Australian Journal of Crop Science AJCS
AJCS 8(5):674-679 (2014) ISSN:1835-2707

Molecular diversity based on AFLP markers and possible natural hybridization among the Australian arid zone *Gossypium* species

Rashmi Sharma Tiwari*[1], Jinfa Zhang[1], and James Mac Stewart[2]

[1]Department of Plant and Environmental Sciences New Mexico State University, Las Cruces, NM 88001 USA
[2]Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR 72701, USA

***Corresponding author: rashmi.tiwari8@gmail.com**

Abstract

In Australia, 17 native wild diploid *Gossypium* species in the subgenus *Sturtia* are divided into three sections, *Grandicalyx* (K genome, 12 species), *Hibiscoidea* (G genome, 3 species) and *Sturtia* (C genome, 2 species). The C and G genomes are sympatric in their distribution ranges. The objectives of the present study were to identify the degree of genetic diversity and possible natural hybridization among Australian arid zone *Gossypium* species, *G. australe*, *G. bickii*, and *G. nelsonii* (section *Hibiscoidea*) based on amplified fragment length polymorphism (AFLP) markers. A few accessions of *G. sturtianum* (section *Sturtia*) sympatric to these species were also analyzed for possible hybridization. Six primer combinations yielded a total of 141 major scorable DNA fragments, of which 133 were polymorphic. Genetic similarity/diversity was evaluated using neighbor-joining analysis. The presence of markers that appeared to be primarily unique to *G. australe* in accessions of *G. nelsonii* and *G. bickii* indicated that the habitat of these species may be invaded by *G. australe*. *Gossypium bickii* shared more DNA markers with *G. australe* than *G. sturtianum* and had higher genetic similarity to *G. australe* than *G. sturtianum*, which strongly supported a monophyletic origin of section *Hibiscoidea*. The molecular evidence showed that natural hybridization has occurred among the arid zone species, suggesting that speciation may be an on-going process among these taxa.

Keywords: Genetic diversity; *Gossypium australe; Gossypium bickii; Gossypium nelsonii;* AFLP.
Abbreviations: AFLP_amplified fragment length polymorphism; RAPD_Random Amplified Polymorphic DNA; RFLP_Restriction Fragment Length Polymorphism; NJ_neighbor-joining

Introduction

The *Gossypium* genus has about 45 diploid species with 26 chromosomes, grouped into eight genomic groups, A-G and K, and 5 allotetraploid species with 52 chromosomes located in arid and semi-arid regions of the world. These latter species are in one tetraploid genomic group AD, based on chromosomal similarities (Cronn et al., 2002; Fryxell 1992; Smith and Cothern 1999; Stewart 1994). In Australia, there are 17 native wild *Gossypium* species and they all are diploid. All of these species are grouped under the subgenus *Sturtia*, which is further divided into three sections, *Grandicalyx* (K genome, 12 species), *Hibiscoidea* (G genome, 3 species) and *Sturtia* (C genome, 2 species) (Cronn and Wendel 2004; Fryxell 1979; Stewart 1994; Wendel et al. 1991). Most of the species occur in the northern region of the country. Eleven species of section *Grandicalyx* occur in the Kimberley region of northwestern Australia and the twelfth, *G. cunninghamii*, occurs in the Cobourg Peninsula of the Northern Territory (Fryxell et al. 1992). Section *Sturtia* species (*G. robinsonii* and *G. sturtianum*, official floral emblem of the Northern Territory) are distributed from Western Australia through central Australia into Queensland, and from the northern parts of South Australia into New South Wales. G*ossypium australe* has a broad distribution from near the east coast to the west coast and from south to north across the continent approximately in line with the southern area of the Northern Territory northward to Katherine in the northern part of the Northern Territory. *Gossypium bickii* occurs primarily within central Northern Territory, and *G. nelsonii* is distributed in a band from central Northern Territory to central Queensland. All the four species have overlapping geographical distribution and grow sympatrically in various combinations (Office of the Gene Technology Regulator 2002; Stewart et al. 1987). However, level of molecular diversity and involvement of natural hybridization is unclear. The genus *Gossypium* shows a complex history of evolution, and many studies have been conducted to reveal the interspecific relationship, evolution, phylogeny, and systematics of the genus (Fryxell 1971).

