Genetic diversity and population structure of cotton (Gossypium hirsutum L. race latifolium H.) using microsatellite markers

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Cotton (Gossypium spp.) is the world’s leading natural fiber crop and is cultivated in diverse temperate and tropical areas. In this sense, molecular markers are important tools for polymorphism identification in genetic diversity analyses. The objective of this study was to evaluate genetic diversity and population structure in tetraploid cotton (Gossypium hirsutum L. race latifolium H.) by means of microsatellite markers. 43 cultivars and inbred lines from Africa, United States and Brazil were analyzed. From a total of 33 SSRs markers, 15 markers revealed 104 polymorphic SSR alleles. Four groups were identified applying different methods (the probabilistic method, Principal Coordinates Analysis and Neighbor Joining tree). American cultivars and inbred lines were included in group I; African cultivars in group II; and Brazilian cultivars in groups II, III and IV. The FST index indicated high genetic variability among the cultivars and inbred lines studied. In general, American cultivars were the most divergent compared to African and Brazilian ones. The dissimilarity index ranged from 0.06 to 0.90 and the lowest genetic divergence was observed between TAMCOT22 and TAM96WD-69s(L). Combination of American cultivars and inbred lines with African and Brazilian cultivars is recommended for obtaining superior segregant in order to improve yield.

Key words: Dissimilarity index, Gossypium hirsutum L., polymorphism, SSRs markers.

INTRODUCTION

The genus Gossypium includes approximately 50 species distributed worldwide, from which five are tetraploids and belong to subgenus Viz karpas (Cronn and Wendel, 2004). Diploid species with 26 chromosomes are divided into eight cytogenetic genomic groups denominated from A to G, and K; whereas tetraploids species with 52 chromosomes are established in group AD (Fryxell, 1984; Wendel and Cronn, 2003; Cronn and Wendel, 2004; Lapace et al., 2007). In cotton industrialization, 1 kg of fiber can be obtained from about 1.65 kg of seed with 21% oil and 23% proteins. This makes cotton the fifth crop for oil production and second for protein source in the world (Wallace et al., 2008; Benbouza et al., 2010). There are 60 countries around the world that cultivate cotton in 34 million hectares. The main areas of production belong to Brazil (1,338 kg.ha⁻¹), United States of America (985 Kg.ha⁻¹), India (550 kg.ha⁻¹), China:
(1,265 kg.ha⁻¹); Mexico (1,247 kg.ha⁻¹); Australia (2,000 kg.ha⁻¹); (Fengguo et al., 2007; Beltrão and Azevedo, 2008; Khadi et al., 2010). Seven races had been described by Hutchinson (1951), six with different stages of domestication “marie-galante”, “punctatum”, “richmondi”, “morrilli”, “palmeri” and “latifolium” and a single wild race “yucaatanense” (Lacape et al., 2007).

In cotton breeding programs, genetic dissimilarity analysis is one strategy for parents’ selection. However, the use of morphological traits has been limited due to the restricted number of markers, and the existence of environment and plant development influence (Lukonge et al., 2007). Biochemical markers like storage proteins and isoenzymes were also used to study genetic diversity in the genus *Gossypium* (Wendel et al., 1992; Brubaker and Wendel, 1994). With the advent of molecular marker technology, it became possible to develop multiple strategies to identify polymorphism. Several studies applying different molecular markers have been carried out including amplified fragment length polymorphisms (AFLPs) (Pillay and Myers, 1999; Abdalla et al., 2001; Lukonge et al., 2007; Iqbal et al., 2001; Zhang et al., 2008; Rakshit et al., 2010), random amplification of polymorphic DNA (RAPD) (Khan et al., 2000; Zhang et al., 2008; Maleia et al., 2010a), restriction fragment length polymorphisms (RFLPs) (Brubaker and Wendel, 1994; Zhang et al., 2008) and simple sequence repeats (SSR) (Gupta and Varshney, 2000; Zhang et al., 2008; Rakshit et al., 2010). Of these markers, SSR was the most outstanding in recent years.

SSR or microsatellites are abundant across genomes, showing high levels of polymorphism. SSRs are co-dominant markers present in coding and noncoding regions distributed throughout the nuclear genome (Collard et al., 2005; Agarwal, 2008; Lin et al., 2010; Kalia et al., 2011).

