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RESEARCH ARTICLE

Phylogeny of *Arachis* based on internal transcribed spacer sequences

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Abstract Phylogenetic analysis, based on nuclear rDNA internal transcribed spacers (ITS) of *Arachis* species, corroborated a broad sub-classification of the genus. Three clustering algorithms were used to generate dendrograms which showed that *Arachis* Sections *Extranervosae, Heteranthae* and *Triseminata* were most primitive, and Section *Arachis* was most advanced, with Sections *Caulorrhizae*, *Erectoides*, *Procumbentes*, *Rhizomatosae*, and *Trierectoides* intermediate in evolutionary terms, in relation to the genus *Stylosanthes*, when it was used as the outgroup.

Keywords *Arachis* · Dendrogram · Groundnut · ITS · Nuclear rDNA · Peanut

Introduction

Native to South America, the cultivated groundnut (peanut), *Arachis hypogaea* L., is an important cash crop worldwide, ranking fifth in vegetable oil production among nine major oilseed crops in the world (Tillman and Stalker 2009). In the developing countries like China and India, 50% or more groundnut is crushed for oil, whereas in the western world including USA, Japan and European Union, most groundnut goes to food (Yu et al. 2008).

Characterized by its narrow gene base, the cultivated groundnut is susceptible to many biotic and abiotic stresses, while resistance can be found in some of its wild relatives (Mallikarjuna 2003). Wild *Arachis* species harbor PCV (Peanut Clump Virus) and PeMoV (Peanut Mottle Virus) resistance not yet identified in the cultigen. Other desirable traits in wild groundnut include high yield factors (Nigam et al. 1991), high oil and high protein (Yu et al. 2008). In addition to their potential use in the genetic improvement of the cultivated groundnut, several wild groundnut species (*A. glabrata*, *A. pintoi*, *A. repens*, *A. stenosperma* and *A. kretschmeri*) are planted as forage/ground cover in USA, Columbia, Brazil, Australia and China (Miavitiz 2002; Yu et al. 2008).

The genus *Arachis* is placed in the tribe Aechynomeneae and subtribe Stylosanthinae together with *Stylosanthes, Chapmannia, Arthrocarpum* and *Pachecoa* (Taubert 1884; Smartt 1990). It has a number of characters in common with *Stylosanthes*, more so than any other related taxa. It was supposed that *Arachis* evolved from *Stylosanthes* (Singh and Simpson 1994). Understanding of genetic relationships among *Arachis* species is helpful for germplasm conservation and utilization of genetic diversity present in wild groundnut relatives (Koppolu et al. 2010).

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Lying between the 18S and 26S nuclear ribosomal DNA (rDNA), the internal transcribed spacer (ITS) has a high copy number and can be easily amplified with universal PCR primers designed from highly conserved flanking sequences. The length and sequences of ITS regions of rDNA repeats are believed to be fast evolving. ITS sequence data is therefore considered most valuable for reconstructing phylogenetic relationships both at a lower level (e.g., between closely related species) and at a higher level, and has been widely used in phylogenetic studies of angiosperms (Wang et al. 1999; Simpson 2006). Information on the systematics of *Arachis*, however, has been largely inferred from research results based on comparative morphology, crossability (Gregory and Gregory 1979), and molecular markers, including isozymes (Lu and Pickersgill 1993), seed-storage proteins (Javaid et al. 2004; Singh et al. 1994), Restriction Fragment Length Polymorphisms (RFLPs) (Kochert et al. 1991), Randomly Amplified Polymorphic DNAs (RAPDs) (Hilu and Stalker 1995; Santos et al. 2003; Creste et al. 2005; Cunha et al. 2008), Simple Sequence Repeats (SSRs) (Yu et al. 2008), and Amplified Fragment Length Polymorphisms (AFLPs) (He and Prakash 2001; Tallury et al. 2005). The proposed relationships between Sections, especially those involving Section *Triseminatae*, are controversial (Gregory and Gregory 1979; Krapovickas and Gregory 1994; Pattee and Stalker 1995). In cultivated groundnut and its wild relatives, rDNA repeat unit polymorphisms have been reported (Singh et al. 2002), as has the cloning and sequencing of an rDNA gene repeat unit (Bhagwat et al. 2001), but to the best of our knowledge, no phylogenetic tree of the *Arachis* genus based on rDNA sequences is currently available.

