila Bela da Santíssima Trindade	15°23'	60°13'	219
	B	VS 14962	BRA, MT, Vila Bela da Santíssima Trindade	15°23'	60°13'	222

Arachis species	Genome	Collectors & numbers[a]	Origin[b]	Lat (S)	Long (W)	Alt (m)
A. helodes Mart. ex Krapov. & Rigoni	A	VSGr 6325	BRA, MT, Santo Antonio do Leverger	15°52'	56°04'	150
	A	VSGr 6324	BRA, MT, Cuiabá	15°36'	56°05'	176
	A	VPoJSv 10470	BRA, MT, Nossa Senhora do Livramento	15°46'	56°09'	153
A. hoehnei Krapov. & W.C.Greg.	?	VPoBi 9094	BRA, MS, Corumbá	19°30'	57°25'	86
	?	VPoBi 9140	BRA, MS, Corumbá	19°15'	57°22'	87
	?	VPoBi 9146	BRA, MS, Corumbá	19°15'	57°16'	85
	?	VMPzW 13985	BRA, MS, Corumbá	19°31'	57°25'	84
	?	VRcMmSv 14547	BRA, MS, Corumbá	19°13'	57°27'	85
	?	KG 30006	BRA, MS, Corumbá	18°15'	57°28'	185
A. hypogaea L. (Xingu type)	AB	VGaRoSv 12549	BRA, MT, São José do Xingu	10°49'	52°39'	342
	AB	Of 101	BRA, MT, P.I. Xingu, Guarujá village	11°28'	53°30'	282
	AB	Of 117	BRA, MT, P.I. Xingu, Ilha Grande village	11°30'	53°27'	290
A. hypogaea L. subsp. hypogaea var. hypogaea	AB	VGaRoSv 12548	BRA, MT, São José do Xingu	10°49'	52°39'	342
	AB	VSgSv 13390	BRA, PE, Palmares	08°38'	35°33'	132
A. hypogaea subsp. hypogaea var. hirsuta H.A.Köhler	AB	Mf 1538 [ex ARG]	ECU, PI, Quito	0°02'	78°26'	2560
A. hypogaea subsp. fastigiata Waldron var. fastigiata	AB	cv. Tatu	BRA, SP, Campinas	22°52'	47°04'	689

Arachis species	Genome	Collectors & numbers[a]	Origin[b]	Lat (S)	Long (W)	Alt (m)
A. hypogaea subsp. fastigiata var. vulgarisHarz	AB	cv. Tatuí	BRA, SP, Campinas	22°52'	47°04'	689
A. hypogaea subsp. fastigiata var. aequatoriana Krapov. & W.C.Greg.	AB	Mf 1678 [ex ARG]	ECU, SU, Shushufindi	0°22'	76°39'	390
A. hypogaea subsp. fastigiata var. peruviana Krapov. & W.C.Greg.	AB	Mf 1560 [ex ARG]	ECU, ES, Quinindé	0°07'	79°25'	260
A. ipaënsis Krapov. & W.C.Greg.	B	KGBPScS 30076	BOL, TA, Ipa	21°00'	63°25'	650
A. kempff-mercadoi Krapov., W.C.Greg. & C.E.Simpson	A	V 13250	BOL, SC, Santa Cruz de la Sierra	17°41'	63°08'	410
A. krapovickasii C.E.Simpson, D.E.Williams, Valls & I.G.Vargas	K	WiDc 1291	BOL, SC, San José Chiquitos	18°14'	60°51'	309
A. kuhlmannii Krapov. & W.C.Greg.	A	VSGr 6352	BRA, MT, Cáceres	15°54'	57°46'	130
	A	VSGr 6380	BRA, MT, Vila Bela da Santíssima Trindade	15°01'	59°56'	200
	A	VKSSv 8887	BRA, MT, Porto Esperidião	15°37'	58°48'	220
	A	VPoBi 9235	BRA, MS, Corumbá	18°52'	56°11'	100
	A	VPoBi 9479	BRA, MS, Aquidauana	19°54'	55°30'	150
	A	VSPmSv 13779	BRA, MT, Cáceres	16°13'	57°23'	190
	A	VOfSv 14691	BRA, MT, Cáceres	16°03'	57°41'	126
A. magna Krapov., W.C.Greg. & C.E.Simpson	B	VSGr 6396	BRA, MT, Vila Bela da Santíssima Trindade	15°29'	60°05'	204
	B	VSPmSv 13748	BRA, MT, Porto Esperidião	16°16'	59°24'	240

