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RESEARCH ARTICLE

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Assessment of genetic diversity in glandless cotton germplasm resources by using agronomic traits and molecular markers

Abstract Seventy-one glandless cotton germplasm resources were firstly evaluated genetically by using nine agronomic traits, 33 simple sequence repeat (SSR) primers and ten amplified fragment length polymorphism (AFLP) primer combinations. Principal component analysis (PCA) of the agronomic traits showed that the first six principal components (PCs) explained a total of 86.352% of the phenotypic variation. A total of 329 alleles were amplified for 33 SSR primers, and 232 polymorphic bands in a total of 389 bands were obtained by using ten AFLP primer combinations. The average polymorphic information content (*PIC*) value was 0.80 and 0.18 for SSR primers and AFLP primer combinations, respectively. The DIST (average taxonomic distance) and DICE (Nei and Li's pairwise distance) coefficients ranged from 0.373 to 3.164 and 0.786 to 0.948, respectively, for agronomic traits and SSR&AFLP data based on UPGMA analysis. This suggested that there was a higher diversity in the evaluated population for both agronomic traits and molecular markers. The Mantel's test showed that the correlation between the dendrograms based on agronomic traits and SSR&AFLP data was non-significant.

Keywords glandless cotton, agronomic trait, AFLP, SSR, principal component analysis (PCA)

1 Introduction

Glandless cotton is a novel type without pigment glandular organs, and gossypol content in the seedlings and leaves exceedingly lower than that in glanded cotton (Bottger et al., 1964). Seeds of glandless cotton could be directly utilized in the processing industry. It is not necessary for cottonseed oil and protein to be refined to remove gossypol. In addition, glandless cotton is not toxic to humans and non-ruminant animals because it could not disturb the function of the stomach (Lee, 1962; Cai et al., 2004). Thus, glandless cotton has all-important economic values.

The first line of glandless upland cotton, namely '23B', was bred by McMichael (1959). The 'Bahtim 110', the first glandless sea island cotton with a dominant gene, was bred by using ^{32}P mutation (Afifi et al., 1966). It has been proven that three alleles (gl_1, gl_2 and gl_3) control the differentia of pigment glandular organs between glandless cotton and glanded cotton. The pigments of stem, petiole, carpel wall, cotyledon and leaf were controlled by gl_1, and those of the cotyledon and leaf were affected by gl_2 and gl_3 (McMichael, 1954, 1960; Roux, 1960). Subsequently, it was deduced that two unimportant alleles, gl_4 and gl_5, could also reduce glands in the cotton plant (Lee, 1962). The gl_6 had the same function as gl_1, but was weaker (Murray, 1965).

Genetic variation among germplasm resources was the groundwork for breeding new super species and germplasm enhancement. The diversity of genetic resources had twofold values in a breeding program. First, genetic heterogeneity could improve the resistance; second, allelic variation could provide and create new favorable gene combinations (van Esbroeck and Bowman, 1998). The DNA-based molecular marker was a useful tool to evaluate germplasm resources, to more effectively open out novel genes or new genotypes and to offer important genetic information for breeding programs. At present, molecular markers such as RFLPs (Reinisch et al., 1994; Shappley et al., 1998), AFLPs & RFLPs (Vroh et al., 1999), SSRs (Saha et al., 2003; Liu et al., 2000a, 2000b; Zhang, 2005a, 2005b), AFLPs (Reddy et al., 1997; Abdalla et al., 2001), AFLP & morphological characteristics (Rana et al., 2005), are utilized to evaluate glanded cotton germplasm resources.

However, the genetic diversity of glandless cottons has not been well characterized. The purpose of this study is to evaluate the genetic diversity of glandless cotton germplasm resources based on agronomic characters, SSR markers and AFLP technology, and to provide elite germplasm for a breeding program.

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2 Materials and methods

2.1 Plant materials and field assay

Seventy-one glandless cottons of various resources were grown in a randomized block design with three replications at the experimental farm of the Agricultural University of Hebei in 2003 (Table 1). Seeds from these genetic resources were planted in single-row plots, 3.5-meters long, spaced 0.65 meter between rows, and apart with individual plants 0.32 meter. The nine agronomic traits recorded were growth period (GP), plant height (PH), number of available bolls (NAB), number of fruit branches (NFB), node of first fruit branch (NFFB), seed index (SI), boll weight (BW), lint percentage (LP) and span length (SL).