According to Wendel and Albert (1992) and Seelanan et al. (1997) each genome group in genus *Gossypium* is monophyletic despite their worldwide distribution and extraordinary morphological and cytogenetic diversity. *Gossypium australe* was originally named in 1875 by Von Muller (Saunders 1961), whereas, the original description of *G. nelsonii* is relatively recent because it was treated as a taxonomic synonym of *G. australe*. On the basis of comparative observation, *G. nelsonii* was recognized as a distinct species relatively recently (Stewart et al. 1987). On the other hand, *G. bickii* shows an unusual evolutionary history.

This species is one of three morphologically similar G-genome cottons, along with *G. australe* and *G. nelsonii*. In contrast to expectations based on this taxonomy, the chloroplast genome of *G. bickii* was shown to be very similar to the plastid genome of *G. sturtianum*, which is a morphologically distant C-genome species in section *Sturtia* (Cronn and Wendel 2004; Wendel et al. 1991). However, phylogeny derived from the nuclear markers indicates that *G. bickii* shares a more recent common ancestor with *G. australe* and *G. nelsonii* than it does with *G. sturtianum*. These studies shows that *G. bickii* has biphyletic ancestry, however, morphological analysis suggests that there is no evidence that *G. bickii* is intermediate between section *Sturtia* and section *Hibiscoidea* species (Wendel et al. 1991). Therefore, to identify evolutionary history of the Australian arid zone *Gossypium* species in section *Hibiscoidea*, the main goals of this research were to identify the degree of genetic similarity/diversity and determine if natural hybridization is occurring among Australian arid zone *Gossypium* species, *G. australe, G. bickii, G. nelsonii*.

Results and Discussion

Characteristics of AFLP markers

AFLP analysis with six primer combinations generated a total of 141 major DNA bands, 133 (22.2 per primer pair) of which (94.3%) were polymorphic across all the selected accessions (Table 2). Minor polymorphic bands were excluded from the analysis because these may be unreliable. The number of DNA fragments obtained varied among primer pairs (Table 2). The efficiency for detecting DNA-level polymorphism in the genus *Gossypium* by AFLP compared favorably with other marker systems. (Khan et al. 2000) detected 578 polymorphisms with 45 RAPD primers (12.8 polymorphism/primer) among 34 *Gossypium* species and ne interspecific hybrid. Rana and Bhat (2005) screened 127 polymorphisms with 20 RAPD primers (6.35 polymorphism/primer) among 18 cultivars of diploid cotton belonging to *G. arboreum* and *G. herbaceum*. RFLP analysis using 80 probe-enzyme combinations revealed a total of 60 polymorphisms among 65 Mesoamerican wild accessions and 23 domesticated lines of *G. hirsutum* ((Brubaker and Wendel 1994). A study of 103 accessions of *G. arboreum* and 31 accessions of *G. herbaceum* using isozymes yielded only 2.36 and 2.00 alleles/locus in *G. arboreum* and *G. herbaceum*, respectively (Wendel, 1989). A survey of 22 polymorphic SSR loci among 109 A-genome accessions (Guo et al., 2006) showed the SSR marker system to be highly polymorphic (5.8 alleles/per primer pair); however, the mean number of polymorphisms per primer was still less than that detected by AFLPs among the diploid entries surveyed in this study. On the basis of these data and DNA marker analysis in other crop species such as soybean (Maughan et al. 1996; Powell et al. 1996) lentil (Sharma et al. 1995), barley (Qi and Lindhout 1997) and wheat (Barrett and Kidwell 1998), AFLP offers superior efficiency for detecting polymorphisms as compared with other DNA fingerprinting systems.

