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Genetic diversity as revealed by AFLP finger-printing and systematic relationships of species in *Sesbania* (Fabaceae)

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Genetic diversity has been revealed, using AFLP finger printing, in 37 accessions representing 21 species of *Sesbania*. The AFLP data has been analyzed and distance trees, illustrating the relationships within and among species, have been constructed using UPGMA and neighbor joining (NJ) methods as implemented in the NTSYS-pc software. Both trees illustrated polymorphism between accessions of the same species, from different origins, but illustrated that intraspecific accessions have closer genetic affinity to each other than to other species. The NJ and UPGMA distance trees clearly delimited the species of the two subgenera Agati and Daubentonia as two separate groups from those in subgenus Sesbania. The AFLP data revealed considerable polymorphism among species of subgenus Sesbania that is not correlated with karyotype resemblances of species in this subgenus and their ability to cross. Based on AFLP data this subgenus may be regarded the center from which species in the other two subgenera have been derived. In the NJ tree *S. quadrata* and *S. rostrata* are distinguished from two large groups, but in the UPGMA tree *S. quadrata* is grouped with *S. bispinosa* and *S. cannabina*, whereas *S. rostrata* is grouped with *S. exaltata* in one of the NJ groups. The delimitation of species based on the analyses of AFLP data is discussed in the light of their systematic delimitation.

***Key words*:** Genetic diversity, AFLP finger-printing, *Sesbania*, Systematics

Introduction

The genus *Sesbania* Scopali (Fabaceae) comprises over 60 species distributed in the tropical and subtropical regions of the World, 32 in Africa,

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10 in Asia, 10 in Australia and eight in the Americas. Few species, e.g. *S. sesban* and *S. sericea* have been naturalized in different parts of the World and had become of a cosmopolitan distribution (Forni-Martins *et al*., 1994). The altitudinal range of the genus varies from sea level to 2000 m, in regions with rainfall distribution between 500 and 2000 mm per year. *Sesbania* species are more commonly found in regions with alternating wet and dry periods than in those with evenly distributed rainfall (Evans, 1990). Several species of this genus are used as animal fodder (Monteiro, 1994) and to maintain soil productivity and improve soil structure for non legume crops (Evans & Rotor, 1987). The species of *Sesbania* were delimited by Bentham (1859) in four sections i.e. *Agati, Doubentonia, Sesbania* and *Glottidium*. More recently however, section *Glottidium* was considered as a distinct monotypic genus and was separated from *Sesbania* and the other three sections have been considered as subgenera of *Sesbania* (Sachet, 1987; Monteiro, 1994).

In addition to the use of morphological traits for the classification of species in *Sesbania*, evidences derived from cytological information have been applied to address systematic and evolutionary relationships of species in the genus. Federov (1974), Goldblatt (1985; 1988) and Goldblatt and Johnson (1991) compiled chromosome counts for about half of the species in *Sesbania*. More recently, Abou-El-Enain *et al*. (1998) and El Shazly & Abou-El-Enain (1999) reported counts and described the karyotype for 39 taxa representing 24 species. The vast majority of the examined species have a basic chromosome number of x=6, but species with numbers based on x=7 and x=8 has been found. Polyploidy was encountered in a number of species; 2n=24 was recorded in *S. aculeata, S. formosa, S. grandiflora, S. sesban, S. tetraptera,* and *S. virgata*, whereas 2n=28 was only found in *S. pachycarpa*. Detailed karyotypic data have been described for several species (Lubis *et al*., 1981; Parihar & Zadoo, 1987; Joshua & Bhatia, 1989; Heering & Hanson, 1993; Salimuddin, 1993; Forni-Martins *et al*., 1994; Abou-El-Enain *et al*., 1998; El Shazly & Abou-El-Enain, 1999).

Seed storage proteins are particularly abundant in the seeds of legumes and have provided valid source of evidence that have successfully been used for addressing the systematic relationships at the species level in many genera of the family Fabaceae. For example, to differentiate between species in *Trifolium* (Badr, 1995), *Lathyrus* (Badr *et al*., 2000), *Astragalus* (Al-Nowaihi *et al*., 2002) and *Lupinus* (El-Shazly *et al*., 2006) In *Sesbania*, Saraswati *et al*. (1993) used electrophoretic patterns of seed proteins for

species identification. The analysis of seed protein electrophoretic data showed that species in the two subgenera Agati and Daubentonia form a heterogeneous group that is distinct from species of subgenus *Sesbania* (Badr *et al*., 1998).