2.2 DNA extraction

The DNA of 71 glandless cottons was extracted from fresh leaves according to the CTAB protocol (Paterson et al., 1993). The quality and quantity of DNA were checked on 0.8% agarose gel and a BECKMAN DU800 spectrophotometer.

2.3 SSR assay

SSR assay was performed in a total of 10 μL reaction mixes containing 80 ng genomic DNA, 1.3 μL 10 × PCR reaction buffer (containing Mg^{2+}), 0.8 μL 2.5 mmol·L^{-1} dNTP, 0.35 μL forward primer (10 ng·μL^{-1}), 0.35 μL reverse primer (10 ng·μL^{-1}), 0.16 μL *Taq* DNA polymerase (TaKaRa, China) and 6.04 μL ddH_2O. PCR amplification was performed on a Tgradient thermal cycler (Biometra, Germany) with the following conditions: at 95°C for 5 min, followed by 30 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 1 min, and a final elongation for 10 min at 72°C. Thirty-three SSR primers containing 21 BNL primers, 7 CIR primers, 4 TMH primers and 1 CML primer were used for PCR (Table 5).

2.4 AFLP analysis

The AFLP analysis was performed as described by Vos et al. (1995) with some minor modifications. A total of 10 polymorphic primer combinations were used to generate selective PCR amplification products (Table 5). The *Eco*RI and *Mse*I (New England Biolabs, USA) were used to double-digest genomic DNA (150 ng) at 37°C for 4 h in a final volume of 20 μL. Five microlitres of the adapter-ligation solution (1.0 μL 50 pmol·μL^{-1} *Mse*I adapter, 1.0 μL 5 pmol·μL^{-1} *Eco*RI adapter, 1.5 U T4 DNA ligase (Promega, Madison, Wis.), 0.5 μL T4 buffer, 1.8 μL 10 mmol·L^{-1} ATP and 0.2 μL ddH_2O) was added to the digested DNA. The reaction mixture was incubated at 16°C for 10 h. These ligates were diluted five times, and five microlitres were used as a template. The pre-amplification mixture included 0.6 μL of the *Eco*RI-00 and 0.6 μL of the *Mse*I-00 primers, 2 μL 10 × PCR reaction buffer (containing Mg^{2+}), 1.6 μL 2.5 mmol·L^{-1} dNTP, 0.1 μL *Taq* DNA polymerase and 10.1 μL ddH_2O. PCR cycles were performed at 95°C for 2 min, then 31 cycles (95°C for 30 s; 56°C for 30 s, 72°C for 1 min), followed by 10 min

Table 1 Glandless cotton genetic resources and their geographic sources

code	germplasm	sources	code	germplasm	sources	code	germplasm	sources
1	Jiwu2031	Hebei	25	Luwu386	Shandong	49	Fenwu34	Shanxi
2	Jiwu3703	Hebei	26	Luwu373	Shandong	50	Fendi99	Shanxi
3	Jiwu3003	Hebei	27	Liaowu19B	Shandong	51	Fenwu195	Shanxi
4	Jiwu252	Hebei	28	Liaowu38B	Shandong	52	GP202gl16	USA
5	Jiwu239	Hebei	29	Luwu4001	Shandong	53	GP203gl56	USA
6	Jiwu302	Hebei	30	Fangwu2	Jiangsu	54	GP205	USA
7	W82-1	Hebei	31	Fangwu7919	Jiangsu	55	GP207gl277	USA
8	Shengwu303	Hebei	32	Fangwu7917	Jiangsu	56	Pamastor464	USA
9	Floss101	Hebei	33	Suwu62	Jiangsu	57	Pamastor784	USA
10	Hanwu23	Hebei	34	Suwu65	Jiangsu	58	Acala63–69	USA
11	Xiawu16	Hebei	35	Guanglingmian	Zhejiang	59	Acala63–75	USA
12	Yuwu302	Henan	36	Guanglingmian1	Zhejiang	60	Acala 63–74	USA
13	Yuwu1309	Henan	37	Zhemian9	Zhejiang	61	Lankart4	USA
14	Zhongwu831	CRICAAS	38	RGlandless	Zhejiang	62	Lankart5	USA
15	Zhongwu151	CRICAAS	39	SuperbractGLcotton	Zhejiang	63	LankartGL-N	USA
16	Zhongwu1051	CRICAAS	40	Wanwu1	Anhui	64	glass	USA
17	Zhongwu383	CRICAAS	41	Wanwu85B5	Anhui	65	Lockett22	USA
18	XiangC40	Hunan	42	Mianyang4176	Sichuan	66	G45E	USA
19	XiangC118	Hunan	43	Mianyang5205	Sichuan	67	Chad 3	Chad
20	XiangC74	Hunan	44	Shannwu9086	Shannxi	68	MaliGLcotton	Mali
21	XiangC70	Hunan	45	Liaowu6051	Liaoning	69	ISABC2	France
22	Jing4585	Hubei	46	Liaowu2152	Liaoning	70	ISABC4	France
23	Luwu403	Shandong	47	Jin7308	Liaoning	71	Mexico45u	Mexico
24	Luwu401	Shandong	48	Jin1386	Liaoning			

