African Journal of Biotechnology Vol. 8 (16), pp. 3691-3697, 18 August, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Genetic diversity analysis of the species of *Gossypium* by using RAPD markers

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Accepted 31 March, 2009

Random Amplified Polymorphic DNAs (RAPDs), a DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence, was utilized to measure the genetic distance among the species of *Gossypium*. Variations among 20 different species of *Gossypium* were analyzed with 63 random 10-mer primers using the polymerase chain reaction (PCR). Out of a total of 370 RAPD bands generated from the 20 wild species, 310 were perceived polymorphic. The study detected a high level of genomic variability of the species of *Gossypium*. The maximum similarity of 64% was observed between *Gossypium arboreum* and *Gossypium herbaceum* and minimum similarity of 3% was observed between *Gossypium stocksii* and *Gossypium longicalyx*. Cluster analysis by the unweighted pair group method of arithmetic means (UPGMA) showed that 20 wild species can be divided into several groups, although it is believed that *Gossypium* is a very widely distributed, wild species having being discovered in all the continents of the world which extends into the subtropical region. The relationship among the species is presented in the dendrogram.

Key words: Random amplified polymorphic DNA, polymerase chain reaction, wild species, genetic diversity, *Gossypium* wild species

INTRODUCTION

The genus *Gossypium* comprises about 51 diploid and tetraploid species of trees, shrubs and herbs (Fryxell, 1971). The study of the genetics and evaluation of *Gossypium* diversity is important for improvement in the existing gene pool. Extensive genetic variation exists among different cultivated and wild species of cotton (Percival and Kohal, 1990). The primary cultivated species namely *G. hirsutum, G. barbadense, G. herbaceum* and *G. arboreum* are cultivated for their oil and fibre. *G. hirsutum* dominates world cotton commerce, with an estimated value of $50 billion per year in the USA alone.

Gossypium species commonly are grouped into eight diploid genomic groups, designated A-G and K, and one tetraploid genomic group, based on chromosomal similarities (Edwards and Mirza, 1979; Endrizzi et al., 1985; Stewart, 1995). Each genome represents a group of morphologically similar species that can only rarely form hybrids with species from other genomic groups. The evolution and domestication of the complex polyploidy cotton species spans the globe from the Old World to the New World. The original lineage of *Gossypium* may have arose from Australia 20-30 million year ago judging from the diverse species of *Gossypium* now growing wild in that region (Percival et al., 1999).

The cotton, genus *Gossypium* L. is an ideal system for examining genome evolution in polyploids. According to Imam (1975), the wild species of the genus originated in

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five different regions of the world, namely the Australian desert, the drier parts of central America from Arizona to Peru, the fringes of the deserts of North and South Africa, the Arabian Desert and South East Asia including Pakistan. According to him, cytogenetic investigation of the species growing in these regions suggests that in each area, distinctly different genotypes evolved.

Morphological and isozyme markers have been used for estimating genetic relatedness in *Gossypium* but they are few or lack adequate levels of polymorphism and are of limited use in this respect (Tatineni et al., 1996). Molecular markers (Parson et al., 1997; Wang and Tansley, 1989; Wu and Tansley, 1993) are efficient in assessing polymorphism. Molecular markers can be extremely useful in plant breeding to solve practical problems faced by the breeder. The assessment of genotypic identity among individuals of a species is central to making valid biological interpretations about population structure, breeding systems, reproductive biology and micro evolutionary processes within and among the species.

Among these markers, RAPD is most widely used because it allows a rapid and inexpensive assay with a large number of markers (Welsh and MacClland, 1990; Williams et al., 1990). There are reports about the use of RAPD to discriminate intra- and inter-specific variation in cotton (Iqbal et al., 1997; Rana and Bhat, 2002).