In recent years, molecular markers derived from DNA using electrophoretic techniques have provided powerful markers for the study genetic relationships of plant species. The amplified fragment length polymorphism (AFLP) that was published by Vos *et al.* (1995) has been found efficient, reliable, and convenient. This method, which defines unique dominant loci in the genome, has the capacity to produce higher number of polymorphic loci and the highest frequency of polymorphism, in a single assay, than other molecular assays (Powell *et al.*, 1996), and has been regarded as the most popular among the molecular DNA finger-printing methods (Bachman, 1998; Martin & Salamini, 2000).

The reliability of this approach for phylogeny reconstruction has been confirmed by the finding that in closely related species of *Hordeum* the co-migrating bands are identical in terms of sequence homology. In that genus, phylogenies based on carefully selected AFLP bands were found to be superior to phylogenies based on ITS sequences (El-Rabey *et al*., 2002). Another approach utilizing AFLPs has been to elucidate the origin and domestication history of some cultivated crops, prominent examples include einkorn wheat (Heun *et al.*, 1997) and barley (Badr *et al.*, 2000). The potential of AFLP in systematics and evolutionary studies was also demonstrated in *Lactuca* (Hill *et al*., 1996), *Solanum* (Kardolus *et al*., 1998), *Bromus* (Massa, *et al*., 2001) and *Lathyrus* (Badr *et al*., 2002). The aim of the present work is to reassess the relationships among 37 species of *Sesbania* that belong to the three subgenera of the genus based on DNA fingerprints as revealed by AFLP.

Material and Methods

Seed material of 37 accessions representing 21 species of *Sesbania* Scopali was kindly provided by gene banks of the International Livestock Center for Africa (ILCA), Ethiopia and the United States Department of Agriculture (USDA). The source, origin, somatic chromosome number and sectional delimitation of the examined material are given in Table 1. Plants of almost all accessions have been grown in open grounds in the Botanical Garden of the Faculty of Education.

Table 1. A list compiling names, source, origin, 2n chromosome number and subgeneric delimitation of *Sesbania* accessions and species used in the present study.

Ser	Species	Source	Origin	2n	Subgenus
01	*S. bispinosa* 1 (Jacq.) Wright	USDA	India	12	Sesbania
02	*S. bispinosa* 2	USDA	Pakistan	12	
03	*S. bispinosa* 3	ILCA	Tanzania	12	
04	*S. cannabina* 1 (Retz.) Pers.	USDA	China	12	Sesbania
05	*S. cannabina* 2	USDA	India	12	
06	*S. exaltata* 1 (Raf.) Rydb. ex Hill	USDA	Australia	12	Sesbania
07	*S. exaltata* 2	USDA	Mexico	12	
08	*S. exaltata* 3	USDA	Uruguay	12	
09	*S. exasperata* 1 Kunth	USDA	Argentina	12	Sesbania
10	*S. exasperata* 2	USDA	Brazil	12	
11	*S. exasperata* 3	USDA	Peru	12	
12	*S. formusa* (F. Muell.) Burb.	ILCA	Australia	24	Agati
13	*S. goetzi* Harms	ILCA	Tanzania	12	Sesbania
14	*S. grandiflora* (L.) Poir	ILCA	Ethiopia	24	Agati
15	*S. greenwayi* Gillett	ILCA	Tanzania	12	Sesbania
16	*S. hirtistyla* Gillett	ILCA	Tanzania	12	Sesbania
17	*S. keniensis* Gillett	ILCA	Ethiopia	12	Sesbania
18	*S. leptocarpa* 1 DC.	USDA	Afghanistan	12	Sesbania
19	*S. leptocarpa* 2	USDA	Turkey	12	
20	*S. macrantha* Phillips. & Hutch.	ILCA	Tanzania	12	Sesbania
21	*S. microphylla* Phillips. & Hutch.	ILCA	Tanzania	12	Sesbania
22	*S. pachycarpa* 1 DC.	ILCA	Senegal	14	Sesbania
23	*S. pachycarpa* 2	USDA	Kongo	14	
24	*S. quadrata* Gill.	ILCA	Tanzania	12	Sesbania
25	*S. rostrata* Bremek. & Oberm.	ILCA	Tanzania	12	Sesbania
26	*S. sericea* 1 (Willd.) Link.	ILCA	Tanzania	12	Sesbania
27	*S. sericea* 2	ILCA	Tanzania	12	
28	*S. sericea* 3	------	Egypt	12	
29	*S. sesban* 1 (L.) Merr.	USDA	India	12	Sesbania
30	*S. sesban 2*	USDA	Taiwan	12	
31	*S. sesban* 3	USDA	Taiwan	12	
32	*S. sesban* 4	USDA	Brazil	12	
33	*S. sesban* 5	------	Egypt	12	
34	*S. speciosa* 1 Taub.	ILCA	Ethiopia	12	Daubentonia
35	*S. speciosa* 1	USDA	Pakistan	12	
36	*S. tetraptera* Hochst. ex Baker	USDA	Swaziland	24	Agati
37	*S. virgata* Cav.) Pers.	USDA	Uruguay	12	Daubentonia