Arachis species	Genome	Collectors & numbers[a]	Origin[b]	Lat (S)	Long (W)	Alt (m)
	B	VSPmSv 13751	BRA, MT, Vila Bela da Santíssima Trindade	16°16'	59°27'	253
	B	VSPmSv 13761-o	BRA, MT, Vila Bela da Santíssima Trindade	15°21'	60°04'	207
	B	VSPmSv 13761-c	BRA, MT, Vila Bela da Santíssima Trindade	15°21'	60°04'	207
	B	VSPmSv 13761-y	BRA, MT, Vila Bela da Santíssima Trindade	15°21'	60°04'	207
	B	VSPmSv 13765	BRA, MT, Glória d'Oeste	15°48'	58°23'	152
	B	VOfSv 14707	BRA, MT, Glória d'Oeste	15°48'	58°23'	152
	B	VOfSv 14724	BRA, MT, Vila Bela da Santíssima Trindade	15°19'	60°03'	204
	B	VOfSv 14727	BRA, MT, Vila Bela da Santíssima Trindade	15°21'	60°04'	207
	B	VOfSv 14744	BRA, MT, Vila Bela da Santíssima Trindade	15°28'	60°07'	222
	B	VOfSv 14750	BRA, MT, Pontes e Lacerda	15°54'	59°31'	298
	B	KGSSc 30097-o	BOL, SC, San Ignacio de Velasco	16°22'	60°58'	410
A. microsperma Krapov., W.C.Greg. & Valls	A	VRGeSv 7681	BRA, MS, Bela Vista	22°06'	56°31'	182
A. monticola Krapov. & Rigoni	AB	SeSnHoCh 2775	ARG, JU, Lozano	24°04'	65°24'	1546

Arachis species	Genome	Collectors & numbers[a]	Origin[b]	Lat (S)	Long (W)	Alt (m)
A. palustris Krapov., W.C.Greg. & Valls	G	VPmSv 13023	BRA, TO, Filadélfia	07°23'	47°36'	195
	G	VKRSv 6356	BRA, TO, Miracema do Tocantins	09°27'	48°34'	233
A. praecox Krapov., W.C.Greg. & Valls	G	VSGr 6416	BRA, MT, Porto Estrela	15°39'	57°15'	190
A. stenosperma Krapov. & W.C.Greg.	A	WPz 421	BRA, TO, Talismã	12°36'	49°20'	218
	A	SvPzSz 3042	BRA, MT, Guiratinga	16°23'	54°01'	343
	A	VSStGdW 7805-AR	BRA, MT, São Félix do Araguaia	11°37'	50°48'	196
	A	VKSSv 9017	BRA, MT, Santo Antonio do Leverger	15°43'	55°40'	205
	A	VSv 10309	BRA, MT, Rondonópolis	16°28'	54°39'	270
	A	VCrSv 14455	BRA, PR, Paranaguá	25°30'	48°31'	8
A. valida Krapov. & W.C.Greg.	B	VPoBi 9153	BRA, MS, Corumbá	19°11'	57°29'	98
	B	VPoBi 9157	BRA, MS, Corumbá	19°10'	57°26'	93
	B	VPzRcSgSv 13514	BRA, MS, Corumbá	19°07'	57°32'	98
	B	VPzRcSgSv 13516	BRA, MS, Corumbá	19°04'	57°29'	94
	B	VS 15096	BRA, MS, Corumbá	19°18'	57°36'	82
A. vallsii Krapov. & W.C.Greg.	?	VRGeSv 7635	BRA, MS, Miranda	20°07'	56°42'	107
	?	VPzRcSgSv 13515	BRA, MS, Corumbá	19°07'	57°32'	98
A. villosa Benth.	A	VMilrLbGvAn 14309	BRA, RS, Uruguaiana	29°47'	57°13'	45

Arachis species	Genome	Collectors & numbers[a]	Origin[b]	Lat (S)	Long (W)	Alt (m)
A. williamsii Krapov. & W.C.Greg.	B	WiDc 1118	BOL, BE, Trinidad	14°48'	64°53'	150
Section Erectoides Krapov. & W.C.Greg.						
A. paraguariensis Chodat & Hassl. subsp. *paraguariensis*	E	VRGeSv 7677	BRA, MS, Bela Vista	22°05'	56°33'	197
A. stenophylla Krapov. & W.C.Greg.	E	VMPzW 14026	BRA, MS, Caracol	21°44'	56°58'	498
Section Procumbentes Krapov. & W.C.Greg.						
A. kretschmeri Krapov. & W.C.Greg.	P	VRcMmSv 14555	BRA, MS, Anastácio	20°25'	56°02'	182
A. lignosa (Chodat & Hassl.) Krapov. & W.C.Greg.	P	VRcSgSv 13570	BRA, MS, Porto Murtinho	21°32'	57°49'	81
A. matiensis Krapov., W.C.Greg. & C.E.Simpson	P	VSPmSv 13718	BRA, MT, Porto Esperidião	16°07'	58°25'	151
A. pflugeae C.E.Simpson, Krapov. & Valls	P	VRcSgSv 13589	BRA, MS, Porto Murtinho	21°44'	57°25'	202