The objectives of the present study were to sequence the nuclear rDNA ITS from selected *Arachis* species, analyze these sequences along with related ITS sequences already deposited in GenBank, and provide insight into groundnut taxonomy and evolution.

Materials and methods

Groundnut materials

We obtained 4 groundnut landraces and 4 accessions of wild *Arachis* species (Table 1) from Professor Yong Shui Chen (Dapigu, Shitouqi), Quanzhou Agricultural Research Institute, Fujian, China, Professor Zu Ming Zhang, Xuzhou Agricultural Research Institute, Jiangsu, China (Suiningerwo, Wulianchengpodun), Professor Rong Hua Tang, China National Wild *Arachis* Germplasm Nursery at Naning, Guangxi, China (wild species). The taxonomic identity of the groundnut materials were verified by the providers. In addition, the identity of the 4 wild *Arachis* species was further confirmed by their PI (Plant Introduction) numbers. Seeds were germinated to produce 6 seedlings of each entry, which were then grown in field nurseries. A minimal distance of 5 m separated adjacent plots (wild species) in all directions to restrict outcrossing and pegging into other plots. For each groundnut genotype, two leaflet samples per plant were collected from 6 different plants in 10 June, 2009.

Preparation of PCR templates

PCR template was prepared from leaflets by using a simple protocol for groundnut developed recently in our laboratory with minor modification (Wang et al. 2009). Briefly, small leaflet discs (6.5 mm^2 diam.) were smashed with a thin-walled PCR tube mounted with a 1 ml pipette tip as a pestle, in 40 µl of 0.25 M sodium hydroxide (NaOH) in an Eppendorf microcentrifuge tube. The mixture was boiled for 30 s. Then 160 µl of 100 mM Tris–HCl (pH 7.6) with 5 mg/ml of polyvinylpyrrolidone (PVP) was added, followed by boiling for 2 min. After centrifugation at 10,000 RPM for 5 min, 90 µl of the supernatant was collected and placed into an Eppendorf tube with 450 µl of TE buffer, and stored at 4°C for use within a week, or stored at −20°C for several months.

PCR

To amplify nuclear rDNA ITS of groundnut, 2 µl DNA template was included in a 25 µl reaction mixture by using Tiangen 2 × *Taq* PCR Master Mix (Tiangen Biotech, Bejing, China) and primers a and b, as recommended by Wang et al. (1999). The amplification was performed on a Biometra™ thermocycler (Biometra, Göttingen, Germany) under the following conditions: 94°C for 3 min, 35 cycles of 50 s at 94°C, 1 min at 55°C, and 1.5 min at 72°C, followed by a final extension at 72°C for 7 min.

Table 1 Sections and species of *Arachis* studied, with the respective origin of ITS sequence information (field nursery or GenBank acc no)

