Genetic diversity among *Hymenaea courbaril* L. genotypes naturally occurring in the north of Mato Grosso State, Brazil

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**ABSTRACT.** *Hymenaea courbaril* L. is a tropical tree species with economic and medicinal potential largely used in programs for the recovery of degraded areas. The use of molecular markers in genetic studies can
help in the definition of forest restoration strategies. This study was conducted to analyze the genetic diversity among *H. courbaril* (‘jatobá’) genotypes naturally occurring in the north of Mato Grosso State, Brazil, using ISSR markers, aiming to contribute to the development of seed collection and seedling production strategies. Twenty-four *H. courbaril* individuals were sampled by collecting leaves for DNA extraction by the CTAB method. The individuals were genotyped using 14 ISSR primers, resulting in the amplification of 87 bands in total, 65 of which were polymorphic. The ISSR markers presented average polymorphism information content of 0.44. Dissimilarity values based on the Jaccard coefficient ranged from 0.12 to 0.52. The most divergent individuals were 3 and 22, whereas 14 and 19 were the most genetically similar. The UPGMA and Tocher clustering methods indicated the formation of four distinct groups, revealing genetic variability among the 24 *H. courbaril* genotypes. Therefore, all the evaluated genotypes can be used for seed collection and seedling production, but the most divergent individuals should be prioritized.

**Key words:** Jatobá; ISSR; Seed collection; Reforestation

**INTRODUCTION**

*Hymenaea courbaril* L. (Leguminosae) is a tropical tree species popularly known as ‘jatobá’ that occurs naturally from the south of Mexico to South America, including Brazil, the French Guiana, Suriname, Guiana, Venezuela, Colombia, Peru, and Bolivia. In Brazil, it is present from the north to the southeast regions (Melo and Mendes, 2005). The wide geographic distribution of this species indicates tolerance and adaptation to different environments; for this reason, it is recommended for programs of recovery of degraded forest areas (Oliveira et al., 2011).

Timber is the most commercialized product from jatobá, used in the manufacture of furniture and construction. The bark of its trunk and its fruit are used in the combat against the flu, bronchitis, anemia, and prostate diseases (Marinho et al., 2011). Rural populations in regions where this plant occurs consume the farinaceous pulp that coats its seeds, which is a highly nutritious edible product (Lorenzi and Matos, 2002).

With the advance of agricultural frontiers, logging, construction of hydroelectric plants, and real estate speculation, Brazilian forest formations have undergone considerable levels of disturbance, which may lead to loss and genetic erosion of forest species like jatobá. As a result, initiatives for the execution of preservation projects and sustainable use and recovery of degraded pastures are growing, especially those involving the use of native species (Pacheco et al., 2013).

Knowledge of genetics and ecology in natural populations of tropical tree species are essential for the development of activities defining the size of reserves, adequate management of species, recovery of degraded areas, and collection of seeds for the planting of native species (Kageyama et al., 2003).

For the restoration of forests, it is important to maintain the genetic diversity found in natural populations to ensure the conservation of new populations inserted in the reforested area (Gois et al., 2014). The success of programs for the recovery of areas depends on the
genetic potential of the material used in reforestation (Sebbenn, 2002). Therefore, genetic studies should be carried out with native plants employed in reforestation, since they help to identify mother trees with the greater genetic variability that can supply seeds for seedling production programs.

Various molecular techniques are available for the analysis and detection of genetic variability at DNA level. Among them are the ISSR (inter-simple sequence repeats) molecular markers, which can be used in the discrimination and molecular characterization of individuals. These markers are genomic regions of 100 to 3000 bp amplified via polymerase chain reaction using a single primer pair (16-20 bp); they are developed from microsatellite sequences (Faleiro, 2007). Some outstanding advantages of the ISSR technique are its low cost, high polymorphism, and the fact that it does not require previous information on the DNA sequence of the organism under study (Santana et al., 2011).

Given the potential use of jatobá for the recovery of degraded pasture, we developed this study to analyze the genetic diversity among *H. courbaril* (jatobá) genotypes occurring naturally in the north of Mato Grosso State, Brazil, using ISSR markers, aiming to provide information for the development of seed collection and seedling production strategies.