For AFLP finger printing, seeds of all accessions were soaked in tap water for two days and germinated in small pots in the glasshouse at Miami University, Oxford, Ohio, USA. Leaves of actively growing seedlings were harvested on ice, frozen in liquid nitrogen, and stored at -80°C for DNA extraction. For DNA extraction, a modified CTAB method (Saghai-Maroof *et al*., 1984) was used. Leaflets were powdered in liquid nitrogen using a mortar and pestle, and homogenized in 0.75 ml hot 4× CTAB buffer containing 1% PVP, 1% Na-bisulphite, and 0.2% β-mercaptoethanol. The tubes were incubated for 30 min in a 60°C water bath with occasional gentle mixing. The mixture was emulsified with 0.5 ml of chloroform-isoamyl alcohol (24:1) and centrifuged at 10,000 g for 5 min and the aqueous layer was pipetted into a new tube, mixed with 0.5 ml cold isopropanol, kept at -20°C for 30 min, and centrifuged at 12,000 g for 10 min. The alcohol was discarded and the pellet was washed in 0.75 ml 76% EtOH/0.01 M NH_4OAC for 5 min followed by washing in 0.75 ml 76% EtOH/0.01 M NaOAC. The pellet was dried and suspended in 0.2 ml TE buffer, and 1 µl RNase was added.

AFLP analysis was performed using the ABI PRISM fluorescent dye labeling and detection protocol (Perkin Elmer, USA) based on the method of Vos *et al*. (1995), with slight modifications. Genomic DNA (500 ng) was double-digested with *Eco*RI and *Mse*I restriction enzymes and ligated to *Eco*RI and *Mse*I adapters by incubating in a total volume of 11 µl for 4 hrs at 37ºC. The restriction/ligation (R+L) product was diluted to 200 µl and stored at 4ºC for pre-amplification, or stored at -20ºC for later use. Five µl of the R+L product were pre-amplified with *Eco*RI + A and *Mse*I + C primers in a total volume of 20 µl in a thermocycler for 25 cycles at 94ºC denaturation (20 sec), 56ºC annealing (30 sec), and 72ºC extension (2 min), with initial hold at 72ºC and a final old at 60ºC for 30 min. The pre-selective amplification product was diluted 15X in 0.1 TE buffer and stored at 4ºC for amplification.

Five µl of the above solution were used as a template for selective amplification using three 5'end labeled *Eco*RI + 3 primers (ACA, blue; AAG, green; and ACC, yellow) and three *Mse*I + 3 primers (CAC, CTC, and CTT). Amplification was conducted in a total volume of 15 µl for 9 cycles at 94ºC (2 min), 56ºC (30 sec), and 72ºC (2 min), reducing the annealing temperature by one degree per cycle, followed by 21 cycles at 94ºC (2 min), 56ºC (30 sec) and 72ºC (2 min), and a hold at 60ºC for 30 min. Two µl of this product were mixed with 20 µl of deionized formamide and 0.5 µl of GeneScan 500 ROX internal size standard in a 0.5-ml tube,

denatured at 95ºC for 5 min, and analyzed by capillary electrophoresis on an automated Perkin Elmer ABI 310 DNA sequencer with an injection time of 12 sec and a run time of 30 min.