Section	Taxon	Cultivar/plant introduction no.	GenBank accession no.	Note
Arachis				
	A. hypogaea L.		AF156675.2	
		Dapigu	[a]	Spanish type
		Shitouqi	[a]	Spanish type
		Wulianchengpodun	[a]	Virginia type
		Suiningerwo	[a]	Virginia type
	A. monticola Krapov. et Rigoni	PI 219824	[a]	GXAAS A7
	A. batizocoi Krapov. et W. C. Greg.		AF203553.1	
			AY615256.1	
	A. cardenasii Krapov. et W. C. Greg.		AY615236.1	
	A. correntina (Burkart) Krapov. et W. C. Greg.		AF203554	
			AJ320394.1	
			AY862309.1	
	A. cruziana Krapov., W. C. Greg. et C. E. Simpson		AY615259.1	
	A. decora Krapov., W. C. Greg. et Valls		AY615237.1	
	A. diogoi Hoehne	PI 276235	[a]	GXAAS A26
	A. duranensis Krapov. et W. C. Greg.		AY615240.1	
		PI 263133	[a]	GXAAS A19
	A. glandulifera Stalker		AY615258.1	
	A. helodes Mart. ex Krapov. et Rigoni		AY615241.1	
	A. hoehnei Krapov. et W. C. Greg.		AY615224.1	
			AJ320395.1	
			AY615223.1	
			AY615222.1	
	A. ipaensis Krapov. et W. C. Greg.		AY615257.1	
	A. kempff-mercadoi Krapov., W. C. Greg. et C. E. Simpson		AY615266.1	
	A. kuhlmannii Krapov. et W. C. Greg.		AY615232.1	
			AY615219.1	
	A. linearifolia Valls, Krapov. et C. E. Simpson		AY615242.1	
	A. magna Krapov., W. C. Greg. et C. E. Simpson		AY615231.1	
			AF203555.1	
			AY615230.1	
	A. microsperma Krapov., W. C. Greg. et C. E. Simpson		AY615221.1	
	A. palustris Krapov., W. C. Greg. et Valls		AY615238.1	
			AJ320396.1	
			AF203557.1	
	A. praecox Krapov., W. C. Greg. et Valls		AY615234.1	
	A. schininii Krapov., Valls et C. E. Simpson		AY615248.1	
	A. simpsonii Krapov. et W. C. Greg.		AY615247.1	
	A. stenosperma Krapov. et W. C. Greg.		AY615252.1	
	A. valida Krapov. et W. C. Greg.		AY615244.1	
	A. villosa Benth.		AF203558	
			AY615215.1	

Table 1 continued

Section	Taxon	Cultivar/plant introduction no.	GenBank accession no.	Note
			AJ320399.1	
	A. villosa Benth.	PI 210555	[a]	GXAAS A8
	A. williamsii Krapov. et W. C. Greg.		AY615255.1	
Caulorrhizae				
	A. pintoi Krapov. et W. C. Greg.		AY615263.1	
			AJ320397.1	
			AF203551.1	
	A. repens Handro		AY615264.1	
Erectoides				
	A. brevipetiolata Krapov. et W. C. Greg.		AY615251.1	
	A. hermannii Krapov. et W. C. Greg.		AF203556.1	
			AY615260.1	
	A. major Krapov. et W. C. Greg.		AY615228.1	
			AF203552.1	
			AY615229.1	
	A. paraguariensis subsp. *capibarensis* Krapov. et W. C. Greg.		AY615217.1	
	A. paraguariensis subsp. *paraguariensis* Chodat et Hassl.		AY615218.1	
Extranervosae				
	A. burchellii Krapov. et W. C. Greg.		AY615262.1	
	A. lutescens Krapov. et Rigoni		AY615246.1	
	A. villosulicarpa Hoehne		AY615265.1	
Heteranthae				
	A. pusilla Benth.		AY615216.1	
Procumbentes				
	A. appressipila Krapov. et W. C. Greg.		AY615254.1	
	A. kretschmeri Krapov. et W. C. Greg.		AY615220.1	
	A. matiensis Krapov., W. C. Greg. et C. E. Simpson		AY615249.1	
	A. pflugeae C. E. Simpson, Krapov. et Valls		AY615233.1	
Rhizomatosae				
	A. burkartii Handro		AY615245.1	Series Prorhizomatosae
	A. glabrata Benth.	PI 262801	[a]	Series Rhizomatosae
	A. glabrata Benth.		AY615250.1	
Trierectoides				
	A. guaranitica Chodat et Hassl.		AY615261.1	
	A. tuberosa Benth.		AY615235.1	
Triseminatae				
	A. triseminata Krapov. et W. C. Greg.		AY615253.1	
			AJ320398.1	
			AF204233.1	