**MATERIAL AND METHODS**

**Collection of plant material**

Young leaves were collected from 24 *H. courbaril* individuals naturally occurring in the north region of Mato Grosso State (Brazil), in the municipalities of Sinop, Itaúba, Colíder, and Nova Canãa do Norte (Figure 1). The collected material was identified and packed with silica gel, and later transported to the Laboratory of Plant Genetics and Molecular Biology at the Universidade do Estado do Mato Grosso, where it was stored in a freezer at -20°C until DNA extraction.
Genomic DNA extraction

The DNA was extracted by the CTAB (cetyltrimethylammonium bromide) method described by Doyle and Doyle (1987), with modifications: the polyvinylpyrrolidone concentration was increased from 1 to 2%; CTAB from 2 to 5%; and β-mercaptoethanol from 0.2 to 2%, in addition to reducing the incubation time in water bath at 65°C from 60 to 30 min. The quality of the extracted DNA was evaluated on 1% agarose gel stained with ethidium bromide. For the quantification, the DNA samples were compared with standard DNA (lambda phage).

Selection of ISSR primers and amplification reactions

We tested 30 ISSR primers obtained from the University of British Columbia for DNA amplification. Of these, we selected 14 primers that produced a larger number of clear bands and reproducible polymorphism. Each amplification reaction contained a final volume of 15 μL, consisting of 1 μL DNA (20 ng/μL), 2 μL 5X buffer (GoTaq colorless), 2 μL MgCl₂ (25 mM), 2 μL primer (0.2 μM), 4 μL dNTPs (1 mM of each dNTP), 0.5 μL BSA, 0.15 μL Taq DNA polymerase, and 3.4 μL autoclaved distilled water.

Reactions were conducted in a thermal cycler (MJ 96) under the following amplification conditions: one initial denaturation cycle at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, 50-59°C (temperature of the primer used) for 1 min, 72°C for 3 min, and one final extension at 72°C for 7 min.

The amplification products were separated by electrophoresis in a horizontal unit using 1.5% agarose gel in 1X TBE buffer at a constant voltage of 90 V. After electrophoresis the gels were stained with ethidium bromide (0.6 ng/μL) for 20 min; next, they were visualized under UV transillumination and photo-documented. The sizes of the amplified fragments were estimated by comparison with the 100-bp DNA Ladder molecular marker (Kapa).

Statistical analyses

The ISSR fragments were coded to build a matrix as binary characters to the presence (1) or absence (0) of bands. The bands that showed weak color and low definition were not evaluated. With the binary matrix, we calculated the percentage of polymorphisms obtained per primer and the genetic diversity of the locus or PIC (polymorphism information content), which is an estimate used for the evaluation of the discriminatory power of a locus. To calculate the PIC of each primer, we used the equation proposed by Rezende et al. (2009). The genetic dissimilarity estimate between each pair of individuals was calculated by the Jaccard coefficient, using the arithmetic complement. A cluster analysis of the genotypes was performed by the UPGMA (unweighted pair-group method arithmetic mean), Ward, and nearest neighbor hierarchical methods. To select the hierarchical method with greatest clustering consistency, we calculated the cophenetic correlation coefficient (CCC) between the genetic similarity matrix and the matrix of cophenetic coefficients. A cluster analysis of the genotypes by Tocher’s optimization approach was also performed, using the distance matrix generated by the arithmetic coefficient of the Jaccard index. Analyses were run in the GENES software (Cruz, 2006).

RESULTS AND DISCUSSION

Eighty-seven (87) fragments were generated with the use of the 14 ISSR primers,
Genetic diversity in *Hymenaea courbaril* L.

65 of which were polymorphic (74.71%). The number of amplified bands ranged from two [Di(GA)$_3^3$ YC] to eleven [Di(AG)$_8^3$ C], with an average of 6.21 per primer (Table 1). These results are similar to those found in other tropical tree species. Silva et al. (2017) found 64 polymorphic bands, with an average of 6.97 bands per primer, using 14 ISSR markers in a study of genetic diversity in *Spondias mombin* L. Rivas et al. (2013) characterized the genetic diversity of *Theobroma subincanum* Mart. populations using 13 ISSR primers and obtained 67.20% polymorphism at the species level, with an average of 4.69 bands per primer.