There are few studies regarding cotton population structure and genetic diversity in Brazil and Africa, especially in Mozambique, using molecular markers. Some research groups have conducted genetic analyses of cultivars from these locations, but no comparison among locations has been carried out (Lacape et al., 2007; Lukonge et al., 2007; Maleia et al., 2010a). Therefore, the objective of this study was to evaluate the genetic diversity and population structure of cotton cultivars and inbred lines from Mozambique and Brazil, using microsatellite markers.

**DNA extraction and quantification**

Genomic DNA was extracted from fresh young leaves, from four days seedlings (Zhang and Stewart, 2000) of each cultivar and inbred line (Table 1). After collection, leaves were immediately placed in eppendorf tubes and maintained in liquid nitrogen to preserve the DNA. DNA concentration was measured by a Fluorometer QuBit™ and samples were diluted with buffer TE 0.1X to a concentration of 30 ng μl⁻¹ and stored at -20°C.

**SSR genotyping**

For the genetic divergence assay, 33 SSR primers were obtained from *Invitrogen-Induslab*, but only 15 were selected since they considered the most polymorphic ones and also because they presented well defined bands (Table 2).

Amplification reactions were conducted in a total volume of 20 μL which contained: 10 mM Tris HCl, pH 8.3; 50 mM KCl; 0.1% Triton X-100; 0.2 mM de dNTP; 1 U Taq DNA polymerase; 4 mM of each primers; MgCl₂ in a concentration compatible to the primer pair; 30 ng genomic DNA; and ultra pure water. Polymorphism was verified comparing the amplified products on Applied Biosystems (AB), and the equipment of Veritti™ following Williams et al. (1990)’s protocol.

The BNL primers amplification was performed using initial desnaturation of 95°C by 12 min, followed by 30 cycles of 93°C by 1 min, annealing at 51 to 55°C by 2 min and extension of 72°C by 3 min. The last cycle was followed by extension of 72°C by 7 min. The NAU and JESP primers amplifications were performed using a initial denaturation of 94°C by 5 min, followed by 35 cycles of 94°C by 30 seg, annealing at 51 to 53°C by 1 min and an extension of 72°C by 1 min. The last cycle was followed by final extension of 72°C by 8 min.

**Statistical analysis**

The software LAB IMAGE 1D, version 1.10, Locus Biotecnologia™ was used to genotype the samples. The higher allelic frequency per locus, the number of alleles per locus, the average of alleles per locus, the average of high allelic frequencies and genetic diversity per locus were obtained using the software Powermarker 3.25 (Liu and Muse, 2005). Population structure analysis of cotton cultivars and inbred lines was conducted using the software Structure 2.3.3. (Pritchard et al., 2000). Cotton variability was also structured using GenAlEx 6.3. (Peakall and Smouse, 2006), which provided a genetic distance matrix used for Principal Coordinates analysis. In addition, genetic diversity among cultivars/inbred lines was also studied by the construction of a Neighbour Joining tree based on C.S. Chord distance (Cavalli-Sforza and Edwards, 1967).

**RESULTS AND DISCUSSION**

A total of 104 alleles were detected analyzing 35 cultivars and eight inbred lines of *G. hirsutum* L. by means of 15 SSR primers. Most of them were polymorphic among the
materials analyzed. The maximum number of alleles found was 10 and corresponded to loci JESPR292, BNL-1694 and JESPR152, with an average of 6.9 alleles per locus (Table 3). The higher allelic frequency values ranged from 0.326 for locus JESPR152 to 0.774 for locus BNL3816, with a mean of 0.453 (Table 3). Similar results were obtained by Liu et al. (2000) who reported an average of five alleles per locus, ranging from 2 to 11 alleles, evaluating 97 cultivars and primitive species of *Gossypium* using 62 SSR markers. The population structure analysis applying the Probabilistic method described by Pritchard et al. (2000) showed the existence of four distinguished groups.

The software Structure was run a single time for each K value ranging from K=2 to K=15. Each run was performed using the admixture model and 10,000 replicates for burn-in and 100,000 during the analysis. The K=5 analysis was of particular interest to identify four groups that consisted of American cultivars and inbred lines, African and Brazilian cultivars, BRS Brazilian cultivars and FM Brazilian cultivars (Figure 1). Structure harvester (Earl, 2010) was used to determine the optimal value for K which resulted to be K=5 (Figure 1). Maleia et al. (2010a), using RAPD markers, found a similar population structure in relation to American cultivar/inbred lines and African cultivars. The principal Coordinates Analysis also revealed the existence of four groups (Figure 2). Populations 1 and 2 corresponded to American cultivars and African and Brazilian cultivars, respectively, while populations 3, 4 and 5 corresponded to Brazilian cultivars.