Due to the technical simplicity and speed of RAPD methodology, it has been successfully used for the generation of genetic similarities and phylogenetic analysis (Gepts, 1993). In recent years RAPD technique was used in cotton. Multani and Lyon (1995) reported RAPD study in cotton in which they studies 14 Australian cultivars. Iqbal et al. (1997) used the RAPD in the generation of DNA fingerprints of cotton varieties. They analyzed the RAPD markers to evaluate the genetic diversity of elite commercial varieties. The present study was undertaken to assess genetic diversity among 20 different species of *Gossypium* using 63 well chosen random oligo-nucleotides.

MATERIALS AND METHODS

Plant material

Twenty representative accessions of wild cotton species in the A, B, C, D, E and F genomes were taken. All the plant materials were collected from Central Cotton Research Institute, Multan. Main features of the selected plant materials are presented in Table 1.

DNA extraction from cotton leaves

The extraction of DNA from the cotton sample is a crucial step for PCR based DNA markers. A reliable DNA extraction method should meet the following criteria: (i) require only a small amount of tissue; (ii) involve simple procedure; (iii) use minimal number and amount of chemicals; (iv) yield high quality DNA; and (v) yield large quantities of DNA.

Healthy young leaves, bulked from 4-6 different plants per specie, were used for DNA extraction according to the method proposed by Doyle and Doyle (1990) with the following modifications: (1) volume of extraction buffer was increased to double from sample weight, (2) the initial incubation temperature was raised to 65°C and (3) the wash buffer was modified to contain 70% ethanol.

Approximately 3 g of fresh leaves were frozen with liquid nitrogen ground with pestle and mortar to form a fine powder and mixed with 6 ml preheated (65°C) DNA extraction buffer (2% CTAB, 1.2 M NaCl, 20 mM EDTA, 0.1M Tris HCl, 1% PVP, 1% × 2 β mercaptoethanol). The mixed suspension was incubated in a water bath at 65°C for 45 min. The suspension was mixed by gentle inversions after every 10 min. Six ml of chloroform was added and mixed gently for 10-15 min and centrifuged at 9000 rpm for 15 min. The aqueous phase was transferred to another tube and an equal volume of chloroform was added. After mixing, the tube was centrifuged for 15 min at 11000 rpm. The supernatant was transferred to a clean tube and mixed with 1/3 ice-cold isopropanol to precipitate DNA. It was rinsed thrice with 70% ethanol and air dried for 2-3 h, and dissolved in 1×TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4°C.

The DNA was further purified by a second extraction. Before cleaning, the DNA was brownish and obviously contained contaminants including polysaccharides, polyphenols and other secondary compounds. In the cleaning procedure a volume of 10 µL RNase was added to DNA solution, which was incubated at 37°C for 30 min, with gentle inversions after every 10 min. Then 4 ml phenol/chloroform/isoamyl-alcohol (25:24:1) mixture was added and mixed by inverting for 10 min. The mixture was centrifuged at 7000 rpm for 10 min and the supernatant was transferred to a clean tube. The DNA was precipitated by adding double volume of 95% ethanol and $1/10^{th}$ volume of 3 M sodium acetate and mixed. The precipitate DNA was rinsed thrice with 70% ethanol, air dried for 30 min and dissolved in sterilized 1×TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4°C. DNA concentration was monitored by subjecting samples to 1% agarose gel electrophoresis in TBE buffer and by visual assessment of band intensities compared with lambda DNA standards.

Purity and concentration of DNA

To measure the concentration of DNA samples, the following procedure was used. In a 1000 µL quartz microcuvette, 10 µL DNA sample and 990 µL of distilled water was mixed. The optical density (OD) at 260 and 280 nm was measured with spectrophotometer (GENESYS 2). The concentration of the DNA sample was calculated as (µg/mL) = O.D 260 × 2.5 (Sambrook et al., 1989). Dilutions of 5 ng/µL were prepared from stock solutions. Pure preparations of DNA have a ratio of approximately 1.8. If there is substantial contamination with protein or phenol, the ratio will be less than 1.8.