at 72°C. The pre-amplification products were diluted five times, and five microlitres were used as a template. The selective amplification mixture included 2 μL 10 × PCR reaction buffer (containing Mg^{2+}), 1.8 μL 2.5 mmol·L^{-1} dNTP, 0.8 μL 50 ng·μL^{-1} *Eco*RI+3 primer, 0.8 μL 50 ng·μL^{-1} *Mse*I+3 primer, 0.15 μL *Taq* DNA polymerase and 9.45 μL ddH_2O. PCR cycles were carried out with the following conditions: 95°C for 2 min, then 13 cycles at 95°C for 50 s, 65°C for 40 s and 72°C for 1 min. The annealing temperature was lowered by 0.7°C for the next 12 cycles, followed by 31 cycles at 95°C for 50 s, 56°C for 40 s and 72°C for 1 min, and an extension at 72°C for 10 min.

2.5 Polyacrylamide gel analysis

Seven microlitres of amplified products from SSR and AFLP were all mixed with a 5 μL formamide-loading buffer, then denatured at 95°C for 10 min. The products were separated on 6% denatured polyacrylamide gel. The pBR322 DNA/*Msp*I marker (Huamei, China) was used. The bands were detected by silver nitrate staining.

2.6 Data analysis

The means over the replications were calculated and normalized prior to cluster analysis for all the agronomic traits. The principal component analysis (PCA) and cluster analysis with the SIMINT module and DIST coefficient were performed for phenotypic data according to the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) Version 2.11a User Guide (James Rohlf, 2000).

For SSR and AFLP, the distinct, major and reproducible bands were scored, and weak polymorphic bands were discarded, which may lead to errors (Schondelmaier et al., 1996; Abdalla et al., 2001). All SSR and AFLP bands were scored as a binary unit character (1 = present, 0 = absent). The *PIC* of SSR was calculated according to $PIC = 1 - \Sigma\ p_i^2$, where p_i was the frequency of the *i*th allele for individual *p* (Ni et al., 2002; Warburton and Crossa, 2002), and *PIC* of AFLP was described in the following equation: $PIC = 1 - [f^2 + (1-f)^2]$, in which *f* was the frequency of the marker in the AFLP data set (De Riek et al., 2001). The assay efficiency index (*AEI*) (Beyene et al., 2005) between SSR and AFLP was calculated with the equation: $AEI = BP/T$, where *BP* was the total number of polymorphic bands or alleles generated, and *T* was the total number of primers used in the study. Genetic similarity was estimated based on Dice (Nei and Li, 1979) coefficients, and cluster analysis was performed with an unweighted pair-group method based on arithmetic average (UPGMA) for SSR & AFLP data matrices. The Mantel's test was used to measure the level of association between the two matrices (Mantel, 1967; Rana et al., 2005; Tara et al., 2006). All statistical analysis was carried out using the NTSYS-pc 2.11a software package.

3 Results

3.1 PCA of agronomic traits

The highly significant differences among PH, GP, BW and SL traits were all revealed by using PCA (Table 2). Correlation analysis from a standardized data matrix showed that the significantly positive correlation existed between PH and GP, PH and NFB, and the coefficients were 0.545 and 0.524, respectively. The significantly negative correlation between SI and LP, LP and SL were revealed with the coefficients of −0.308 and −0.223 (Table 3). The first six PCs explained a total of 86.352% of the phenotypic variation. In the first PC, which explained 32.27% of the total variation, the most important traits were PH, NFB, GP, NFFB, BW and SL. The second PC, which accounted for 17.08% of the total variation, was dominated by SI. In the third PC, which explained 11.52% of the total variation, the predominant trait was LP. In the fourth PC, which accounted for 10.02% of the total variation, the important trait was NAB. The fifth PC explained 8.34% of the total variation, and the sixth PC explained 7.12% of the total variation (Table 4).