AFLP fragment profiles produced by the nine primer pair combina-tions were printed on photographic paper for manual scoring. The presence (1) or absence (0) of bands from 50 to 350 bp was scored (Fig. 1). Only poly-morphic bands scored in at least two accessions were considered for analysis; uncertain fragments were scored as uncertain (?). Distance trees were constructed using Dice and Jaccard similarity coefficients using UPGMA (Sokal & Michener, 1958) and Neighbor-joining (Saitou & Nei, 1987) tree building methods with the software NTSYS-pc 2.1 (Rohlf, 1993).

Results and Discussion

The nine primer pair combinations for *Eco*RI and *Mse*I produced considerable variation in the AFLP banding profiles. Over 400 AFLP bands have been scored in the examined 37 accessions of *Sesbania*. Of these bands, 138 have been found polymorphic and were used for the analyses to construct NJ and UPGMA trees. The AFLP banding profile for the 37 accessions of *Sesbania* following DNA digestion with *Eco*RI and *Mse*I, and amplifying the fragments in the presence of the CTC *Mse*I adapter and the AAG *Eco*RI adapter are illustrated in Fig. 1.

The neighbor joining (NJ) tree based on the analysis of AFLP polymorphism (Fig. 2) clearly distinguished the examined species in two major groups; a small one comprising species in the two subgenera Agati and Daubentonia and a large one that comprises species in subgenus Sesbania (see table 1). The small group is clearly divided into two subgroups at a high level of distance coefficient; one composed of the two accessions of *S. speciosa* and *S. virgata* that belong to subgenus Daubentonia and the other comprised of *S. formusa, S. grandiflora* and *S. tetraptera* that belong to subgenus Agati.

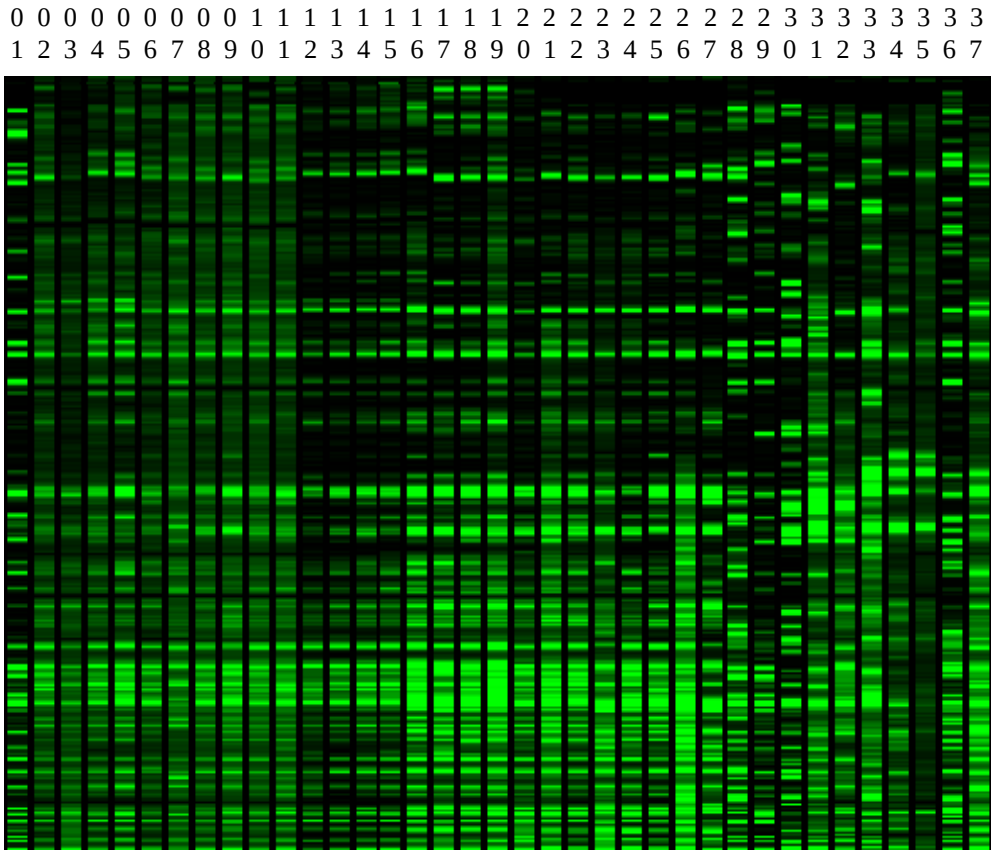

Figure 1. AFLP banding profile for the 37 accessions and species of *Sesbania*, DNA was digested with *Eco*RI and *Mse*I, and fragments were amplified using PCR in the presence of the *Mse*I adapter CTC, and the AAG *Eco*RI adapter.