Oligonucleotides

10-mer arbitrary primers were obtained from Operon Technologies Inc. 10-mer primers were screened against *G. thurberii* and *G. hirsutum.* Those 10-mer primers which revealed proper amplification were selected for further studies.

The PCR-RAPD reaction

The following method was optimized for the amplification reaction (Williams et al., 1990). Most amplification reactions were performed in a 25 µL volume overlaid with 50 µL of light mineral oil (BDH) and

Table 1. List of species of cotton (*Gossypium*) used in the present study.

No.	Species	Genome	Year	Location
1	*G. herbaceum*	A_1	1753	Old World cultigen
2	*G. arboreum*	A_2	1755	Old World cultigen
3	*G. hirsutum*	AD_1	1763	New World cultigen
4	*G. barbadense*	AD_2	1753	New World cultigen
African				
5	*G. triphyllum*	B_2	1862	Southern Africa
6	*G. barbosanum*	B_3	1963	Cape Verde Island
7	*G. capitis viridis*	B_4	1950	Cape Verde Island
Australian				
8	*G. sturtianum*	C_1	1863	Central to Eastern Australia
9	*G. robinsonii*	C_2	1875	Western Australia
10	*G. australe*	C_3	1958	Northern Australia
11	*G. costulatum*	C_5	1963	North-Western Australia
12	*G. populifolium*	C_6	1863	North-Western Australia
13	*G. nelsonii*	C_9	1974	North Australia
American				
14	*G. thurberii*	D_1	1854	Sonora, Mexico/Arizona
15	*G. aridum*	D_4	1911	West Central Mexico
16	*G. raimondii*	D_5	1932	West Central Peru
17	*G. gossypioides*	D_6	1913	Oaxaca, Mexico
18	*G. lobatum*	D_7	1956	Michoacan Mexico
Arabian/African				
19	*G. stocksii*	E_1	1874	Arabia, Pakistan
Redesignations				
20	*G. longicalyx*	F_1	1958	North Eastern Africa

using a genetic thermocycler (GTC-2). Each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH=9 at 25°C), 200 µM of each of the dNTPs, 0.25 µM primer, 50 ng template DNA, 2.0 U *Taq* polymerase and 2.5 mM $MgCl_2$. The amplification program consisted of denaturation at 94°C for 2 min, followed by 35 cycles comprising a denaturation step at 94°C for 1 min, an annealing step at 37°C for 1 min and an extension step at 72°C for 2 min. The cycling program was terminated by a final extension step at 72°C for 9 min.

The amplification products were separated by loading 12.5 µL of each reaction onto a 1% ultra pure agarose gel containing ethidium bromide (0.17 ng/mL) in TBE buffer (0.089M Tris-borate, 0.002M EDTA, pH 7.8) and running at 5V/cm for 4 h. The sizes of the fragments obtained were estimated by running alongside a 1 kb ladder marker (Gibco-BRL). Bands were viewed using a UV transilluminator (Maniatis et al., 1982).

Data analysis

DNA fragments banded on agarose were scored for all tested species of *Gossypium* as 1 for presence and 0 for absence of the band. The resulting matrix was used to calculate the Jaccard's Similarity Coefficient (Sokal and Sneath, 1963). Un-weighted pair-group arithmetic mean average (UPGMA) analysis was used to construct the dendrogram. All statistical analysis was carried out with MVSP, computer package (Version 2.1).

RESULT AND DISCUSSION

The RAPDs generated were used to determine the genetic diversity among 20 species of *Gossypium* presented in Table 1. Numerous polymorphisms were observed among the species of *Gossypium* (Figures 1 and 2). Amplification of genomic DNA extracted from these species using all the sixty three 10-mer primers produced a variety of RAPD patterns. A total of 370 bands were amplified with an average of 6.2 bands per primer. Out of 63 random primers used, almost all of them showed polymorphisms among the species of *Gossypium*. The monomorphic bands are constant bands and cannot be used to study diversity while polymorphic bands revealed differences and can be used to examine and establish systematic relationships among the genotypes (Hadrys et al., 1992).