[a] ITS sequences generated by the authors

DNA analysis

PCR products were recovered by using the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, Norcross, USA), and sequenced on an ABI 3730XL sequencer (Foster City, USA). Additional ITS sequences for diverse *Arachis* taxa representing all nine sections (a total of 43 species, including 24,2,4,3,1,4,2,2 and 1 from Sections *Arachis*, *Caulorrhizae*, *Erectoides, Extranervosae, Heteranthae, Procumbentes, Rhizomatosae, Trierectoides* and *Triseminatae*, respectively) (Table 1) were downloaded from GenBank.

Phylogenetic trees were constructed with MEGA version 4 (Tamura et al. 2007) and ITS sequences of *Stylosanthes leiocarpa* and *S. montevidensis* serving as the outgroup. The robustness of the phylogenetic trees was evaluated by comparing dendrograms obtained from three clustering algorithms, neighbor-joining (NJ), minimal evolution (ME), and maximum parsimony (MP), and bootstrap analysis with 1,000 replicates.

Results and discussion

Bootstrap consensus rooted phylogenic trees were constructed based on neighbor-joining (NJ), minimal evolution (ME), and maximum parsimony (MP) clustering algorithms. These trees displayed no significant differences in the arrangement of groups or major subgroups among the 3 methods; thus, only the NJ tree is illustrated (Figs. 1 and 2).

Three species of Section *Extranervosae, A. burchellii, A. villosulicarpa* and *A. lutescens*, grouped together ($P = 99\%$). These three species, along with *A. pusilla* of Section *Heteranthae* and *A. triseminata* of Section *Triseminatae,* were distantly related to other *Arachis* taxa, and formed the most primitive