Genetic diversity levels and patterns

The percentage of polymorphic bands within the species in section *Hibiscoidea* (*G. australe, G. bickii,* and *G. nelsonii*) ranged from 57% to 69% with an average of 63%. However, section *Hibiscoidea* accessions showed higher level (89%) of polymorphism among the three species. A neighbor-joining dendogram (Fig. 2) clearly showed four groups (clades), which corresponded to the four species. The mean pair-wise genetic distance (Supplementary Table 2) between the G-genome species ranged from 0.151 (*G. australe* vs. *G. nelsonii*) to 0.429 (*G. australe* vs. *G. bickii)*. All of the *G. australe* accessions, except for three accessions (i.e., 2, 6, 7) collected from the western area of Western Australia, and one accession (i.e., 31) from near Kathrine in the Northern Territory, formed a common cluster with mean pair-wise genetic distance ranging from 0.031 to 0.149. Uniformity among the widely distrtibuted *G. australe* suggests that the original populations arose from seeds that were wind-dispersed through relatively recent transportation activity as large populations of the species occurred on roadsides (Office of the Gene Technology Regulator, 2002). The eastern accessions (i.e., 10, 29, and 30) of Western Australia clustered with northern accessions. The presence of one northern accession (i.e., 31), with a mean pair-wise genetic distance of 0.187 to one western accession (i.e., 7), in the cluster of western accessions indicates that seeds of some western accessions were dispersed from western location to northern site. The western clade had the genetic similarity with a mean pair-wise distance of 0.10 (between accession 2 and 6) to 0.20 (between accession 6 and 7). Since all three G genome species arose from a common node with a high genetic similarity level, a monophyletic origin of the species is suggested (Stewart et al. 1987). *Gossypium nelsonii* occupies a position on the same branch as the western accessions of *G. australe,* with mean pair-wise genetic distance of 0.168 (between accession 6 and 48) to 0.308 (between accession 7 and 51), and the presence of bands that are primarily unique to *G. australe* in accessions of *G. nelsonii* is an indication that previously isolated areas of *G. nelsonii* may be invaded by *G. australe*. Since *G. australe* has wind dispersed seed and shows the most diverse group of the genome with higest mean pair-wise genetic distance of 0.265 within the species, it can be suggested that the seeds were dispersed through human road-building activity and that gene introgression has occurred by natural hybridization, a powerful evolutionary process for speciation (Grant 1981; Potts and Reid 1988). This implies that speciation is still an ongoing process. The occurrence in the *G. nelsonii* clade of accessions collected as possible hybrids between *G. australe* and *G. nelsonii* with a mean pair-wise genetic distance of 0.168 (e.g., accession 57 and 50), which is equal to the genetic distance between *G. australe* and *G. nelsonii* (e.g., accession 6 and 48), also supports this statement. The accessions of *G. sturtianum* were isolated from G-genome species is not unexpected since they are from the C-genome. The placement of *G. bickii* on a separate branch of the G-genome species tree and the similarity of DNA fragments to the other two species of the G-genome with the mean pair-wise genetic distance with *G. australe* ranged from 0.184 to 0.429 and with *G. nelsonii* ranged 0.253 to 0.348, supports the supposition that *G. bickii* shares a common ancestor with *G. australe* and *G. nelsonii*. The accessions grouped within one cluster of the *G. bickii* clade appear to have affinity with *G. australe*. One of the selected *G. bickii* accessions (acc. 38) was collected from an area where *G. australe* was not seen before, whereas accession 34 was collected from an area where one of the *G. australe* accessions (acc. 25) was also collected. Both accession 34 and 38 of *G. bickii* (growing with *G. australe* and without *G. australe*, respectively) had a genetic similarity with a mean pair-wise genetic distance of 0.067 between them, which was similar to the genetic distance between plants of the same accession (34A and 34B). The genetic distance of accession 34 and 38 to *G. australe*

Table 1. *Nucleotide sequence of AFLP adapters and primers.*

Component	5'----3' sequence
EcoRI forward adapter	CTCGTAGACTGCGTACC
EcoRI reverse adapter	AATTGGTACGCAGTC
MseI forward adapter	GACGATGAGTCCTGAG
MseI reverse adapter	TACTCAGGACTCAT
EcoRI +1 primer	GACTGCGTACCAATTCA
Mse +1 primer	GACGATGAGTCCTGAGTAAC
EcoRI +3 primer 1	GACTGCGTACCAATTCAGC
EcoRI +3 primer 2	GACTGCGTACCAATTCACG
EcoRI +3 primer 3	GACTGCGTACCAATTCACA
EcoRI +3 primer 4	GACTGCGTACCAATTCACC
EcoRI +3 primer 5	GACTGCGTACCAATTCAGG
MseI +3 primer 1	GACGATGAGTCCTGAGTAACAA
MseI +3 primer 2	GACGATGAGTCCTGAGTAACAC
MseI +3 primer 3	GACGATGAGTCCTGAGTAACTG
MseI +3 primer 4	GACGATGAGTCCTGAGTAACTT