[a]Collector abbreviations: An = A. Carneiro; B = D.J. Banks; Bi = L.B. Bianchetti; Ch = J. Chalian; Cr = C.M. Castro; Dc = D. Claure; G = W.C. Gregory; Ga = M.L. Galgaro; Gd = I.J. Godoy; Ge = M.A.N. Gerin; Gr = A. Gripp; Gv = F.R. Galvani; Ho = D. Hojsgaard; Ir = B.E. Irgang; J = L. Jank; K = A. Krapovickas; Lb = L.R.M. Baptista; M = J.P. Moss; Mi = S.T.S. Miotto; Mm = M. Moraes; Of = F.O. Freitas; P = J.R. Pietrarelli; Pm = R.N. Pittman; Po = A. Pott; Pz = E.A. Pizarro; R = V.R. Rao; Rc = R.C. Oliveira; Ro = D.M.S. Rocha; S = C.E. Simpson; Sc = A. Schinini; Se = J.G. Seijo; Sg = A.K. Singh; Sn = V.G. Solis Neffa; St = H.T. Salker; Sv = G.P. Silva; Sz = R. Schultze-Kraft; V = J.F.M. Valls; Vg = I.G. Vargas; Vp = V.J. Pott; W = W.L. Werneck; Wi = D.E. Williams.

[b]Origin: Countries: ARG = Argentina; BOL = Bolivia; BRA = Brazil; ECU = Ecuador. Argentinian Province: JU = Jujuy. Bolivian Departments: BE = El Beni; SC = Santa Cruz; TA = = Tarija. Brazilian States: GO = Goiás; MS = Mato Grosso do Sul; MT = Mato Grosso; PE = Pernambuco; PR = Paraná; RS = Rio Grande do Sul; SP = São Paulo; TO = Tocantins. Provinces of Ecuador: ES = Esmeraldas; PI = Pichincha; SU = Sucumbíos. Other abbreviations: cv = cultivar, Instituto Agronômico de Campinas, São Paulo, Brazil; Mf = Estación Experimental de Manfredi, Córdoba, Argentina; P.I. Xingu = Parque Indígena do Xingu (Xingu Indigenous Park), Mato Grosso, Brazil.

Arachis magna accessions include plants representing a single field population but showing three flower colors, V 13761-orange, yellow and cream, as well as the additional sample V 14727, from the same site but collected in the wild six and a half years later. Also from a single location, V 13765 and V 14707 were collected with this same time interval. A similar situation occurs with the accession V 6389 of *A. gregoryi*, collected again in the wild as V 14957, in what was believed by the same original collectors to be the

same geographic location, respectively in 1981 and 2004. This long period of time, associated with obvious changes in the dirt road system and local land use recommended the initial designation of these germplasm samples as distinct accessions.

Arachis batizocoi includes a field accession (K 9484) as well as a mutant of the same observed when cultivated by Walton C. Gregory in Raleigh, at North Carolina State University, showing a thick, ribbed epiphyll, and locally designated "Corduroy". Likewise, the two samples of *A. cruziana* showed small differences in anthocyanin pigmentation and hairiness, observed during the initial seed multiplication.

The geographic coordinates and altitude of the Brazilian accessions were obtained directly on site with GPS equipment, or accurately verified, based on field notes. Geographic information on accessions from Argentina, Bolivia and Ecuador was extracted from the literature (Krapovickas and Gregory 1994) or from herbarium labels, or retrieved from germplasm databases.

Of the 30 wild species, 14 are represented by plants directly multiplied from seeds rescued from nature at the same site of collection of the herbarium vouchers later designated as type specimens. In addition, *A. pflugeae*, a species with very limited seed production, was sampled from plants transplanted directly from the wild population documented by the type specimen in its original collection.

DNA extraction and PCR amplification

Total DNA was extracted from young leaflets as described by Grattapaglia and Sederoff (1994) modified by the inclusion of an additional precipitation step with 1.2 M NaCl. The DNA was diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and the concentration estimated in 1% agarose gels.

PCR products were obtained using microsatellite primer pairs designed for *A. hypogaea* or *A. stenosperma* (Moretzsohn et al. 2005). PCRs were performed in 6 µl volumes, containing 0.6 µl 1X PCR buffer (10 mM Tris−HCl pH 8.4, 50 mM KCl, 2 mM $MgCl_2$), 1.2 µl BSA (2.5 mg/ml), 1.0 µl dNTP (2.5 mM), 0.1 µl primer pair (5 µM each), 0.2 µl *Taq* DNA polymerase (5 units/µl), 0.9 µl ultra-pure water, and 2.0 µl DNA (5 ng/µl). The amplification cycle consisted of heating at 94° C for 5 minutes, followed by 30 cycles of 94° C for 1 minute, annealing temperature specific for each primer pair (48° – 60° C) for 1 min, and 72° C for 2 minutes, and a final extension at 72° C for 7 minutes. The reactions were performed on ABI 9700 thermocyclers (Applied Biosystems, CA, USA). The forward primer was labelled with one of the fluorescent dyes HEX, 6-FAM or NED (Applied Biosystems). The primers were multiplexed according to the fluorescence, annealing temperature and size of the amplified alleles. PCR products were denatured and size fractioned by electrophoresis on an ABI 3730 DNA Analyzer (Applied Biosystems). Allele sizing of the electrophoretic data was performed using GeneMapper 4.1 software (Applied Biosystems).