The number of RAPD fragments generated per primer ranged from 2 fragments to a maximum of 18 fragments. The majority of primers were, however amplified between 4 and 10 fragments. The variation in the number of bands amplified by different primers influenced by variable fac-

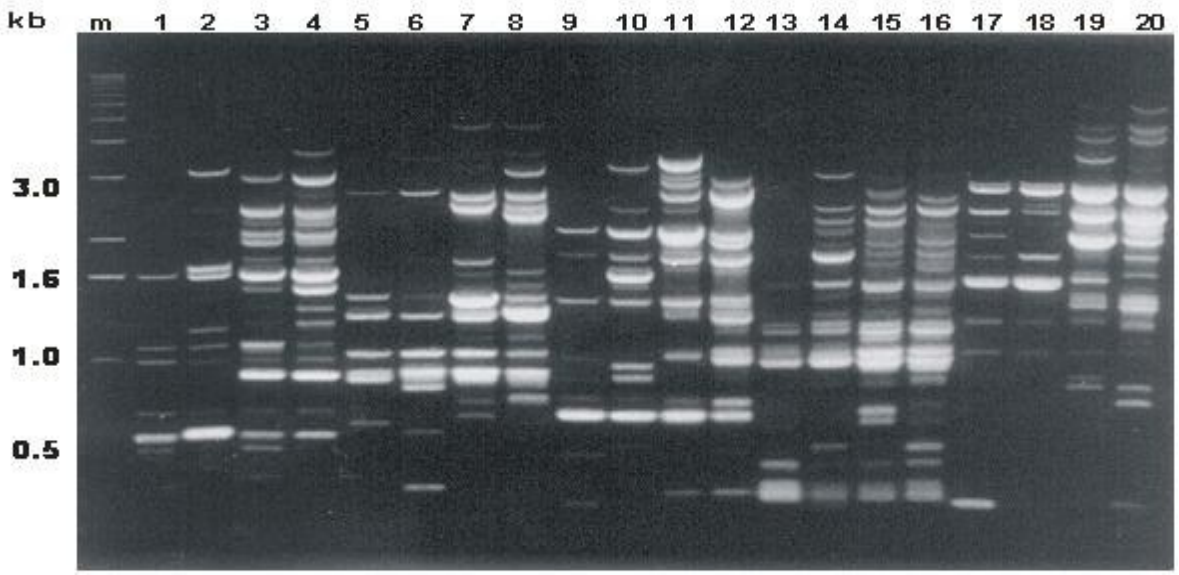

Figure 1. RAPDs generated from 20 species of *Gossypium* by using primer OPI-05, lane m: 1kb ladder, lanes 1-20 *Gossypium* species presented in Table 1.