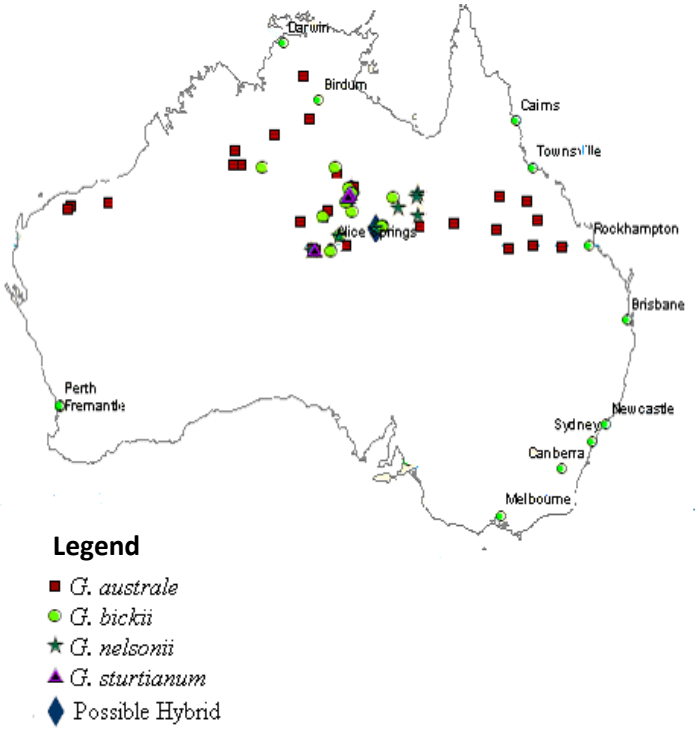

Fig 1. Distribution of all selected accessions, G. australe, G. nelsonii, G.bickii, G. sturtianum, and possible hybrids.

accession 25 was 0.336 and 0.353, respectively. However, accession 41 collected in close proximity to *G. nelsonii* had a genetic similarity with a mean pair-wise genetic distance of 0.274 with *G. nelsonii* accession 47. This value is close to the genetic distance of *G. nelsonii* and two possible hybrids (accession 48 and 56). This suggests that this accession (i.e., acc. 41) may be a hybrid between *G. bickii* and *G. nelsonii*. Previous phylogenetic studies based on nuclear markers (Cronn and Wendel 2004; Wendel et al. 1991) supported the common nuclear origin of the three species. Also, phenetic analysis reported by (Fryxell 1971) indicated the common ancestry of G-genome species with the placement of the Australian *G. austale* and *G. bickii* in a common lineage. However, the presence of a few bands that were unique to *G. sturtianum* in accessions of *G. bickii* and cladistic analysis of cpDNA which unite *G. bickii* and *G. sturtianum* (Wendel et al. 1991), are indications of ancient hybridization where a *G. sturtianum*-like species served as the maternal parent with a *G. austale*-like plant as the paternal and recurrent parent (Cronn and Wendel 2004; Wendel et al. 1991).

The data show that *G. australe*, the most widely distributed of the arid zone *Gossypium* species, is also the most molecularly diverse species in the group. Molecular evidence shows that natural hybridization is occurring among the arid zone species, an indication of ongoing speciation. This information should be helpful to eliminate redundancy in the selection of accessions for cotton improvement.

Materials and methods

Plant materials

Fifty-seven accessions of four *Gossypium* species were included in this study (Supplementary Table 1), including 33 accessions of *G. australe*, 12 accessions of *G. bickii*, 7

Table 2. Total number of amplified fragment length polymorphism (AFLPs) generated with six selective AFLP primer pair combinations.

Primer Combination	Total AFLP bands scored	No. of monomorphic bands	No. of polymorphic bands	% of Polymorphic bands
E-ACA/M-CAC	23	0	23	100
E-AGC/M-CAA	22	0	22	100
E-AGG/M-CTT	25	2	23	92
E-ACC/M-CTG	21	3	18	85.71
E-ACG/M-CAC	25	1	24	96
E-AGG/M-CAA	25	2	23	92
SUM	141	8	133	
MEAN				94.285