Data analysis

A total of 26 microsatellite markers were included in this analysis. Pairwise genetic distances were estimated from the allelic data using the modified Rogers' genetic distance (Goodman and Stuber 1983) and the software BOOD (Coelho 2000). The resulting diagonal matrix was then submitted to cluster

analysis using UPGMA (unweighted pair-group method analysis). The reliability of the generated dendrogram was tested by bootstrap analysis also using the BOOD program with 10,000 iterations. The consistency of the resulting dendrogram was also evaluated by the cophenetic correlation coefficient (r), evaluated by Mantel's test at 5% significance level and with 1,000 permutations. All these analyses were performed using NTSYS 2.21 (Rohlf 2009). The total number of amplified alleles (A), observed (Ho) and expected heterozygozities (He) were estimated for each species using GDA (Lewis and Zaykin 2001).

Results

Genetic diversity was analyzed using 26 microsatellite markers and 93 accessions belonging to 31 species of the genus *Arachis*. Polymorphism levels were estimated for each species (Table 2) by the mean number of alleles per locus, and the expected (He) and observed (Ho) heterozygosities. Out of the 29 diploid species analyzed, 16 had only one accession available or included in this study. For the remaining 13 diploid species with more than one accession analyzed, Ho was lower than He, with the single exception of *A. batizocoi*, with two accessions and equal Ho and He values. This low proportion of heterozygotes suggests that most species analyzed are autogamous. Exceptions could be *A. microsperma, A. stenophylla, A. pflugeae*, and *A. kretschmeri*, with Ho values considerably higher than the other species (Ho > 0.35), but with only one analyzed accession each (Table 2). For the two tetraploid species, *A. hypogaea* and *A. monticola*, the values obtained in the present study are overestimated, since most of the markers amplified alleles on duplicated loci. Thus, they cannot be compared to those obtained for diploid species.

Table 2
Number of accessions by species analyzed (n); total number of amplified alleles (A), expected heterozygozity (He) and observed heterozygoziy (Ho) based on the analysis of 93 accessions of 31 *Arachis* species for 26 microsatellite markers.

Species	n	A	He	Ho
A. batizocoi	2	1.120	0.060	0.060
A. benensis	1	1.000	0.000	0.000
A. cardenasii	1	1.174	0.174	0.174
A. cruziana	2	1.160	0.080	0.060
A. decora	3	2.154	0.469	0.090
A. diogoi	1	1.250	0.250	0.250
A. glandulifera	2	1.625	0.347	0.083
A. gregoryi	13	6.269	0.654	0.063
A. helodes	3	1.833	0.328	0.139
A. hoehnei	6	3.200	0.501	0.029
A. ipaënsis	1	1.000	0.000	0.000
A. kempff-mercadoi	1	1.000	0.000	0.000
A. krapovickasii	1	1.083	0.083	0.083
A. kretschmerii	1	1.381	0.381	0.381
A. kuhlmannii	7	5.000	0.771	0.130
A. lignosa	1	1.100	0.100	0.100
A. magna	13	5.960	0.701	0.008
A. matiensis	1	1.045	0.046	0.046
A. microsperma	1	1.417	0.417	0.417
A. palustris	2	1.667	0.361	0.083
A. paraguariensis	1	1.048	0.048	0.048
A. pflugeae	1	1.375	0.375	0.375
A. praecox	1	1.105	0.105	0.105
A. stenophylla	1	1.353	0.353	0.353

Supplementary file 1. Matrix of pairwise genetic distances estimated by the modified Rogers' genetic distance (Goodman and Stuber 1983) and the software BOOD (Coelho 2000). Bootstrap values estimated with 10,000 iterations are also shown.

Species	n	A	He	Ho
A. stenosperma	6	3.680	0.616	0.073
A. valida	5	2.885	0.513	0.019
A. vallsii	2	1.304	0.181	0.109
A. villosa	1	1.087	0.087	0.087
A. williamsii	1	1.000	0.000	0.000
Mean		**1.941**	**0.276**	**0.116**
A. hypogaea	10	5.808	0.677	0.709
A. monticola	1	1.250	0.250	0.250

Supplementary file 1. Matrix of pairwise genetic distances estimated by the modified Rogers' genetic distance (Goodman and Stuber 1983) and the software BOOD (Coelho 2000). Bootstrap values estimated with 10,000 iterations are also shown.

Modified Rogers' genetic distances were estimated in pairwise comparisons of the 93 accessions, with values ranging from 0.15 to 0.71 (Supplementary File 1). Therefore, all the accessions were differentiated using the 26 loci. Based on this data, a dendrogram was constructed using the UPGMA method (Fig. 1). The cophenetic correlation coefficient (r) was 0.84 and significant by the Mantel's test (p < 0.01), indicating that the tree reflected the original genetic distance matrix. Eleven major groups were evident (Fig. 1), although distances between groups were usually very low, with low bootstrap values (Supplementary File 1).