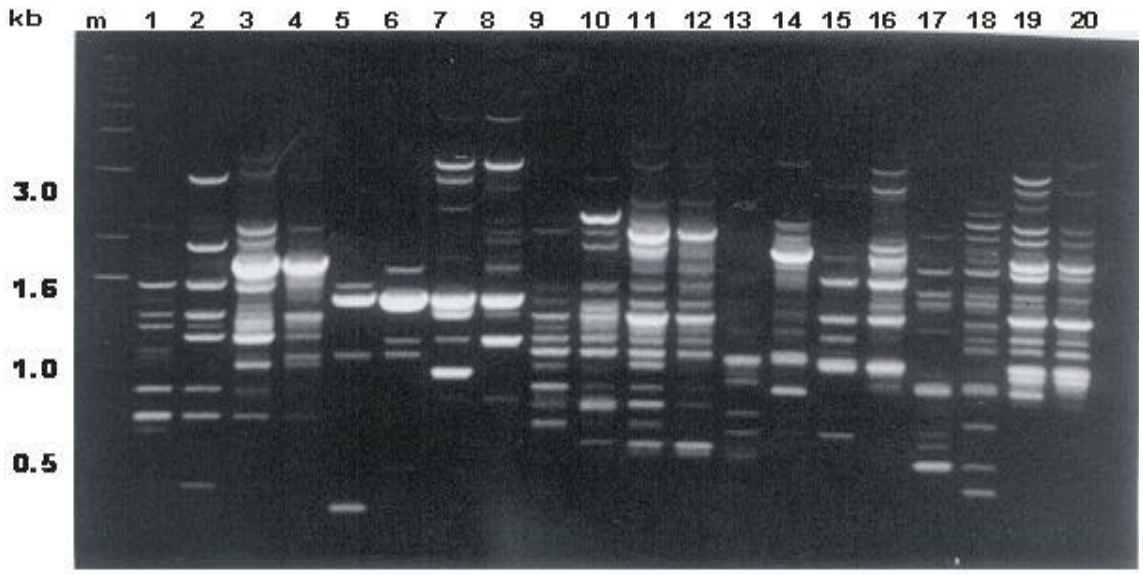

Figure 2. RAPDs generated from 20 species of *Gossypium* by using primer OPZ-20, lane m:1 kb ladder, lanes 1-20 *Gossypium* species presented in Table 1.

tors such as primer structure, template quantity and less number of annealing sites in the genome (Kernodle et al., 1993).

The similarity matrix is based on Jaccard's similarity coefficients. The similarity coefficients ranged from 0.03 to 0.64 (Table 2). Maximum similarity of 64% was observed between *G. herbaceum* and *G. arboreum* and lowest similarity of 3% was observed between *G. stocksii* and *G. longicalyx*.

The dendrogram (Figure 3), based on similarity coefficients, was constructed by using the Unweighted Pair Group of Arithmetic Means (UPGMA). Twenty species clustered into five distinct groups and two species, *G. longicalyx* and *G. stocksii* stand separately in the dendrogram. The position of *G. longicalyx* in the dendrogram is closer to the cluster of A genome species. This conclusion is similar to the findings of Phillips and Strickland (1966) that *G. longicalyx* on the basis of cytogenetic data

Table 2. Similarity coefficients among 20 species of *Gossypium* generated through RAPD data. Alphabets on the top of the table represent the same species as listed in the first vertical column.

***Gossypium* species**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**
G. arboreum	0.55																		
G. hirsutum	0.46	0.41																	
G. barbadense	0.42	0.38	0.64																
G. triphyllum	0.50	0.25	0.25	0.25															
G. barbosanum	0.08	0.10	0.14	0.13	0.03														
G. capitis virdis	0.18	0.22	0.14	0.21	0.12	0.42													
G. sturtianum	0.26	0.41	0.37	0.27	0.15	0.14	0.33												
G. robinsonii	0.33	0.25	0.25	0.25	0.14	0.28	0.17	0.15											
G. australe	0.45	0.40	0.35	0.42	0.20	0.18	0.18	0.26	0.50										
G. costulatum	0.25	0.30	0.20	0.26	0.22	0.20	0.20	0.28	0.22	0.50									
G. populifolium	0.21	0.25	0.25	0.31	0.08	0.16	0.21	0.25	0.18	0.41	0.33								
G. nelsonii	0.30	0.10	0.14	0.16	0.28	0.11	0.11	0.14	0.28	0.18	0.20	0.16							
G. thurberii	0.44	0.22	0.33	0.30	0.28	0.11	0.11	0.14	0.28	0.18	0.20	0.07	0.42						
G. aridum	0.55	0.33	0.41	0.38	0.42	0.10	0.22	0.30	0.25	0.21	0.30	0.25	0.37	0.57					
G. raimondii	0.33	0.16	0.46	0.42	0.33	0.04	0.05	0.18	0.09	0.14	0.15	0.21	0.30	0.44	0.55				
G. gossypioides	0.18	0.22	0.14	0.21	0.12	0.11	0.42	0.25	0.12	0.30	0.33	0.40	0.11	0.11	0.22	0.08			
G. lobatum	0.37	0.50	0.21	0.25	0.40	0.14	0.33	0.21	0.16	0.22	0.42	0.20	0.14	0.33	0.50	0.22	0.33		
G. stocksii	0.20	0.25	0.25	0.23	0.14	0.12	0.50	0.25	0.14	0.20	0.22	0.30	0.12	0.12	0.25	0.20	0.50	0.40	
G. longicalyx	0.21	0.20	0.30	0.28	0.25	0.10	0.22	0.21	0.25	0.40	0.30	0.25	0.10	0.22	0.33	0.40	0.22	0.28	0.42