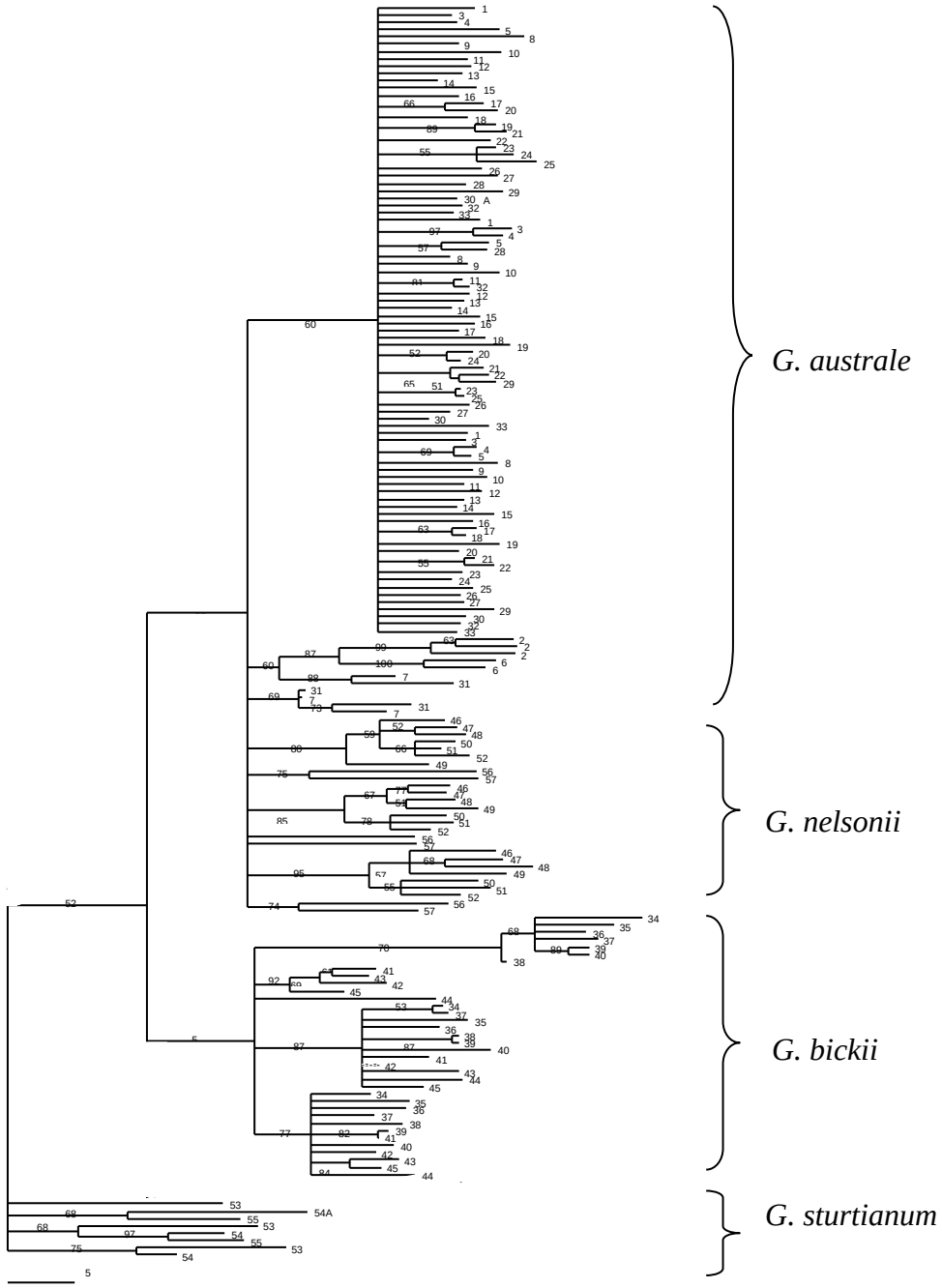

Fig 2. Unrooted neighbour-joining dendogram for *G. australe*, *G. bickii*, *G. nelsonii*, *G. sturtianum*, and possible hybrids from Australia, based on 141 AFLP markers. A, B, and C indicates three different plants of each accession. Numbers at the branch points indicate bootstrap support for accessions clustered to the right of the number.

accessions of *G. nelsonii*, 3 accessions of *G. sturtianum*, and 2 accessions of possible natural hybrid origin (Fig. 1). The accessions of *G. sturtianum* were included because they were collected at the same location as one of the other three species. Thus these three accessions have the potential to form hybrids with one of the G-genome species. Three plants were grown in the greenhouse for each accession. The accessions were identified by Plant Introduction (PI) number or by the Australian Plant Genetic Resources Information System number. Seeds of accessions were collected from different regions of Queensland, Northern Territory and Western Australia by J. McD. Stewart or were obtained from the Australian Centre for Plant Biodiversity.