Table 2 The statistics of nine agronomic characters in 71 glandless cotton germplasm resources

trait	mean	Std. Dev.	min.	max.
PH/cm	83.310	12.438	52.000	119.000
NAB	13.268	3.902	6.000	23.000
NFB	11.648	1.288	8.000	15.000
NFFB	6.423	1.051	4.000	9.000
GP/d	117.606	7.719	107.000	145.000
SI/g	11.577	1.865	7.320	22.830
BW/g	6.270	0.840	3.710	8.050
LP/%	36.865	2.853	31.080	48.290
SL/mm	29.015	2.240	21.400	33.900

Table 3 Correlation coefficients among agronomic characters of glandless cotton

	PH	NAB	NFB	NFFB	GP	SI	BW	LP	SL
PH	1								
NAB	0.300	1							
NFB	0.524	0.261	1						
NFFB	0.431	0.286	0.175	1					
GP	0.545	0.115	0.372	0.454	1				
SI	0.093	−0.018	0.241	0.040	0.044	1			
BW	0.200	0.107	0.333	0.021	0.154	0.445	1		
LP	−0.096	−0.135	−0.042	0.061	−0.089	−0.308	−0.149	1	
SL	0.385	0.221	0.266	0.387	0.276	0.193	0.116	−0.223	1

The plots of the eigenvectors and the outlier projections revealed that 'Fenwu34' was more of an outlier, and the test values for outliers were 42.850 (*T*2), 4.217 (*Fs*) and 0.000 (Prob) (results omitted) (Fig. 1). Correspondingly, the test values (*T*2, *Fs* and Prob) of 'Jin1386' were 20.378 (*T*2), 2.005 (*Fs*) and 0.536 (Prob) (results omitted).

3.2 SSR and AFLP analysis

Seventy-one glandless cotton germplasm resources were analyzed genetically by using 33 SSR primers distributed over 17 chromosomes and 10 AFLP primer combinations. A total of 329 alleles were generated in SSR, and the number of alleles per SSR primer ranged from 2 to 22, with an average of 10. The selected AFLP primer combinations generated a total of 389 amplification products, of which 232 were found to be polymorphic. The averaged *PIC* value for 33 SSR primers was 0.80 and ranged from 0.21 (BNL3449) to 0.95 (BNL3649). The *PIC* value for AFLP ranged from 0.08 (M62-E40) to 0.27 (M50-E36), with a mean of 0.18. The assay efficiency index (*AEI*) was 23.2 for AFLP primer combinations and 10 for SSR primers (Table 5).

Table 4 Eigenvectors, eigenvalues, individual and cumulative percentage of variation explained by the first six principal components after assessing agronomic characters in 71 glandless cotton germplasm resources

trait	PC1	PC2	PC3	PC4	PC5	PC6
PH	0.793	0.216	0.125	0.004	0.223	0.078
NAB	0.461	0.163	−0.345	0.751	−0.103	−0.167
NFB	0.683	−0.101	0.389	0.253	0.206	0.358
NFFB	0.604	0.459	−0.120	−0.206	−0.414	−0.290
GP	0.678	0.271	0.182	−0.341	0.312	−0.290
SI	0.356	−0.738	0.028	−0.186	−0.295	−0.025
BW	0.432	−0.601	0.361	0.146	−0.187	−0.245
LP	−0.264	0.513	0.636	0.091	−0.441	0.151
SL	0.624	0.033	−0.409	−0.224	−0.260	0.477
eigenvalue	2.904	1.537	1.037	0.902	0.756	0.641
individual percent	32.267	17.080	11.520	10.023	8.340	7.122
cumulative percent	32.267	49.347	60.867	70.890	79.230	86.352