is closer to A genome species than E genome species. In contrast, Khan et al. (2000) suggested the placement of *G. longicalyx* as a sister cluster to the D genome diploid species. The first cluster comprises of *G. barbosanum*, *G. triphyllum* and *G. capitis viridis*; these three species belong to African group. This result was also supported by the conclusion of the Wendel and Albert (1992) that cpDNA demonstrated a remark-able similarity among these three species of B genome. The second cluster consists of four cultivated species of the *Gossypium* i.e. *G. arboreum*, *G. herbaceum*, *G. barbadense* and *G. hirsutum*. These diploid species of A genome have similarity to the allotetraploids AD genome, similar result was reported by Khan et al. (2000) that A genome has a more UPGMA clustering similarity to the AD genome. The diploid species are closely related to each other as compared to these two tetraploids. The American species (D Genome), *G. thurberii*, *G. aridum*, *G. gossypioides*, *G. ramondii* and *G. lobatum* are grouped into third cluster of the dendrogram.

The Australian species *G. sturtianum* and *G. robinsonii* fall in fourth cluster and rest four, *G. populifolium*, *G. costulatum*, *G. australe* and *G. nelsonii* make the fifth cluster. The Australian species, *G. nelsonii* was more divergent in the group. It has maximum difference of 93% from another Australian species, *G. robinsonii.* Similar findings were reported by Khan et al. (2000). Other two Australian species *G. populifolium* and *G. costulatum* have maximum similarity of 50% between them as compared to the rest of the species of C genome.

The cultivated diploid species *G. arboreum* and *G. herbaceum* have similarity of 64%. This result is close to the findings of Rana and Bhat (2002) who reported 56.7% polymorphism in 18 diploid Indian *G. arboreum* and *G. herbaceum* cultivars. Beasley (1942) reported that *G. arboreum* and *G. herbaceum* comprised the A genome, based on chromosome pairing relationship. In the present study these two species are together in one cluster having maximum similarity. Similar observ-