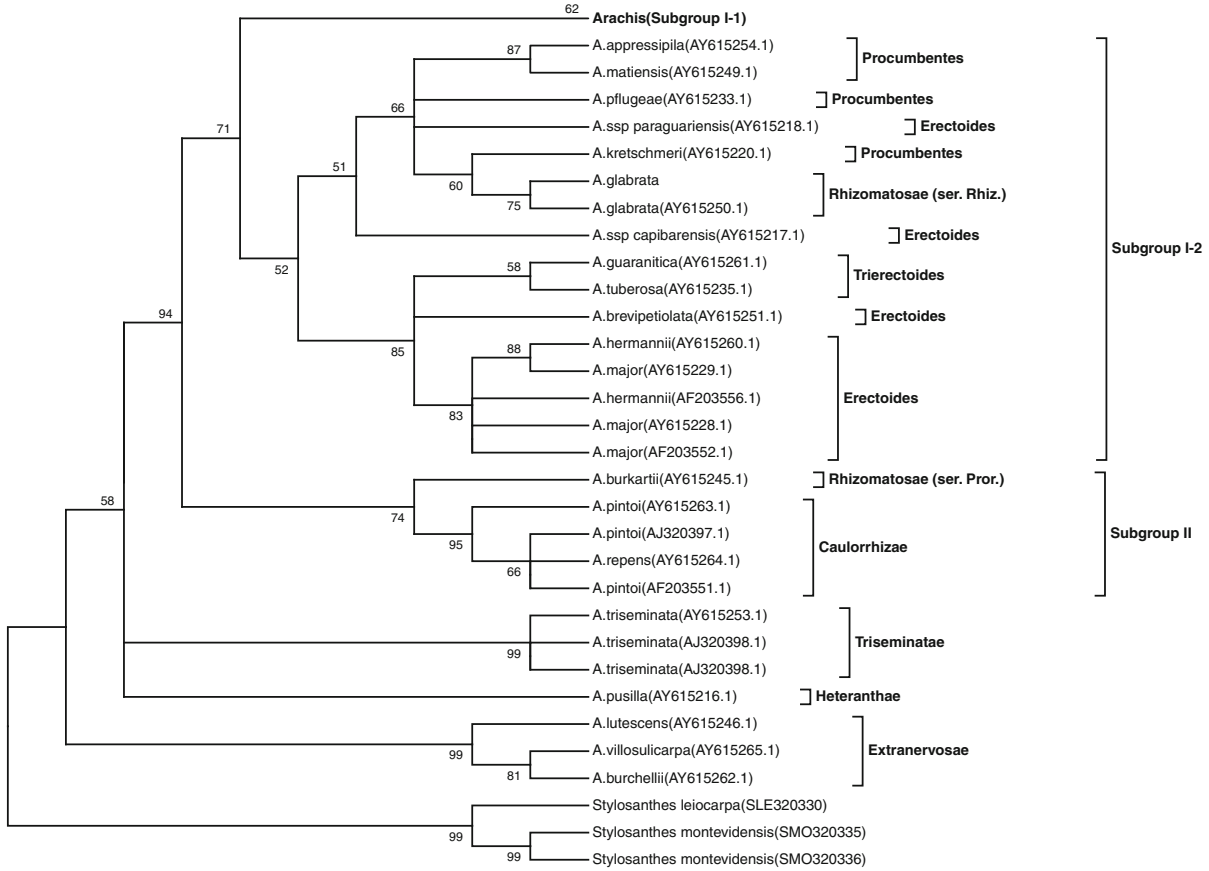

Fig. 1 A bootstrap neighbor-joining tree based on nuclear rDNA ITS sequences of *Arachis* species. Bootstrap *P* values are given at the corresponding node for each cluster