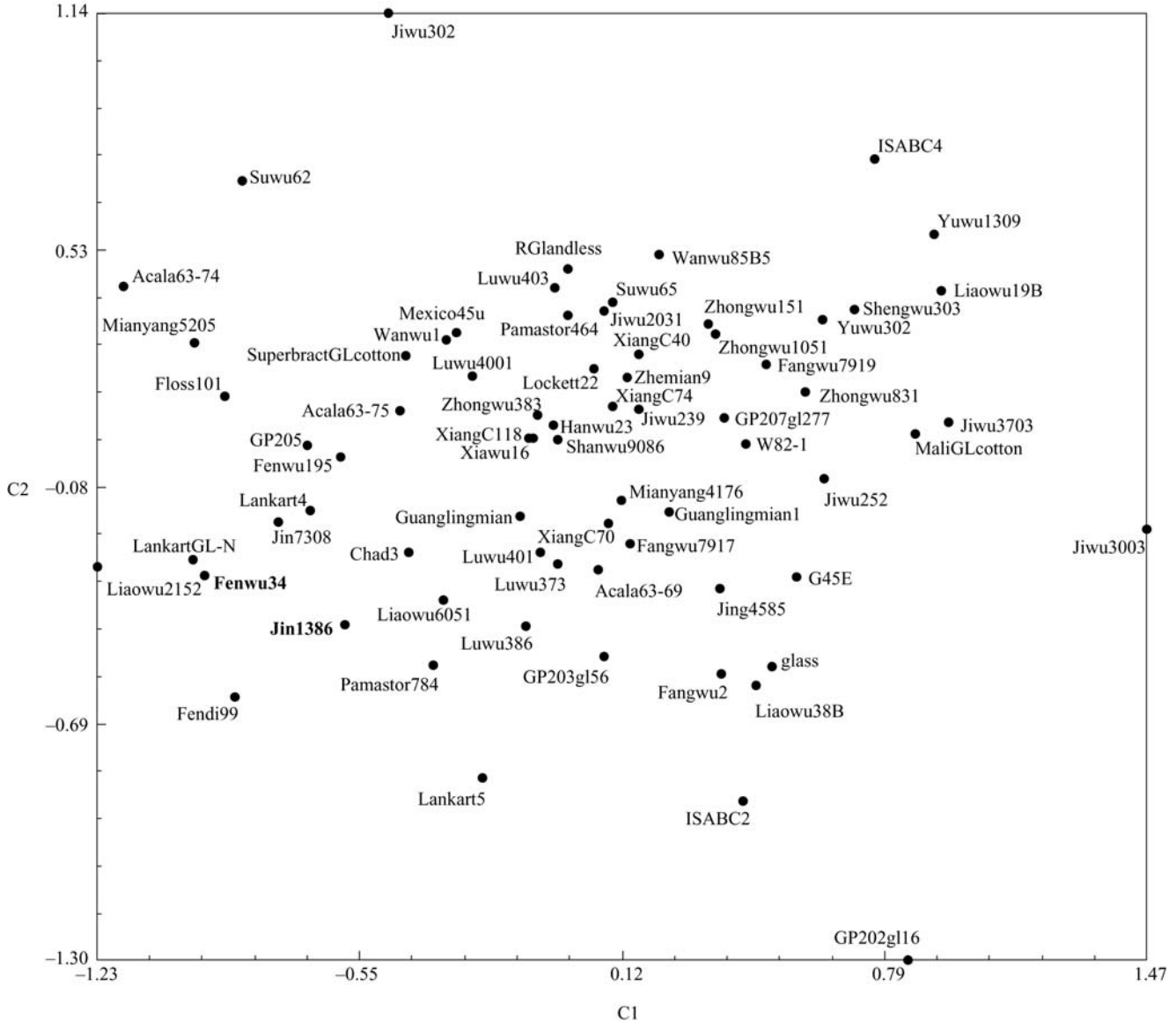

Fig. 1 The plots of the eigenvectors and the projections for outliers

Table 5 The information obtained with 33 SSR primers and 10 AFLP primer combinations in 71 glandless cotton germplasm resources