Group 1 consisted of the only know accession of *A. ipaënsis* (B genome) and the tetraploid accessions, including representatives of the six botanical varieties of *A. hypogaea*, the landraces cultivated by Brazilian Indians (Xingu), and *A. monticola*. The accessions of *A. hypogaea* clustered into two subgroups, separating the two subspecies, with the exceptions of the subsp. *fastigiata* var. *peruviana* (accession Mf 1560), which clustered with the subsp. *hypogaea* accessions, and subsp. *hypogaea* var. *hirsuta* (accession Mf 1538), which clustered with the subsp. *fastigiata* accessions. The Xingu accessions grouped together, close to the *hypogaea* var. *hypogaea* and *fastigiata* var. *peruviana* accessions. The other accessions of B genome species formed a large group (Group 2), and a smaller group (Group 4), this one with only three accessions of *A. magna* (V 13765, V 14707, V 13751).

Accessions of the same species tended to group together, with the exception of some few *A. magna* and *A. gregoryi* accessions (Fig. 1). All accessions of the three K genome species (*A. batizocoi*, *A. cruziana*, and *A. krapovickasii*) and the only known D genome species (*A. glandulifera*) formed a clear group (Group 3), closely related to the B genome species group. Group 5 contained the two accessions of *A. vallsii*, which was originally described as a member of the section *Procumbentes* by Krapovickas and Gregory (1994). The six accessions of *A. hoehnei* were joined in Group 6, closely related to a group containing most of the A genome species analyzed, and the only F genome species included in the

present study, *A. benensis* (Group 7). Group 8 contained the six accessions of the three G genome species (2n = 18), while Group 9 had three accessions of *A. kuhlmannii* and the only *A. kempff-mercadoi* accession included in this study. Five of the six accessions of sections *Procumbentes* and *Erectoides* clustered on Group 10, while *A. kretschmeri* (section *Procumbentes*) formed an external group (Group 11).

Discussion

The present analysis of genetic relationships based on microsatellite markers showed, in general, the grouping of accessions according to their assignment to species and genome types, corroborating the taxonomic and cytogenetic classification.

The landraces of *A. hypogaea* cultivated by the Brazilian Indians in the Xingu Indigenous Park have some unique morphological traits, especially in the pods, that exceed the variation described for the six formal botanical varieties. However, the ten accessions of *A. hypogaea* grouped together (Group 1), showing the strong affinity of its six varieties, as well as the three Xingu Indigenous Park types, identified as White Kayabi, Nambikwara and Xingu (Freitas et al. 2007). The *A. hypogaea* accessions were very closely related to *A. monticola*, a species probably involved in the origin and domestication of peanut (Gregory and Gregory 1976; Raina and Mukai 1999; Lavia et al. 2008; Bertioli et al. 2019)d *ipaënsis*, which shows high cytogenetic homology (Kochert et al. 1996; Seijo et al. 2004; Fávero et al. 2006; Seijo et al. 2007; Robledo et al. 2010) and an extremely high DNA similarity (99.96%) with the B genome of *A. hypogaea* (Bertioli et al. 2016). *Arachis ipaënsis* is considered the donor of the B genome to *A. hypogaea* (Kochert et al. 1996; Seijo et al. 2004; Fávero et al. 2006; Bertioli et al. 2016). Our results corroborate this assumption, since *A. ipaënsis* was the only B genome species placed in the group containing the *A. hypogaea* accessions (Group 1).

This group was closely associated to Group 2, which contained most of the B genome accessions. Only a single accession of *A. ipaënsis* is available in germplasm collections worldwide, which was collected in Bolivia, from where it may now have disappeared (Williams 2022). The genetic similarity of *A. ipaënsis* and the species of Group 2, composed by a significant number of accessions of *A. magna, A. gregoryi, A. valida*, and the only known accession of *A. williamsii*, and the knowledge of their genetic relationships, open new possibilities for the incorporation of useful genes into cultivated peanut. Some of the accessions of *A. gregoryi* and *A. magna* included in the present study had never been analyzed by molecular markers.

The five accessions of *A. valida* grouped together. In contrast, accessions of *A. magna* and *A. gregoryi* grouped into different subgroups, and three *A. magna* accessions, including the two representatives of the easternmost natural population, formed a differentiated group (Group 4). The intraspecific variability of these two species has been shown by the number and distribution of rDNA loci (Custodio et al. 2013) and by microsatellite markers and sequences of single-copy genes (Moretzsohn et al. 2013) and their taxonomic status needs further investigation.