**Table 1.** Inter-simple sequence repeat primers used and respective sequences, annealing temperature (AT), total number of amplified bands (TNB), number of polymorphic bands (NPB), polymorphism percentage (%P), and polymorphism information content (PIC) for 24 *Hymenaea courbaril* genotypes with natural occurrence in the north of the Mato Grosso State.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>AT</th>
<th>TNB</th>
<th>NPB</th>
<th>%P</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di(GA)$_3^3$ C</td>
<td>AGAGAGAGAGAGAGAGGC</td>
<td>50</td>
<td>11</td>
<td>10</td>
<td>90.90</td>
<td>0.56</td>
</tr>
<tr>
<td>Di(CA)$_3^3$ G</td>
<td>CACACACACACACACAG</td>
<td>51</td>
<td>4</td>
<td>4</td>
<td>100.00</td>
<td>0.52</td>
</tr>
<tr>
<td>Tri(ATG)$_6^6$</td>
<td>ATGATGATGATGATGATG</td>
<td>50</td>
<td>6</td>
<td>2</td>
<td>33.00</td>
<td>0.18</td>
</tr>
<tr>
<td>Di(GA)$_3^3$ G</td>
<td>AGAGAGAGAGAGAGAGGG</td>
<td>50</td>
<td>7</td>
<td>5</td>
<td>71.43</td>
<td>0.44</td>
</tr>
<tr>
<td>Di(CA)$_3^3$ A</td>
<td>CACACACACACACACAA</td>
<td>50</td>
<td>5</td>
<td>5</td>
<td>100.00</td>
<td>0.74</td>
</tr>
<tr>
<td>Di(AC)$_6^6$</td>
<td>ACACACACACACACACT</td>
<td>51.4</td>
<td>7</td>
<td>6</td>
<td>85.71</td>
<td>0.83</td>
</tr>
<tr>
<td>Di(CA)$_3^3$ C</td>
<td>ACACACACACACACACCC</td>
<td>50</td>
<td>4</td>
<td>3</td>
<td>75.00</td>
<td>0.36</td>
</tr>
<tr>
<td>Di(GA)$_3^3$ YC</td>
<td>AGAGAGAGAGAGAGGAGACAYYC</td>
<td>50</td>
<td>2</td>
<td>1</td>
<td>50.00</td>
<td>0.22</td>
</tr>
<tr>
<td>Di(AC)$_3^3$ YG</td>
<td>ACACACACACACACACYG</td>
<td>52</td>
<td>5</td>
<td>5</td>
<td>100.00</td>
<td>0.58</td>
</tr>
<tr>
<td>Tri(AC)$_6^6$ DRO</td>
<td>DBRMDACACACACACAL</td>
<td>50</td>
<td>10</td>
<td>8</td>
<td>80.00</td>
<td>0.51</td>
</tr>
<tr>
<td>Di(CA)$_3^3$ CY</td>
<td>CYCACACACACACACACA</td>
<td>58.8</td>
<td>9</td>
<td>5</td>
<td>55.56</td>
<td>0.23</td>
</tr>
<tr>
<td>Tri(AC)$_3^3$ RC</td>
<td>ACACACACACACACARC</td>
<td>50</td>
<td>6</td>
<td>6</td>
<td>100.00</td>
<td>0.70</td>
</tr>
<tr>
<td>Di(GA)$_3^3$ CY</td>
<td>CYGAGAGAGAGAGAGAAGCA</td>
<td>50</td>
<td>6</td>
<td>2</td>
<td>83.00</td>
<td>0.03</td>
</tr>
<tr>
<td>Di(AC)$_8^8$ BDH</td>
<td>HBHACACACACACACA</td>
<td>50</td>
<td>5</td>
<td>3</td>
<td>60.00</td>
<td>0.28</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>6.21</td>
<td>4.64</td>
<td>73.95</td>
<td>0.44</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>87</td>
<td>65</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Y = C or T; B = C, G or T; R = A or G; D = A, G or T.

Among the ISSR markers used, those that generated the largest part of the polymorphism contained CA, AC, and AG repetitions. Reddy et al. (2002) stated that primers with repetitions (AG), (GA), (CT), (TC), (AC), and (CA) present more polymorphism than those with other repeated di-, tri-, or tetranucleotides; this explains the results found here.

The PIC ranged from 0.03 to 0.83, and the primers Di(AC)$_3^3$ T, Di(CA)$_3^3$ A, and Tri (ACA)$_3^3$ RC presented the highest values: 0.83, 0.74, and 0.70, respectively (Table 1). According to Botstein et al. (1980), markers with PIC values higher than 0.5 are considered very informative; those with values between 0.25 and 0.5 are considered moderately informative, and markers with PIC values lower than 0.25 are little informative. In the present study, the average PIC was 0.44, and so we can consider that the ISSR primers were moderately informative to reveal polymorphism in the jatobá individuals; this confirms that the markers utilized were efficient to estimate genetic diversity in *H. courbaril* since ISSR classified as moderately informative have been successfully used in other species, e.g., *Ficus bonjesulapensis* R.M. Castro (Duarte et al., 2015), *Hancornia speciosa* Gomes (Costa et al. 2015), *Mimosa caesalpiniaefolia* Benth. (Araújo et al., 2016), and *Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum (Silva et al., 2016).