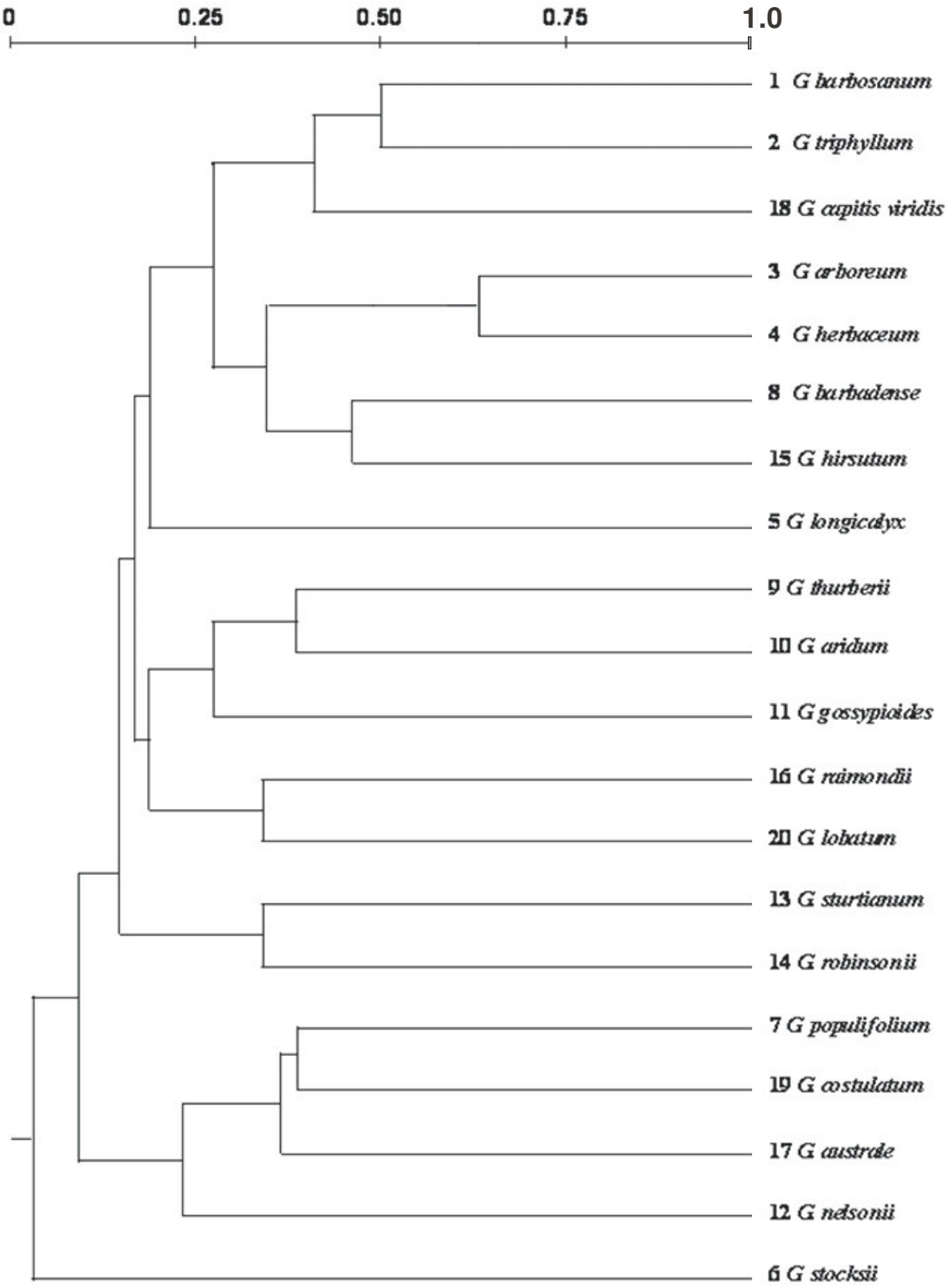

Figure 3. UPGMA cluster analysis-based dendrogram depicting genetic relationships among the 20 species of *Gossypium* Table 1.

ation were by Endrizzi et al. (1985), Khan et al. (2000) and Wendel et al. (1989).

The expected ancestor of cultivated cotton, *G. raimondii* of D genome has more similarity of 55% with

the cultivated *G. hirsutum* as compared to the other expected ancestor, *G. thurberii* which has only 25% similarity with *G. hirsutum*. These findings support the conclusion of Mahmood et al. (2004) that the *G. herbaceum* and *G. ramondii* are true ancestors of *G. hirsutum*.

The use of random primers in a PCR is a powerful tool that reveals extensive DNA polymorphism, and it has become valuable in genetic analysis. Since RAPD-PCR does not require prior sequence information and an arbitrary chosen short primer is used at lower annealing temperature than routine PCR to amplify one or multiple DNA segments from genomic DNA, a large number of polymorphic DNA markers may be easily generated.

Wild cotton species has proved to be a valuable source of the genetic material. Wide spectrum of a biological and economical index could be useful for the modeling of varieties corresponding to modern requirements and conditions. The general conclusion from this study is that RAPD profiling is efficient in revealing usable level of DNA polymorphisms among the species of *Gossypium*. These results promote the initiative to integrate these RAPD markers in cotton breeding applications, and DNA fingerprinting.

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