On the other hand, Lacape et al. (2007) found an average of 5.6 alleles per locus (from 2 to 17), while analyzing 47 accessions of *Gossypium* with SSR markers.

In this study, microsatellite markers exhibited PIC values that varied from 0.367 to 0.771, with a mean value of 0.646. The locus with higher diversity among cultivars and inbred lines was JESPR152 with a value of 0.797, followed by loci BNL1053 and BNL4035 with values of 0.770 and 0.753, respectively; whereas the locus with the lowest genetic diversity was BNL3816 with 0.409 (Table 3). These values are higher than those found by Liu et al. (2000), while testing a collection of 97 cultivars and primitive species of *Gossypium* through 62 SSR markers that obtained PIC values from 0.05 to 0.82 (means of 0.31). In other research, Lacape et al. (2007), evaluating 47 accessions of *Gossypium*, obtained PIC values from 0.08 to 0.89 (mean of 0.55), while Lukonge et al. (2007), evaluating 26 cotton cultivars using AFLP markers, found values between 0.35 and 0.57 (mean of 0.47). This aspect showed a high genetic diversity among the
### Table 2. List of SSR primers utilized in polymerase chain reaction (PCR) amplification and genomic DNA analysis.

<table>
<thead>
<tr>
<th>Number of order</th>
<th>SSR marker</th>
<th>Motif</th>
<th>Nucleotides sequence (5’-3’)</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BNL1694</td>
<td>(AG)19, (TC)19</td>
<td>GTTTGTTTTTCGTGTAACAGG TGTTGGATTCAATCCAAAG</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>2</td>
<td>BNL3408</td>
<td>(GT)2AT(GT)12</td>
<td>ATCCAAACCATTGACCACT GTTACGTTGAGAAGTACATCTGC</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>3</td>
<td>BNL2495</td>
<td>(AG)14, (TC)14</td>
<td>ACCGGCCATTACTGGAACAAAG AATGGAAATTTGAACCACGTCA</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>4</td>
<td>BNL2572</td>
<td>(GA)23</td>
<td>GTCCTTATTACTAAAAATGTATTAGCC CGATGGTTAATCAATCGGTCA</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>5</td>
<td>BNL1053</td>
<td>(AC)16</td>
<td>AGGGTCTGTATGTGTTGAG CATGCATGCTAGCTGTTA</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>6</td>
<td>BNL3649</td>
<td>(TC)20</td>
<td>GCAAAACGAGTGGACACCAT CCTGTTTCTACAGCGTTC</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>7</td>
<td>BNL2543</td>
<td>(AG)11</td>
<td>ACCGGCCATTACTGGAACAAAG AATGGAAATTTGAACCACGTCA</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>8</td>
<td>BNL2494</td>
<td>(AG)14, (TC)14</td>
<td>ATCGATCATCGTGTTCC</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>9</td>
<td>BNL3816</td>
<td>(TG)15, (TG)5TA(TG)15</td>
<td>GTTAGGCCACGTTGTTCTATG ATCGATCATCGTGTTCC</td>
<td>Forward</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>10</td>
<td>BNL3904</td>
<td>(GT)11</td>
<td>ATGCATTAATGAGTCATAGGC GCACAAACAAAACACGTTTACG</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>11</td>
<td>BNL3998</td>
<td>(AC)11</td>
<td>CGCCGAAGAAGTGGAAAGATC TGTCGAAATGGGTGGTAA</td>
<td>Forward</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>12</td>
<td>BNL4035</td>
<td>(TG)10</td>
<td>TCCTTTCAGCTTGGGTGAATCC</td>
<td>Forward</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>13</td>
<td>NAU864</td>
<td>(TG)10</td>
<td>GGATTAAATAGCCCCCACCACAT TCTTTTCAGCTTGTTTCT</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>14</td>
<td>JESPR292</td>
<td>(CTT)7</td>
<td>GCTTGCAATCTCTTACACC GAATATGTTTACATGAGGCC</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>15</td>
<td>JESPR152</td>
<td>(GAA)50</td>
<td>GATGCACAGATCTTTTTATTAG GGTACATCGGAATCATCAGTG</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
</tbody>
</table>

Source: Cotton Marker Database (2010).