DNA extraction

DNA was extracted from leaves of three individual plants (labeled as A, B, and C) for each accession based on Zhang and Stewart (2000) with minor modifications Or QIAGEN DNeasy® Plant Mini kit. The DNA was checked by its appearance in a 1% agarose gel for quality and was quantified using a spectrophotometer.

AFLP analysis

The standard procedure for AFLP was similar to the one reported by Vos et al. (1995) with some modifications. DNA samples were digested with restriction enzymes, *Eco*RI and *Mse*I at 37°C for 3 h and 70°C for 15 min. The digested DNA fragments were ligated to *EcoRI* and *MseI* adapters (Table 1) using T4 ligase at 20°C for 2 h. The ligated DNA was then used for pre-amplification with primer *EcoRI* +1 and *MseI* +1. The pre-amplification was performed in a thermocycler using the following parameters: denaturing at 95°C for 1 min; followed by 20 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 1 min; and 72°C for 2 min. Selective amplification was performed using six primer combinations (Table 1). The PCR reaction was incubated in a thermocycler as follows: 95°C for 1 min; 10 cycles of 95°C for 30 s, 65°C to 56°C (0.7°C reduction/cycle) for 30s, and 72°C for 1 min; and 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The amplified products were electrophoresised with a constant 1,800 volts through a 5% denaturating acrylamide gel and silver stained for visualization.

Data analysis

Polymorphic bands between accessions were manually scored as present (1) and absent (0) for each DNA sample and only major bands were considered in the analysis. The data matrix for all 171 individual plants (3 plants per accession) and the 6 primer combinations was used for analysis of data by neighbor-joining (NJ) method and an unrooted dendogram was generated using PAUP version 4b2 (Swofford 2003) for Macintosh. Support for the tree was obtained by performing one hundred bootstrap operations on the data set generated by distance analysis.

Conclusion

In summary, classification of species based on morphological characters may not reflect the diversity of the species. On the other hand, molecular analyses allow direct comparison of species at the DNA level. By using a molecular method we have obtained a snapshot of the evolution of Australian arid zone *Gossypium*. Six primer combinations produced 141 major DNA fragments of which 133 were polymorphic. These DNA analyses confirmed the relationship among *G. australe*, *G. bickii*, and *G. nelsonii*. The western accessions of *G. australe* were similar to northern accessions with a mean pair-wise genetic distance of 0.132 between the two accession (6 and 18). This indicates that seeds of some western accessions were probably dispersed from the western location to the northern site. *Gossypium nelsonii* showed lower level of diversity, with mean pair-wise genetic distance of 0.054 to 0.132, in compared to G. *australe* (0.031 to 0.265) and *G. bickii* (0.036 to 0.199). Also, the presence of *G. australe* alleles in *G. nelsonii*, and the fact that *G. nelsoni* was more genetically similar to *G. australe* with a mean pair-wise genetic distance of 0.151 to 0.328 than *G. bickii*, which had a mean pair-wise genetic distance of 0.253 to 0.355. This supports the suggestion of invasion of the habitat of *G. nelsonii* by *G. australe* (J. Stewart, personal communication). *Gossypium bickii* had more DNA fragments in common with *G. australe* than *G. sturtianum* and had higher genetic similarity to *G. australe* than *G. sturtianum*. These observations strongly support monophyletic origin of the section *Hibiscoidea*. However, cytoplasmic introgression from a species aligned with section *Sturtia* into a species aligned with section *Hibiscoidea* probably occurred in the evolution of this species (Wendel et al. 1991). The origin of *G. bickii* most likely is polyphyletic.

Acknowledgements

The authors would like to thank Dr. Chunda Feng for his assistance during the PAUP*.