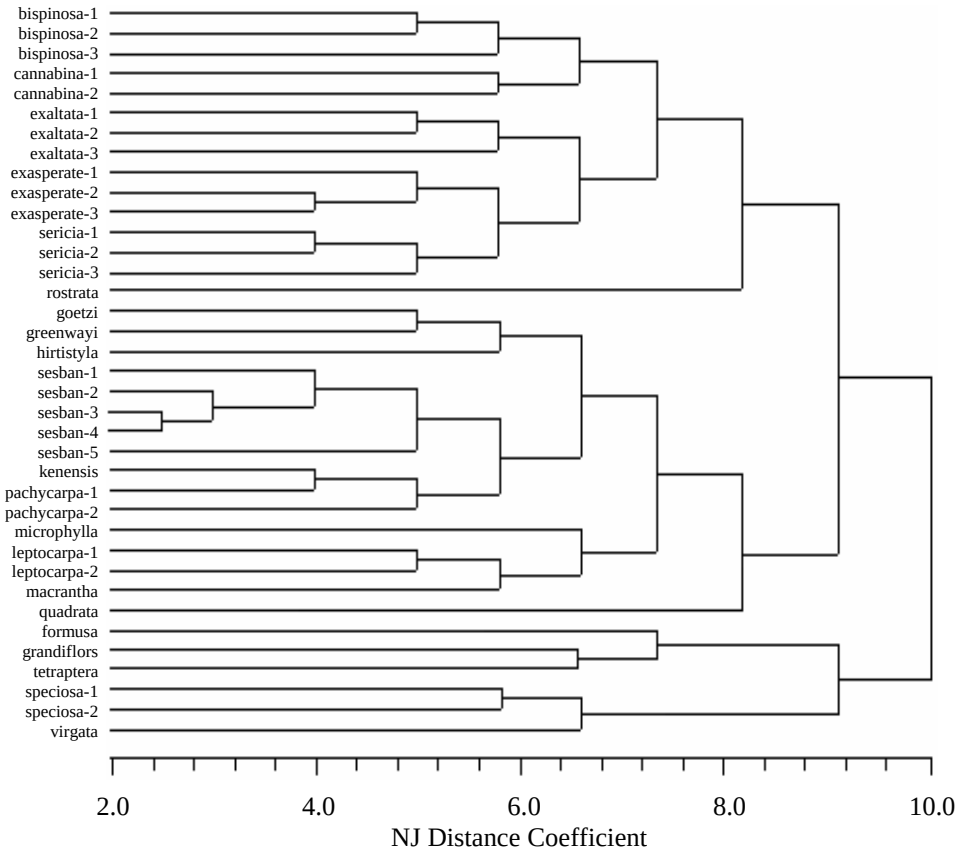

Figure 2. The relationship between accessions and species of *Sesbania*, based on AFLP data, as demonstrated by the NJ average distance tree.

The major group in the NJ tree that comprises species of subgenus Sesbania is divided into two groups. In one of these groups *S. quadrata* is clearly distinguished from a group comprised of 15 other accessions of mostly African species clustered in two subgroups, a small one that includes *S. macrantha*, two accessions of *S. leptocarpa* and *S. microphylla* and a large one that includes two clusters; one comprising the two accessions of *S. pachycarpa* and the five accessions of *S. sesban* and the other *S. goetzi*, *S. greenwayi* and *S. hirtistyla*. In the second group, *S. rostrata* is distinguished from 14 accessions of mostly non African species (Table 1; Fig. 1) that are also delimited in two subgroups; one of nine accessions in two clusters; one comprising *S. sericia* (3 accessions) and *S. exasperate* (3 accessions) and the other the three accessions of *S. exaltata*. The other subgroup is composed of two clusters; one comprising the accessions of *S. bispinosa* and the other accessions of *S. cannabina*.

In the UPGMA tree (Fig. 3) the species in subgenus Daubentonia (*S. speciosa* and *S. virgata*) are separated together as a small group from a major group that comprises the species in the other two subgenera. However, the species in the two subgenera Agati and Sesbania are divided into two groups. In one of these groups, the species of subgenus Agati (*S. formusa, S. grandiflora* and *S. tetraptera*) are delimited together and are clearly separated from another two subgroups comprising 15 accessions of subgenus Sesbania. One of these subgroups includes the two accessions of *S. leptocarpa* (two accessions), *S. hirtistyla*, *S. greenwayi* and *S. microphylla*. The other larger group is comprised of the five accessions of *S. sesban*, and *S. keninses* as well as the two accessions of *S. pachycarpa*, in addition to *S. goetzi* and *S. macrantha*, which are only associated at a high distance coefficient.