The American cultivars and inbred lines were placed in very distinguished groups from the African and Brazilian cultivars according to the First Principal Coordinate (34.51%). The African and some Brazilian cultivars were in separate groups according to the Second Principal Coordinate that represented 20.35% of the sample variability. Brazilian cultivars BRS and Brazilian cultivars FM formed isolated groups. The first two Principal Coordinates represented 54.86% of the total variation of the sample.

The $F_{ST}$ value according to Wright's method was 0.236 showing a high genetic variability among the cultivars and inbred lines evaluated. Population subdivision determined
Table 3. Estimated indexes of genetic diversity per microsatellite locus evaluated.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele number</th>
<th>Haf</th>
<th>GD</th>
<th>PIC</th>
<th>Minimum (bp)</th>
<th>Maximum (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNL-3408</td>
<td>5</td>
<td>0.395</td>
<td>0.728</td>
<td>0.685</td>
<td>136</td>
<td>214</td>
</tr>
<tr>
<td>BNL-3816</td>
<td>4</td>
<td>0.743</td>
<td>0.409</td>
<td>0.367</td>
<td>116</td>
<td>223</td>
</tr>
<tr>
<td>BNL-3904</td>
<td>4</td>
<td>0.465</td>
<td>0.613</td>
<td>0.535</td>
<td>154</td>
<td>187</td>
</tr>
<tr>
<td>BNL-4035</td>
<td>7</td>
<td>0.326</td>
<td>0.753</td>
<td>0.712</td>
<td>135</td>
<td>213</td>
</tr>
<tr>
<td>BNL-3649</td>
<td>8</td>
<td>0.419</td>
<td>0.74</td>
<td>0.706</td>
<td>80</td>
<td>178</td>
</tr>
<tr>
<td>BNL-2544</td>
<td>5</td>
<td>0.512</td>
<td>0.671</td>
<td>0.632</td>
<td>195</td>
<td>297</td>
</tr>
<tr>
<td>BNL-1053</td>
<td>7</td>
<td>0.372</td>
<td>0.77</td>
<td>0.739</td>
<td>164</td>
<td>297</td>
</tr>
<tr>
<td>NAU-864</td>
<td>6</td>
<td>0.465</td>
<td>0.67</td>
<td>0.614</td>
<td>139</td>
<td>192</td>
</tr>
<tr>
<td>JESPR-292</td>
<td>10</td>
<td>0.419</td>
<td>0.708</td>
<td>0.665</td>
<td>161</td>
<td>206</td>
</tr>
<tr>
<td>BNL-1694</td>
<td>10</td>
<td>0.432</td>
<td>0.737</td>
<td>0.707</td>
<td>114</td>
<td>235</td>
</tr>
<tr>
<td>BNL-2572</td>
<td>8</td>
<td>0.432</td>
<td>0.707</td>
<td>0.666</td>
<td>219</td>
<td>291</td>
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<tr>
<td>BNL-3998</td>
<td>4</td>
<td>0.512</td>
<td>0.645</td>
<td>0.592</td>
<td>118</td>
<td>156</td>
</tr>
<tr>
<td>JESPR-152</td>
<td>10</td>
<td>0.326</td>
<td>0.797</td>
<td>0.771</td>
<td>110</td>
<td>198</td>
</tr>
<tr>
<td>BNL-2495</td>
<td>9</td>
<td>0.432</td>
<td>0.715</td>
<td>0.677</td>
<td>123</td>
<td>214</td>
</tr>
<tr>
<td>BNL-2494</td>
<td>7</td>
<td>0.512</td>
<td>0.664</td>
<td>0.623</td>
<td>107</td>
<td>269</td>
</tr>
<tr>
<td>Means</td>
<td>6.9</td>
<td>0.453</td>
<td>0.688</td>
<td>0.646</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Haf=Higher allelic frequency; GD=genetic diversity; PIC=polymorphism information content, allele size (bp) values.

Figure 1. Population structure of cotton cultivars and inbred lines. K=5 groups obtained using software Structure 2.3.3. (Pritchard et al., 2000).

by structure analysis was coincident with the clusters observed in the Neighbor-joining tree generated based on CS Chord genetic distance (Cavalli-Sforza and Edwards, 1967) (Figure 3). Cultivars and inbred lines from USA (cluster I) were divergent from cultivars from Africa (cluster III) and Brazil (clusters II, III and IV). Most of the American cultivars and inbred lines have common ancestors as DP 90, DP 50 and other unrealized inbred lines (Thaxter and Smith, 2005; Wallace et al., 2008). Divergence between American and African cultivars was also reported by Maleia et al. (2010a) while evaluating the genetic divergence of 21 cultivars and inbred lines, using RAPD markers.