SSR				AFLP			
primer	location	number of alleles	*PIC*	primer combinations	number of polymorphic bands	total number of bands	*PIC*
CIR060	Chr.23	3	0.56	M50(-CAT)-E36(-ACC)	50	64	0.27
CIR063	Chr.20	2	0.29	M50(-CAT)-E38(-ACT)	48	63	0.27
CIR183	Chr.22	8	0.86	M59(-CTA)-E33(-AAG)	33	44	0.20
CIR234	Chr.15 unl-D03	12	0.90	M59(-CTA)-E35(-ACA)	7	25	0.12
CIR251	Chr.17	6	0.76	M59(-CTA)-E37(-ACG)	8	17	0.08
CIR291	A01, A02, Chr.4, Chr.6, Chr.9, Chr.10	16	0.90	M59(-CTA)-E40(-AGC)	15	31	0.15
				M59(-CTA)-E41(-AGG)	25	39	0.18
CIR013	D02	6	0.76	M61(-CTG)-E32(-AAC)	33	55	0.24
TMHB29-K1	–	10	0.88	M61(-CTG)-E41(-AGG)	7	27	0.13
TMHB82-B11	–	12	0.92	M62(-CTT)-E40(-AGC)	6	24	0.12
TMHC2-G8	–	3	0.67				
TMHC7-N5	–	3	0.54				
CML60	7Lo	2	0.5				
BNL840	Chr.26	6	0.71				
BNL1053	Chr.03	20	0.94				
BNL1317	Chr.9	11	0.90				
BNL1414	9Lo	18	0.93				
BNL1721	Chr.18	12	0.89				
BNL2572	Chr.04	9	0.82				
BNL2634	Chr.16	9	0.87				
BNL3255	5sh	8	0.85				
BNL3408	3sh17Lo	10	0.82				
BNL3449	Chr.16	2	0.21				
BNL3479	18Lo	16	0.93				
BNL3563	10Lo	6	0.83				
BNL3599	12sh	17	0.93				
BNL3627	LGA02/Chr.5	6	0.83				
BNL3649	LGA03/Chr.16	22	0.95				
BNL530	Chr.4	11	0.90				
BNL1551	Chr.16	15	0.92				
BNL686	BNLTM1X3-79LG21/Chr.9	15	0.91				
BNL2440	Chr.01	9	0.86				
BNL3482	Chr.20/26Lo	11	0.86				
BNL3816	12sh	13	0.89	total	232	389	
total		329		mean	23.2	38.9	0.18
mean	10 (alleles)		0.80				
AEI	10			*AEI*	23.2		

3.3 Cluster analysis

Based on the nine agronomic traits above, 71 glandless cotton germplasm resources were clustered with the DIST coefficient. The coefficients ranged from 0.373 ('Shanwu9086' and 'Mianyang4176') to 3.164 ('Gp202gl16' and 'Jiwu302') (results omitted). 'GP202gl16' and 'Mexico45u' were found to be highly distinct from the rest of the materials in the dendrogram. The rest of the glandless cotton germplasm resources could be separated into two groups with the DIST coefficient of 1.58. 'Mianyang4176' and 'Shanwu9086' had a high level of similarity (Fig. 2).

The dendrogram of 71 glandless cotton accessions based on SSR & AFLP data was generated with the DICE coefficient ranging from 0.786 ('Fenwu34' and 'Mianyang4176') to 0.948 ('Liaowu2152' and 'Pamastor464') (results omitted). The results showed that 'Mianyang4176' was quite distinct from other materials and that 'Pamastor464' and 'Liaowu2152' had a high genetic similarity. The rest of glandless cotton accessions could be differentiated based on SSR & AFLP data. These materials, however, could not be obviously divided into several unattached groups (Fig. 3).

The correlation between the DIST distance matrix based on these agronomic traits and the DICE distance matrix from SSR & AFLP data was found to be non-significant ($r = 0.092$, $P = 0.86$).

4 Discussion

Analysis on genetic diversity of the glandless cotton germplasm resources was important for parental selection of a breeding program, construction of core germplasms and