The other group is divided into two subgroups; a large one that comprises ten accessions and small one of six accessions. In the large subgroup, the three accessions of *S. sericia* are distinguished from another two clusters; one includes the three accessions of *S. exasperata* and the other *S. quadrata* and the three accessions of *S. exaltata*, in the latter cluster *S. quadrata* is clearly differentiated from the accessions of *S. exaltata* at a high distance coefficient. The other small subgroup is delimited in two clusters; one comprising the three accessions of *S. bispinosa* and the other includes *S. rostrata* and the two accessions of *S. cannabina* that are clearly distinguished from each other at high distance coefficient.

The AFLP finger printing of *Sesbania* species show that accessions of the same species, from different origins, have closer genetic affinity to each other than to accessions of other species. This is reflected in the topology of both the NJ and UPGMA trees. This observation is correlated with intarspecific resemblances in karyotype features (Abou-El-Enain, *et al.*, 1998; El-Shazly & Abou-El-Enain, 1999) and similarities in storage seed protein electrophoretic profiles (Saraswati *et al*., 1993; Badr *et al*., 1998). However, close inspection of AFLP profiles and of the topology of the distance trees shows more genetic diversity between different accessions of the same species that is not manifested in the karyotype features or seed protein electrophoretic profile. Considerable AFLP polymorphism is particularly evident among accessions of *S. bispinosa*, *S. cannabina*, *S. exaltatat*, *S. sesban*, and *S. speciosa* in both the NJ tree and UPGMA trees. This polymorphism may be useful markers for selection of desired traits in breeding of selected lines of *Sesbania* species.

The NJ and UPGMA trees clearly delimited the species in subgenus Daubentonia (*S. speciosa* and *S. virgata*) and the species in subgenus Agati (*S. formusa S. grandiflora* and *S. tetraptera*) as two different groups from other species that have been delimited in subgenus Sesbania. This is in agreement with the current systematic treatment of the genus. Species of subgenus Agati are characterized by large flowers, elliptic standard and reniform oblong seeds (Monteiro, 1994). The delimitation of subgenus Agati is also correlated with the karyotype criteria; species of this subgenus are mostly polyploid with a tetraploid number of $2n=24$ while those in the other two subgenera are mostly diploid with $2n=12$ (Abou-El-Enain *et al*., 1998; El-Shazly & Abou-Enain, 1999). The separation of subgenus Agati is also supported by evidence of seed protein electrophoretic data (Badr *et al.*, 1998). In line with these evidences the AFLP data, as illustrated in the UPGMA tree reflect the view of Baily (1924) and Maberly (1987) suggesting the treatment of subgenus Agati as a separate genus related to *Sesbania*. However, the topology of NJ indicate the retention of this subgenus as part of genus *Sesbania* as suggested by Monteiro (1994) supporting the view of Gillet (1963), Burbidge (1965) and Sachet (1987).