References

Barrett BA, Kidwell KK (1998) AFLP-based genetic diversity assessment among wheat cultivars from the Pacific Northwest. Crop Sci 38: 1261-1271

Brubaker CL, Wendel JF (1994) Reevaluating the origin of domesticated cotton (*Gossypium hirsutum*; malvaceae) using nuclear restriction fragment length polymorphisms (RFLPs). Am J Bot 81: 1309-1326

Cronn RC, Wendel JF (2004) Cryptic trysts, genomic mergers, and plant speciation. New phytol 161: 133-142

Cronn RC, Wendel JF, Haselkorn T, Small RL (2002) Rapid diversification of the cotton genus (*Gossypium*: Malvaceae) revealed by analysis of sixteen nuclear and chloroplast genes. Am J Bot 89: 707-725

Fryxell PA (1971) Phenetic analysis and the phylogeny of the diploid species of *Gossypium* L. (Malvaceae). Evolution 25: 554-562

Fryxell PA (1979) The natural history of the cotton tribe. Texas A&M University Press. College Station, TX

Fryxell PA (1992) A revised taxonomic interpretation of *Gossypium* L. (Malvaceae). Rheedea 2: 108-165

Fryxell PA, Stewart JM, Craven LA (1992) A revision of *Gossypium* sect. Grandicalyx (Malvaceae), including the description of six new species. Syst Bot 17: 91-114

Grant V (1981) Plant speciation. Columbia University Press. New York, USA

Guo W-Z, Zhou B-L, Yang L-M, Wang W, Zhang T-Z (2006) Genetic diversity of landraces in *Gossypium arboreum* L. race sinense assessed with simple sequence repeat markers. J Integr Plant Biol 48: 1008-1017

Khan SA, Malik KA, Zafar Y, Stewart JM, Hussain D, Askari E (2000) Molecular phylogeny of *Gossypium* species by DNA fingerprinting. Theor Appl Genet 101: 931-938

Maughan P, Saghai Maroof M, Buss G, Huestis G (1996) Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance, and near-isogenic line analysis. Theor Appl Genet 93: 392-401

Office of the Gene Technology Regulator. 2002. The biology and ecology of cotton (*Gossypium hirsutum*) in Australia. (http://www.ogtr.gov.au/pdf/ir/biologycotton.pdf).

Potts BM, Reid JB (1988) Hybridization as a dispersal mechanism. Evolution 42: 1245-1255

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breeding 2: 225-238

Qi X, Lindhout P (1997) Development of AFLP markers in barley. Mol Gen Genet 254: 330-336

Rana MK, Bhat KV (2005) RAPD markers for genetic diversity study among Indian cotton cultivars. Curr Sci India 88: 1956-1961

Saunders J (1961) The wild species of *Gossypium* and their evolutionary history. London Oxford Univ. Press, NewYork.

Seelanan T, Schnabel A, Wendel JF (1997) Congruence and consensus in the cotton tribe (Malvaceae). Syst Bot 22: 259-290

Sharma SK, Dawson IK, Waugh R (1995) Relationships among cultivated and wild lentils revealed by RAPD analysis. Theor Appl Genet 91: 647-654

Smith CW, Cothren JTE (1999) Cotton: origin, history, technology, and production. John Wiley & Sons Inc, New York

Stewart JM (1994) Potential for crop improvement with exotic germplasm and genetic engineering. pp. 13–327. In: Challenging the Future: Proceedings of the world cotton research conference-1, edited by Constable G. A., Forrester N. W. CSIRO, Melbourne, Brisbane, Australia, pp. 13-327

Stewart JM, Fryxell PA, Craven LA (1987) The recongnition and geograpic distribution of *Gossypium nelsonii* (Malvaceae). Brunonia 10: 215-218

Swofford DL (2003) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinauer Associates

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kulper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23(21): 4407-4414

Wendel JF (1989) New World tetraploid cottons contain old world cytoplasm. P Natl Acad Sci U S A 86: 4132-4136

Wendel JF, Albert VA (1992) Phylogenetics of the cotton genus (*Gossypium*): Character-state weighted parsimony analysis of chloroplast-DNA restriction site data and its systematic and biogeographic implications. Syst Bot 17: 115-143

Wendel JF, Stewart JM, Rettig JH (1991) Molecular evidence for homoploid reticulate evolution among Australian species of *Gossypium*. Evolution 45: 694-711

Zhang J, Stewart JM (2000) Economical and rapid method for extracting cotton genomic DNA. J Cotton Sci 4: 193-201