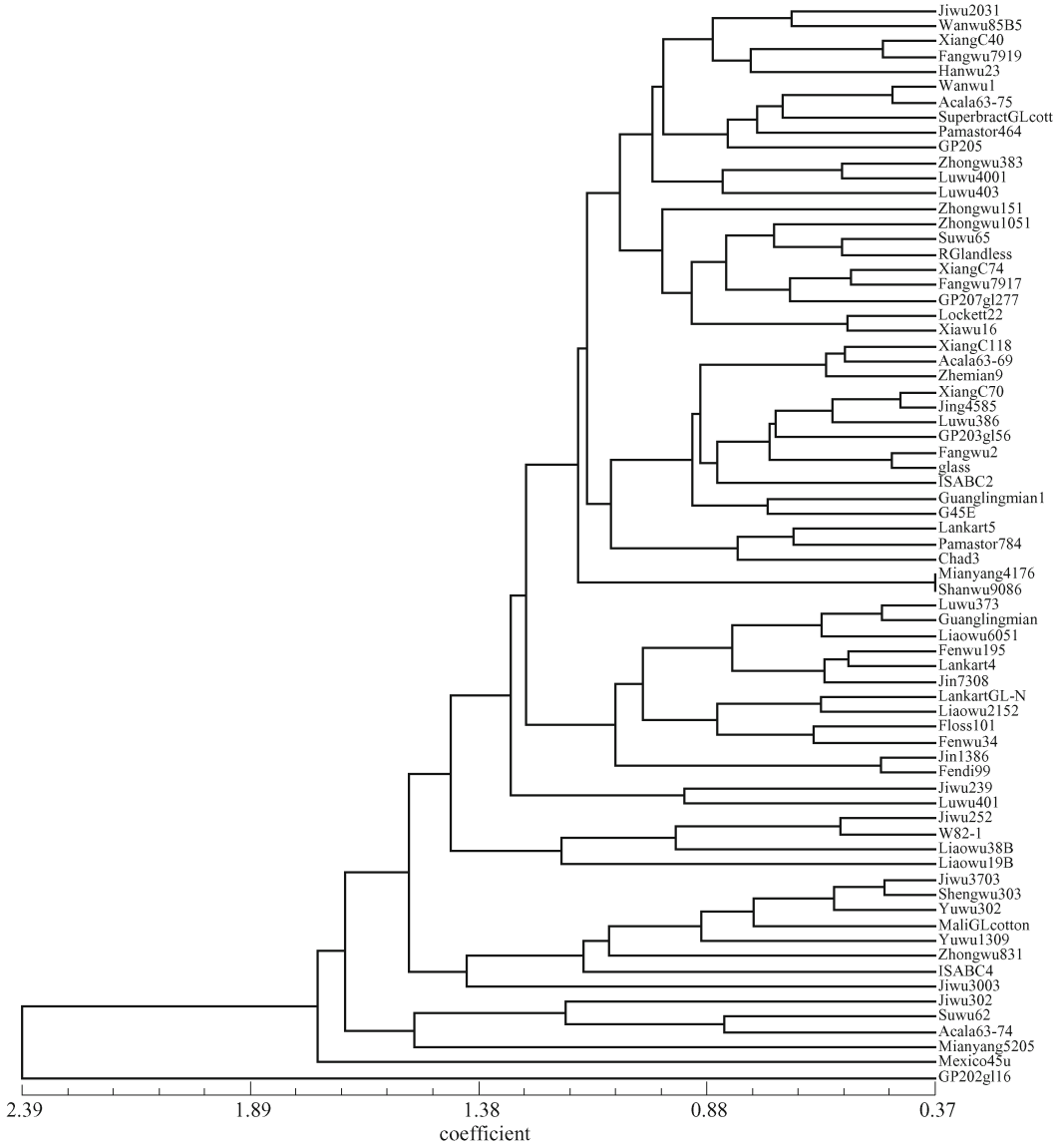

Fig. 2 Dendrogram of 71 glandless cotton germplasm resources derived by using UPGMA from the agronomic characters data

exploitation of heterosis. In our study, the PCA of 71 glandless cotton germplasm resources showed that the first six PCs explained a total of 86.352% of the phenotypic variation. A total of 329 alleles were generated in SSR analysis, and 232 polymorphic bands were obtained by using AFLP primer combinations. Cluster analysis based on agronomic traits showed that all the genetic resources were divided into two groups with the DIST coefficient of 1.58, except for 'GP202gl16' and 'Mexico45u'. Moreover, the glandless cotton germplasm resources could be differentiated based on SSR & AFLP data in this research. The extent of agreement between dendrograms derived from these agronomic traits and SSR & AFLP data was found to be 0.092, which indicated that there was no consistency for the two data types. The result was similar to the previous report on glanded upland cotton (Rana et al., 2005).

For the analysis based on two kinds of molecular marker techniques, SSR revealed the difference of alleles, which reflected the dissimilarity number of simple sequence repeats in glandless cotton genomes. By contrast, AFLP represented the difference of digested sites between two kinds of restriction endonucleases in the whole genome (Vos et al., 1995). Thus, the combination of a co-dominant SSR marker and dominant AFLP marker could completely reveal the genetic diversity of the glandless cotton germplasm resources.