All the groups identified applying different methods were consistent with pedigree data. African and Brazilian cultivars share ancestors in their pedigrees (cultivars DP61, Sicala and Deltapine) (Bertini et al., 2006). Cultivar Sicala originated from CSIRO cultivars selected from DP16; this cultivar is one of the genitors of CA 324 and belongs to African groups. While, cultivars ISA 205 and Stam-42, both from the African group originated from cultivar Deltapine (Dessauw [1]). The highest dissimilarity Cs Chord index between the materials analyzed was 0.9, and corresponded to TAMCOT 22, TAM 96WD-69s, TAMCOT Pyramid, TAM 98D-102 (L), TAM 96WD-18 (L), TAM 94J-3 (L), TAM 94J-3 (L), TAMCOT Sphinx, TAM 98D-99ne (L), TAM 94WE-37s (L), TAM 94L-25 (L), FM 993, and FM 966. These cultivars tend to promote heterosis effects when used in cotton breeding programs (Falconer and Mackay, 1996). Similar results, in relation to dissimilarity indexes were obtained by Bertini et al. (2006), while evaluating a sample of 53 cultivars using SSR markers.

Figure 2. Population structure between cotton cultivars and inbred lines by Principal Coordinates analysis (PC1 vs PC2). Pop1= USA Cultivars and inbred lines; Pop 2=African cultivars; Pop 3= Brazilian cultivars; Pop 4= Brazilian (BRS and one FMT); Pop 5= Brazilian cultivars (FM).

Figure 3. Distribution of 43 cotton cultivars and inbred lines according to Neighbor-Joining tree composing four groups. Each main branch is in agreement with K=5 formed groups by Structure software.

For Cotton Breeding Programs in Africa, particularly in Mozambique, hybridizations between TAMCOT 22 x Albar sz 9314, TAM 98D-99ne (L) x Albar FQ 902, TAM 96WD-69s (L) x FMT 705, TAMCOT Pyramid x CA-222, TAMCOT Pyramid x Albar BC 853, TAMCOT Sphinx x Stam-42, TAM 96WD-69s (L) x IRMA 12-43, TAMCOT Sphinx x ISA-205, TAMCOT 22 x REMU-40, TAMCOT Sphinx x CD 404, TAMCOT Pyramid x FM 993, FM 966 x
BR3 293, TAMCOT 22 x BRS Buriti, TAMCOT 22 x CA-324 would be the most promising and recommended combinations. Cultivars Albar sz 9314, Albar FO 902, Albar BC 853, CA-222, IRMA 12-43, ISA-205 and REMU-40 show wide adaptability and high tolerance to jassids (Empoasca facialis). Moreover, CA324 and Stam42 demonstrated specific adaptability to high and low quality environments and mean and low tolerance to jassids, respectively (Maleia et al., 2010). In addition, TAMCOT Sphinx showed a high resistance to nematodes (Rotylenchus reniformis) (El-Zik and Thaxton, 1996) and the inbred line TAM 96WD-69s (L) is pilose, facilitating mechanized harvest. TAMCOT Pyramid is a cultivar that originated from the cross between TAMCOT Sphinx and CD3HGCBU8S-1-91 (unreleased inbred line), and shows improved characteristics as high yield, moderate pilosity, good fiber quality and high adaptability to diverse environments in Texas (Thaxton and Smith 2005; Wallace et al., 2008). TAMCOT 22 originated from crosses involving TAM 88G-104 is a cultivar with high yield and medium cycle and has high resistance to whitefly (Bemisia argentifolii). This inbred line has high-yielding and excellent fiber properties (Thaxton and Smith, 2005; Wallace et al., 2008).

Conclusions

A great variability was observed among the 43 cotton cultivars and inbred lines (Gossypium hirsutum) analyzed. The cultivars from USA were more divergent in relation to cultivars from Brazil and Africa as revealed by the probabilistic method, Principal Coordinates Analysis and Neighbor-Joining tree. Also, some Brazilian cultivars (BR and FM) showed low genetic similarity with other cultivars analyzed.

For Cotton Breeding Programs in Africa, particularly in Mozambique, hybrid combinations between African and Brazilian cultivars with USA cultivars/inbred lines could be applied to acquire high productivity.

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