Arachis batizocoi, *A. cruziana*, and *A. krapovickasii* grouped together (Group 3), associated to the B genome species. The placement of the three K genome species, formerly classified as B genome species, in a consistent group supports the validity of the genome reassignment made by Robledo and Seijo (2010). *Arachis glandulifera* was also placed in Group 3. This D genome species has a unique karyotype within section *Arachis* (Stalker 1991; Fernández and Krapovickas 1994; Samoluk et al. 2019), but FISH mapping of rRNA loci and DAPI banding have shown homologies between *A. glandulifera* and *A. batizocoi* (Robledo and Seijo 2008). Additionally, *A. glandulifera* tends to group with the K genome species, when analyzed by molecular markers (Moretzsohn et al. 2004; Tallury et al. 2005; Bechara et al. 2010; Moretzsohn et al. 2013). These four species are native to Bolivia, where *A. glandulifera*, the only one also found in Brazil, might have originated from one of the K genome species after fixing chromosomal rearrangements.

The two accessions of *A. vallsii* grouped together, in Group 5, closely related to the B and K genome species. *Arachis vallsii* was originally assigned to section *Procumbentes* (Krapovickas and Gregory 1994). However, its reclassification into section *Arachis* has been proposed, based on morphological and chromosomal features (Lavia et al. 2009), as well as in its annual life cycle, a feature absent in the *Procumbentes*. Analysis of interspecific and intersectional crossability showed that *A. vallsii* produces hybrids with representatives of different species of section *Arachis*, including *A. hypogaea* and *A. monticola* (unpublished data from our research group). These results strongly suggest that *A. vallsii* should be classified in the section *Arachis*, as proposed by Lavia and coworkers (2009) and corroborated by Moretzsohn and coworkers (2013).

The six accessions of *A. hoehnei* were joined in a consistent group (Group 6). Most of the genetic studies based on molecular markers have shown the close genetic relationship and clustering of *A. hoehnei* accessions, corroborating its taxonomic status (Bravo et al. 2006; Cunha et al. 2008; Bechara et al. 2010; Moretzsohn et al. 2013). Despite this, cytogenetic studies of different accessions of *A. hoehnei* found conflicting results. Accession K 30006, representative of the type collection, was described as not having the small 'A' chromosome pair based on classical cytology (Feulgen staining) (Fernández and Krapovickas 1994), chromosome painting of telomeric repeats, and by GISH/FISH (Du et al. 2016; Du et al. 2019). The last study also showed the absence of the 'A' pair in the accession V 9094. In contrast, FISH analysis of the accession V 9146 showed the presence of a pair of small chromosomes similar in size to the "A" chromosomes, but with a different chromatin condensation from the other A genome species (Custodio et al. 2013). The presence of the 'A' pair was also found in the accessions K 30006 and V 9094, in an unpublished study of Germán Robledo, cited by Robledo and Seijo (2010). The great majority of genetic studies also showed that *A. hoehnei* is more closely associated to the A genome species (Bravo et al. 2006; Gimenes et al. 2007; Cunha et al. 2008; Koppolu et al. 2010; Bechara et al. 2010; Moretzsohn et al. 2013; Vishwakarma et al. 2017), in accordance with our data. Crossings between *A. gregoryi*, which has a B genome, and the accession K 30006 of *A. hoehnei* were unsuccessful (Custodio, unpublished data). These results reinforce that *A. hoehnei* is distant from the B genome species. However, more cytogenetic studies of a diverse panel of accessions are still necessary to determine the genome constitution of *A. hoehnei*.

Most accessions of A genome species grouped together (Group 7). The A genome species have been previously arranged into three subgroups (La Plata River Basin, Chiquitano, and Pantanal) based on the variability of DAPI heterochromatic bands and 18S–26S rRNA loci (Robledo et al. 2009). A total of 14 out of the 17 accessions of the Pantanal subgroup included in the present study were accordingly located in this subgroup. The three accessions of *A. helodes*, which occurs naturally in a very restricted area in the surroundings of Cuiabá (Mato Grosso, Brazil) grouped together, closely related to *A. diogoi* Vp 5000 and one of the two Chiquitano species included (*A. cardenasii* GKP 10017). *Arachis stenosperma*, probably cultivated by indigenous peoples in ancient times, and quite possibly subject to human migration (Custodio et al. 2005), has a wide area of occurrence and a long disjunction between Central Brazil and the Atlantic coast. Despite this, all accessions of this species grouped together, within Group 7. *Arachis kuhlmannii* is found in a more restricted area, the West Central region of Brazil, but four of its accessions were located in Group 7, while three accessions formed a differentiated group with the unique accession of *A. kempff-mercadoi* (Chiquitano group) included in this study (Group 9). Krapovickas and Gregory (1994) mentioned some morphological differences between accessions of *A. kuhlmannii*. In addition, studies using different molecular markers have shown the high genetic variability of *A. kuhlmannii* (Milla et al. 2005; Koppolu et al. 2010; Moretzsohn et al. 2013; Fávero et al. 2017).