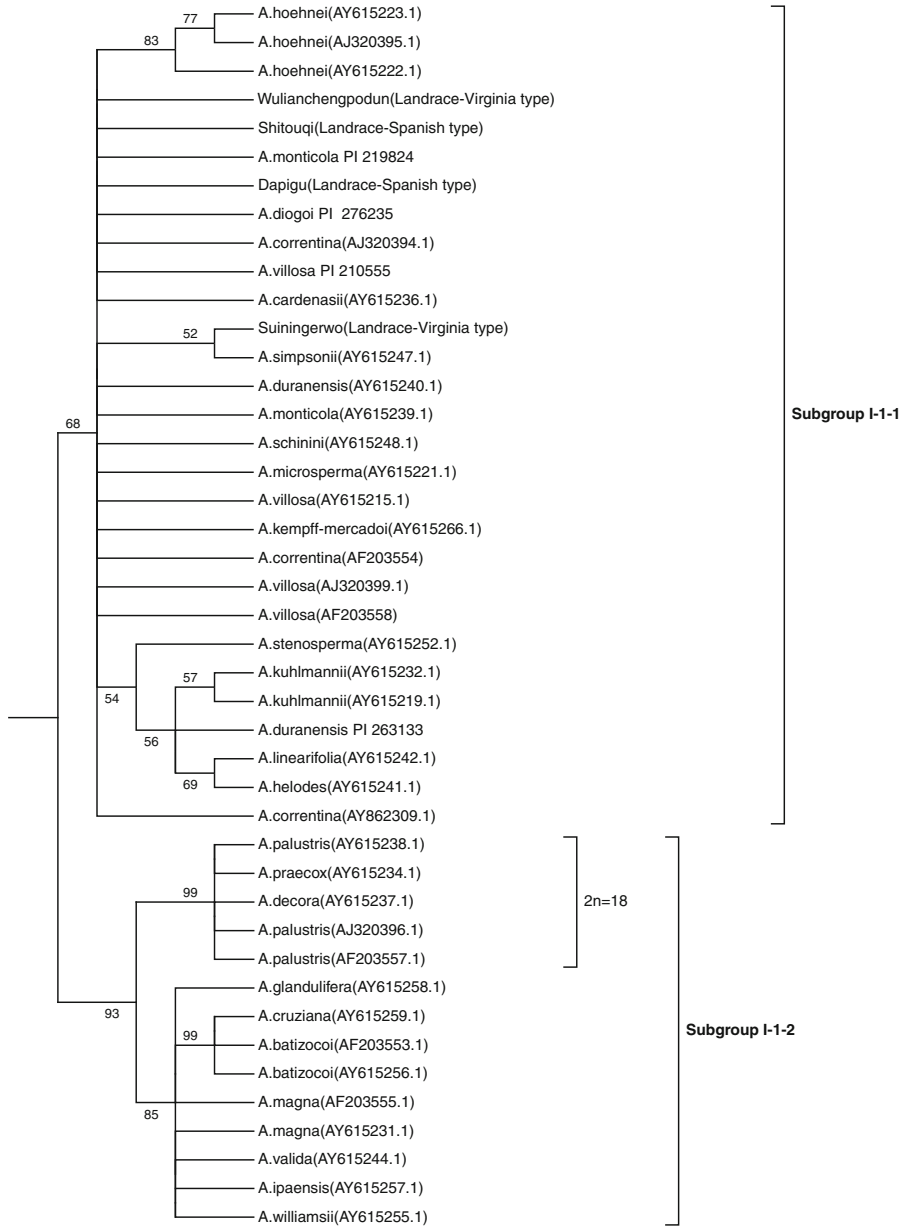

Fig. 2 Partial bootstrap NJ tree based on nuclear rDNA ITS sequences of section *Arachis* species, corresponding to *Arachis* Subgroup I-1 in Fig. 1

groups (Fig. 1), most closely related to *Stylosanthes*. Other accessions were clustered into 2 major subgroups, with Section *Caulorrhizae* and *A. burkartii* of Series *Prorhizomatosae* of Section *Rhizomatosae* basal to all other species (Subgroup II of Fig. 1). The species of Section *Arachis*, including the tetraploid cultivated groundnut, *A. hypogaea* L., and the tetraploid species, *A. monticola*, formed the most phylogenetically advanced group (Subgroup I-1 of Figs. 1 and 2), with species representing Sections *Erectoides, Trierectoides, Eurhizomatosae* and *Procumbentes* and Series *Rhizomatosae* of Section *Rhizomatosae* located in an intermediate position in evolutionary terms (Subgroup I-2 of Fig. 1).

Section *Arachis* can be further divided into 2 subgroups (Subgroup I-1-1 and Subgroup I-1-2)

($P = 62\%$) (Fig. 2). In this study, the 3 *Arachis* species with $2n = 18$ (*A. palustris, A. praecox* and *A. decora*) formed a robust subgroup ($P = 99\%$) (Fig. 2). Previous cytogenetic studies confirmed that they lack the pair "A" Chromosome (Creste et al. 2005). These 3 species, along with 1 D-genome species (*A. glandulifera*) (Stalker 1991) and 6 B-genome species (Cunha et al. 2008) together constituted a less advanced subgroup (Subgroup I-1-2) directly under Subgroup I-1 (Fig. 2). Earlier workers have noted the B- and D- genomes were more closely related to each other than to A-genome (Tallury et al. 2005). In contrast to an earlier report by Tallury et al. (2005), where all species of Section *Arachis* tested generally grouped according to their genomes, in the present study, here subgroup I-1-1 was composed of a combination of A-, B- and AABB-genome species, which represented a recently evolved group. Cunha et al. (2008) also found the B-genome species, *A. hoehnei* (Fernandez and Krapovickas 1994; Holbrook and Stalker 2003) grouped to A-genome species and shared the smallest number of bands with *A. batizocoi* (Also a B-genome species). Sequence data of the *trn*T-F region (Tallury et al. 2005) also placed *A. hoehnei* in the A-genome clade, so did SRAP (Sequence-related amplification polymorphism) analysis by Ren et al. (2010). Further cytogenetic studies may reveal the genomic classification of *A. hoehnei* (Cunha et al. 2008).