Some genetic resources such as 'Luwu4001' and 'Luwu403' were clustered in the dendrogram based on agronomic traits and SSR & AFLP data. It suggested that they might share similar genetic backgrounds. However, for most of glandless cotton germplasm resources, the dendrogram based on agronomic traits was inconsistent with that based on SSR & AFLP data. The Mantel's test

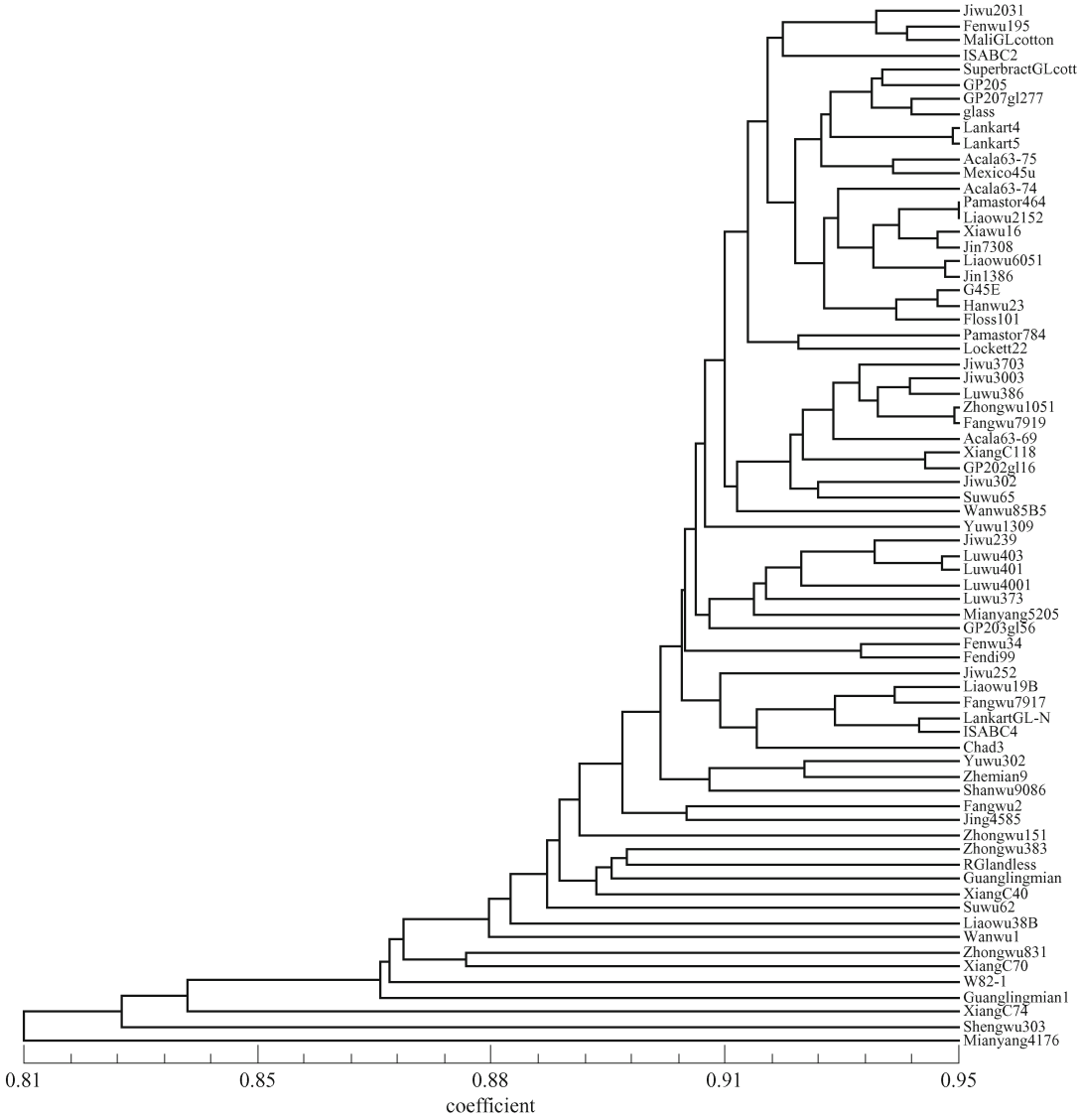

Fig. 3 Dendrogram of 71 glandless cotton germplasm resources derived by using UPGMA from the SSR&AFLP data

(0.092) further proved the poor fit of these two data types. Although 71 glandless cottons could not be obviously divided into several unattached groups, some valuable information about the genetic relationship of some germplasms was obtained from the dendrogram based on SSR & AFLP data. For example, 'Luwu401' and 'Luwu403', 'Lankart4' and 'Lankart5' and 'Fenwu34' and 'Fendi99' were clustered together according to the geographic sources. However, multitudinous glandless cotton germplasm resources could not be clustered together in the dendrogram according to the geographic sources. This indicated that breeders might introduce some heterogeneity materials from other breeding centers in the breeding program.

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