Using RAPD markers and several of the same accessions tested here, Fávero et al. (2017) assigned the accessions of *A. kuhlmannii*, with minor exceptions, to three geographic groups: group 1 - from the state of Mato Grosso, west of the Paraguay river, including V 6352, V 6380, and V 8887, these three accessions gathered here with the Bolivian *A. kempff-mercadoi* in group 9; group 2 - also from the state of Mato Grosso, but east of the Paraguay river, including V 13779, here included in group 7; and group 3 - from Mato Grosso do Sul, again east of the river, which included V 9235 and V 9479, also gathered here in group 7. Thus, the *A. kuhlmannii* accessions included in group 9 come from natural sites west of the Paraguay River, while those in group 7, together with the easternmost *A. stenosperma*, occur east of the river. Accession V 14691, not available for Fávero et al. (2017) survey, fits the pattern, as it was collected in the urban area of Cáceres, very close to the east bank of the Paraguay River. In any case, additional taxonomic studies seem to be necessary for the material currently classified as *A. kuhlmannii*.

Group 7 also contained the only F genome species included in this study, *A. benensis*. This species was formerly considered to belong to the B genome group, but it was reassigned to the F genome, due to differences in amount and distribution of heterochromatin and the presence of centromeric bands (Robledo and Seijo 2010). *Arachis benensis* is a peculiar species, since it has the type 9 satellited chromosome that is typical of section *Procumbentes* (Fernández and Krapovickas 1994). The genetic relationships of the F genome species (*A. benensis* and *A. trinitensis*) with species from the other genome types of section *Arachis* are not clear, since different studies have found different results (Moretzsohn et al. 2004; Milla et al. 2005; Tallury et al. 2005; Friend et al. 2010; Tallury et al. 2005; Moretzsohn et al. 2013). However, in each of these studies, they were separated from the B genome species. Additionally, crossings between *A. gregoryi* (B genome) and *A. benensis* (F genome) resulted in hybrids with only 1% pollen viability (Custodio, unpublished data). These results show that *A. benensis* is genetically distant

from the B genome species and also support the validity of the genome reassignment made by Robledo and Seijo (2010).

The six accessions of the three species with 2n = 2x = 18 chromosomes (*A. decora*, *A. palustris*, and *A. praecox*) grouped together (Group 8). These species usually form a well-differentiated group in most genetic relationship studies using molecular markers, what shows they are very closely related. *Arachis decora*, from Goiás and Tocantins States, and *A. palustris*, from Tocantins and Maranhão, were more closely related, while *A. praecox*, from Mato Grosso, was genetically differentiated from the other two species. The origin of these species is controversial, and sometimes they are associated with the A genome group (Tallury et al. 2005; Bravo et al. 2006; Koppolu et al. 2010) or, more often, with the B, D, F and K genome species (Moretzsohn et al. 2004; Gimenes et al. 2007; Bechara et al. 2010; Friend et al. 2010; Moretzsohn et al. 2013). Studies based on rDNA loci and position of heterochromatin suggested these G genome species were derived from an A genome species (Silvestri et al. 2015). In our study, they grouped outside a major group containing the A, B, D, F, and K genome species and thus the result was not informative about the origin of the G genome species.

Group 10 contained three species of section *Procumbentes* and two of *Erectoides.* Therefore, the microsatellite markers used here could not separate these species according to their sections, but did show a clear differentiation of *A. kretschmeri*, of section *Procumbentes* (Group 11). Genetic studies, based on ITS and the plastid *trn*T– *trn*F sequences (Bechara et al. 2010; Friend et al. 2010), and on microsatellite (Hoshino et al. 2006) and InDel (Vishwakarma et al. 2017) markers have shown that species of these two taxonomic sections (and also from section *Trierectoides*) tend to be placed in a same clade or similarity group. Additionally, species that currently belong to these three sections were formerly classified in the section *Erectoides* (Krapovickas 1969; Gregory et al. 1973). Their separation into three sections was based on cytogenetic (Fernández and Krapovickas 1994) and hybridization studies (Gregory and Gregory 1979; Krapovickas and Gregory 1994), which included only one (*A. lignosa*) of the four species of section *Procumbentes* included in this study. These results evidenced that more studies are needed to understanding the circumscriptions of sections *Erectoides*, *Procumbentes*, and *Trierectoides*.

Conclusions

In summary, the results presented here, based on 26 microsatellite loci, shed more light on the genetic relationships of *Arachis* species. In general, results were consistent with the current classification, but suggest the need of further taxonomic revision of a few taxa, especially some accessions of *A. gregoryi*, *A. magna* and *A. kuhlmannii* and the circumscriptions of sections *Erectoides* and *Procumbentes*.

Knowledge of the genetic relationships between accessions of *A. ipaënsis*, *A. gregoryi, A. magna, A. valida* and *A. williamsii*, some of which had never been analyzed by molecular markers, expands the number of available accessions for incorporation of useful genes from species associated with the peanut B genome.