Two *Trierectoides* species (*A. guarantica* and *A. tuberosa*) and 3 *Erectoides* species (*A. brevipetiolata, A. hermannii* and *A. major*) were placed together in a well-defined cluster ($P = 85\%$), suggesting that they are closely related (Fig. 1). *Arachis paraguariensis* subsp. *capibarensis* and *A. paraguariensis* subsp. *paraguariensis*, both of section *Erectoides,* however, could be found in close relationship with rhizomatous groundnut *A. glabrata* (series *Rhizomatosae*) and members of Section *Procumbentes* (Fig. 1), raising questions about the taxonomic placement of *A. paraguariensis*, and even about the two subspecies themselves, necessitating check of the real identification of the materials used to generate the GenBank sequences, and where applicable, nomination of the species and subspecies may be reconsidered. Our study supported the primitive status of Series *Prorhizomatosae* relative to Series *Rhizomatosae* of Section *Rhizomatosae* as proposed by Gregory and Gregory (1979).

In conclusion, we constructed a phylogenetic tree of the *Arachis* genus for the first time by using *Stylosanthes* as the outgroup, which provided information essentially supporting the current sub-generic classification of the *Arachis* genus (Krapovickas and Gregory 1994) with some exceptions. Within subgroup I-2, species from Section *Erectoides* scattered in more than one subgroup, so did the 2 series of Section *Rhizomatosae*. Taken from a practical standpoint, for the genetic improvement of the cultivated groundnut, wild relatives from Section *Arachis*, especially the A-genome species, are readily accessible; Other species are from the tertiary gene pool (Smartt 1990), among which the species within Subgroup I-2, i.e., those from Sections *Procumbentes*, *Erectoides* and *Trierectoides* and Series *Rhizomatosae* of Section *Rhizomatosae* may be more easily accessible than species from Subgroup II and the most primitive Sections, *Triseminatae, Heteranthae* and *Extranervosae* (Fig. 1).

The evolutionary relationships suggested by our study partially differed from previous reports. Gregory and Gregory (1979) and Krapovickas and Gregory (1994) proposed that Sections *Extranervosae* and *Erectoides* and Series *Prorhizomatosae* were primitive, and Sections *Arachis, Triseminatae, Caulorrhizae, Heteranthae* and *Procumbentes* and Series *Rhizomatosae* were advanced. Studies of pachytene chromosomes indicated that species in Sections *Erectoides*, *Extranervosae* and *Triseminatae* were more ancient than were species in Sections *Arachis* or *Rhizomatosae* (Pattee and Stalker 1995). Creste et al. (2005) postulated that the 3 species with $2n = 18$ might suggest they represented a new branch with a recent origin while using RAPD (random amplified ploymorphism) to study the relationship between annual species from Sections *Arachis* and *Heteranthae*. In our study, Sections *Extranervosae, Heteranthae* and *Triseminatae* were basal, and Section *Arachis* was the most advanced, whereas the other Sections can be viewed as intermediate in evolutionary terms. The 3 species with $2n = 18$ were advanced relative to D-genome and most B-genome species, and basal to all A-genome and AABB genome species. There were still problems with resolution within Section *Arachis* and confusion in Subgroup I-2, suggesting that at least a few other conservative genes or gene regions should be analyzed. Although several chloroplast DNA genes, *atpB, rbcL, matK* and *ndhF*,

along with some nuclear genes such as *Adh*, have proven to be of high utility in plant systematic (Simpson 2006), they have not yet been used in *Arachis*. Analysis with these genes is absolutely necessary, since it may provide us with additional information on phylogeny of the genus.

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