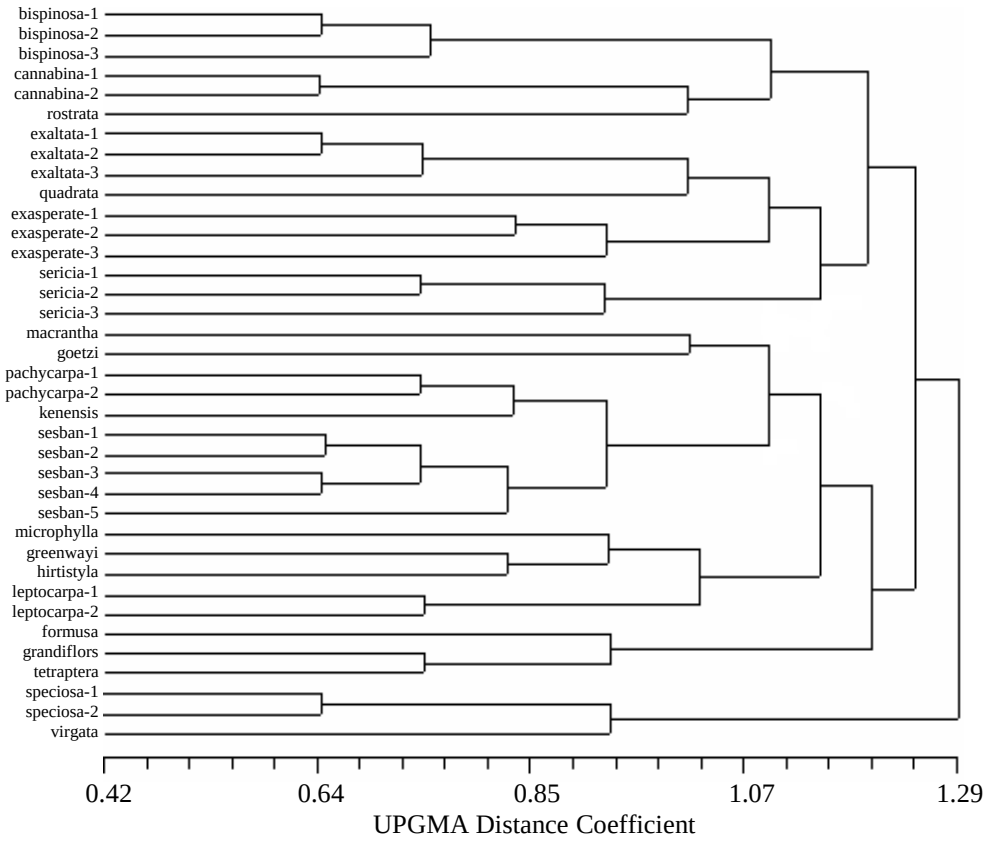

Figure 3. The relationship between accessions and species of *Sesbania*, based on AFLP data, as demonstrated by the UPGMA tree.

Compared to subgenus Agati species in the two subgenera, Sesbania and Daubentonia, have smaller flowers, suborbicular standard and cylindrical to oblong seeds (Monteiro, 1994). The species of the two subgenera are mostly diploid with 2*n*=12 but the species in subgenus Daubentonia have shorter chromosomes and more asymmetric karyotypes (Abou-El-Enain *et al*., 1998; El-Shazly & Abou-El-Enain, 1999). Species in this subgenus were also recognized as a separate group based on evidence from seed protein electrophoretic data (Badr *et al*., 1998). The AFLP polymorphism, as illustrated in the NJ and UPGMA trees, may be considered as support for the separation of subgenus Daubentonia in *Sesbania* and support its delimitation from the other two subgenera as based on similarities in morphological criteria, (Gillet, 1963; Burbidge, 1965; Sachet, 1987).

The AFLP data revealed considerable genetic polymorphism among species in subgenus Sesbania. This is not congruent with the possibility of hybridization between some species in this subgenus (Lewis, 1988; Heering & Hanson, 1993). The high level of genetic diversity as revealed by AFLP data may be due to the different origins from, which the examined materials were obtained. Heering & Hanson (1993) noted that crossing, among species, in subgenus Sesbania, may be encouraged by the overlapping distribution of closely related species and is indicative of a close phylogenetic relationship between them (Lewis, 1988). The delimitation of species in the subgenera Agati and Daubentonia from species in subgenus Sesbania, particularly in the NJ tree, support the view of Badr *et al*. (1998) and El-Shazly & Abou-El-Enain (1999) that subgenus Sesbania may be the center from which species of the other two subgenera have been derived.

Goldblatt (1981) considered the genus as a monophyletic group in the tribe Robinieae. The monophyly of the genus is supported by the occurrence of seven common bands in the seed protein electrophoretic profile of 15 species (Saraswati *et al.*, 1993) and of eight bands common to 24 species (Badr *et al*., 1998) of the genus. The results reported by the latter authors supported the view that species in the subgenera Agati and Daubentonia are distinguished as two groups that could have been derived from species in subgenus Sesbania. However, seed protein data did not reveal the level of polymorphism illustrated by the AFLP data. This may be due to the limited possible variation among the types of seed storage proteins that are controlled by few genes compared the AFLP polymorphism that results from nucleotide changes across the whole genome (Vos *et al*., 1995; Powell *et al.*, 1996). The grouping of species in

subgenus Sesbania indicate relationships that point out the need for further investigation on the phylogeny and biogeography of the genus.

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