It is also relevant to note that the SSR profiles were consistent with the proximity of accessions of *A. batizocoi, A. cruziana*, and some of *A. gregoryi and A. magna*, currently kept as distinct in gene banks, due to small morphological peculiarities perceived during their initial increase under management, or obtained through recurrent collections in the wild, basically from the same original populations, but after long intervals of time.

Declarations

Competing Interests

The authors declare no conflict of interest.

Author Contributions

A.R.C., M.C.M., and J.F.M.V. conceived and planned the study and wrote the manuscript; J.F.M.V. provided the germplasm accessions, which were multiplied by A.R.C. for the experiment; A.R.C. and A.B.S. carried out the genotyping; M.C.M. and A.R.C. analyzed the data. All authors read and approved the final manuscript.

Data Availability

An additional dataset is available as a Supplementary Material. Any other data generated during the current study is available from the corresponding author on reasonable request.

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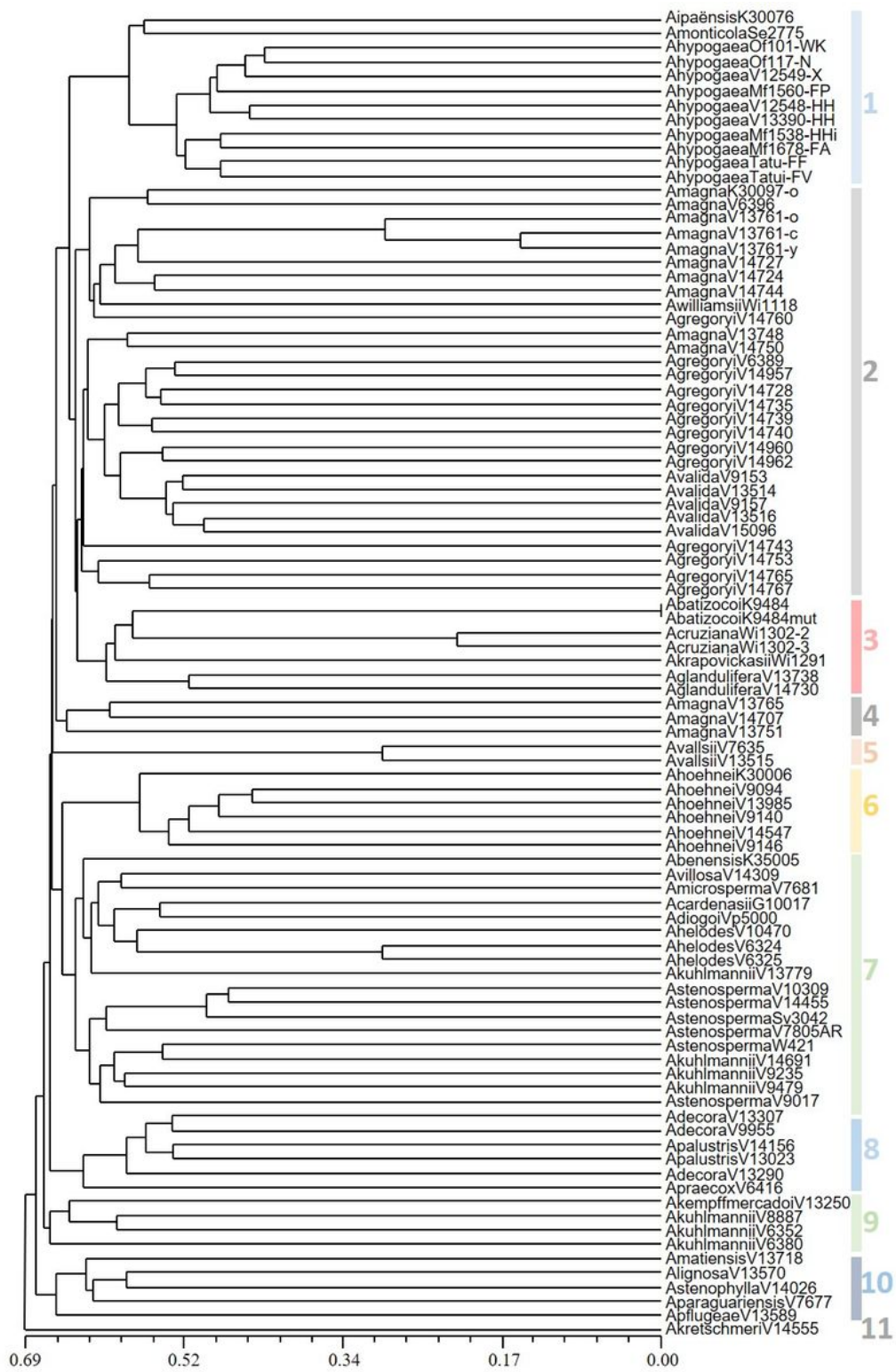
Figures

Figure 1

Dendrogram based on genetic distances estimated by the modified Rogers' coefficient of 93 accessions belonging to 31 species of the genus *Arachis* generated by UPGMA.

Supplementary Files

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- CustodioetalSSRArachisSuppl.File1.xlsx