The genetic dissimilarity values ranged from 0.12 to 0.52 (Table 2). The genotypes that showed the lowest dissimilarity were 14 and 19, whereas the most divergent were 3 and 22. Rodrigues et al. (2016) evaluated the genetic variability of *Byronima crassifolia* (L.) H.B.K. from the germplasm database of Embrapa Amazônia Oriental and identified dissimilarity

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values between 0.10 and 0.59. Lorenzoni et al. (2014) studied the genetic diversity among *Rollinia mucosa* [Jacq.] Baill. genotypes with ISSR markers and found dissimilarity values ranging from 0.09 to 0.51, indicating high genetic variability among the accessions. We can thus infer that the dissimilarity results found in this study demonstrate the existence of a considerable genetic diversity among the *H. courbaril* genotypes.

<table>
<thead>
<tr>
<th>Distortion (%)</th>
<th>2.17</th>
<th>41.81</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress (%)</td>
<td>14.74</td>
<td>27.60</td>
</tr>
<tr>
<td>CCC</td>
<td>0.75**</td>
<td>0.50**</td>
</tr>
</tbody>
</table>

**Significant at a 1% level, by the t-test.

The dendrogram of genetic dissimilarities based on ISSR, obtained by the UPGMA method, is shown in Figure 2. The analysis allowed for the division of the individuals into four groups, based on a cutoff point of 0.33, according to the criterion proposed by Mojena (1977). Group I is composed of 21 genotypes (14, 19, 10, 15, 23, 11, 18, 20, 17, 4, 1, 21, 6, 7, 8, 16, 5, 12, 2, 9, and 24); groups II, III, and IV are formed by only one genotype each (3, 13, and 22, respectively).
Four groups were formed by the Tocher optimization method (Table 4). Of the 24 genotypes, 20 were allocated to the first group. Individuals 9 and 13 were allocated to the second group. The third and fourth groups were composed of genotypes 3 and 22, respectively.

Results from the Tocher clustering method were consistent with those obtained with UPGMA with similarities in the composition of groups despite the different criteria each method adopts to classify the genotypes. Most (95.24%) of the individuals clustered in the group I by the UPGMA method were also allocated in the group I by the Tocher method. Both methods agreed in allocating individuals 3 and 22 in separate groups. The high agreement between the two methods (UPGMA and Tocher) in the distinction of the genotype groupings provides bases for more certain inferences about genetic diversity among the evaluated individuals. The use of more than one clustering method, given the differences in hierarchization, optimization, and ordering of groups, allows for their classifications to be complementary, as a function of the criteria each method uses, preventing erroneous inferences from being adopted in the allocation of materials within a certain group of genotypes (Arriel et al., 2006). Ramalho et al. (2016) studied genetic diversity in *Bertholletia excelsa* Bonpl. genotypes with the use of ISSR markers and also observed that cluster analysis by the UPGMA and Tocher methods agreed partially.

![Figure 2. Dendrogram of the genetic dissimilarity among 24 *Hymenaea courbaril* trees, using unweighted pair group method with arithmetic mean (UPGMA) method based on the arithmetic complement of Jaccard’s index.](image-url)

Table 4. Clustering of 24 *Hymenaea courbaril* genotypes based on Tocher’s method, using Jaccard’s dissimilarity matrix.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>14, 19, 15, 10, 23, 21, 17, 18, 20, 8, 7, 4, 1, 6, 12, 5, 11, 16, 2, and 24</td>
</tr>
<tr>
<td>II</td>
<td>9 and 13</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
</tr>
<tr>
<td>IV</td>
<td>22</td>
</tr>
</tbody>
</table>

In programs aimed at seedling production for the restoration of degraded areas, information about the genetic diversity of trees that provide seeds is useful. The existence of large genetic distances among *H. courbaril* individuals indicates a strong genetic diversity that can be used for the selection of more divergent genotypes for seed collections in projects.
for the reforestation of degraded areas. The results found in this study can also be used in environmental actions aimed at the conservation and sustainable use of the species since the genetic diversity in natural populations of *H. courbaril* has been affected by the logging activity (Carneiro et al., 2011; Lacerda et al., 2008). Therefore, we recommend the *in situ* conservation of the evaluated genotypes aiming to maintain the genetic variability of the species in the north of Mato Grosso State.

In conclusion, the ISSR markers were efficient to characterize the genetic diversity among the *H. courbaril* genotypes, detecting polymorphism. Both the UPGMA and Tocher methods revealed the formation of four distinct groups, demonstrating that there is genetic dissimilarity among the analyzed genotypes. A collection of seeds for seedling production in programs for the recovery of degraded areas can include all the evaluated individuals, and wherein the most genetically divergent (3 and 22) should be prioritized.

**Conflicts of interest**

The authors declare no conflict of interest.

**ACKNOWLEDGMENTS**

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