Discovery of novel SSR markers from transcriptome data of Astronium fraxinifolium Schott, a threatened tree species in Brazil

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Abstract

Background

*Astronium fraxinifolium* is an endangered tree species from Brazil. Due to its high importance for environmental reforestation, as well as for the use of its wood, it is necessary to implement management programs for conservation of this species. Simple sequence repeats (SSR) or microsatellite markers have been widely used in population genetic studies across diverse organisms. In this study, we reported for the first time SSR markers for *A. fraxinifolium* as well as its frequency and distribution from transcriptome data.

Results

More than 125 thousand RNA-seq sequences derived microsatellites, with predominant distribution of trinucleotides repeats. From the initial screening, we selected 20 microsatellite loci, validated and evaluated genetic indexes in two natural populations. All loci were polymorphic, ranging from four to eleven alleles per locus. The observed and expected heterozygosities ranged from 0 to 1.0 and from 0.533 to 1.0, respectively. Genetic differentiation between populations (= 0.363) showed higher diversity within than among populations.

Conclusions

The developed SSR loci from RNA-seq consists in a base for future studies of genetic diversity and population structure, mating system and gene flow in *A. fraxinifolium* populations as well as related species, aiming the conservation and management of the species.

Background

Forest fragmentation directly impacts the genetic diversity, population structure, mating system and gene flow of tree populations. Population genetic studies based on genetic markers are keys to understand the effects of anthropogenic interventions on natural populations, conservation and improvement of trees species. *Astronium fraxinifolium* Schott (Anacardiaceae) tree occur discontinuously distributed on rocky terrains of different Brazilian biomes, from Cerrado to Caatinga [1, 2]. It is an insect pollinated dioecious tree, used for restoration of degraded areas [2]. Due to the intense fragmentation of its biomes, *A. fraxinifolium* is placing as threatened of extinction and remaining populations are found as isolated trees along highways margins or in small forest fragments [3, 4]. Thus, the development of genetic markers as microsatellite loci (SSR) is urgent to be used as tool for genetic investigation of genetic diversity, population structure, mating system, and gene flow of the remaining species populations.

Genomic studies in *Astronium* genus are scarce. More recently, with the development of high throughput sequencing technologies has increased the development of molecular markers in a broad range of organisms [5, 6]. The search against these datasets has increased the chances of finding SSR without any prior enrichment. From this, the development of SSR markers from transcriptome sequences has become an effective tool for population genetic investigations [7, 8], in special for endangered species [8]. Here, we developed a set of 20 polymorphic microsatellites loci for *A. fraxinifolium* and evaluated its frequency and distribution based on Illumina sequencing from a RNA-Seq data. These loci were then validated to be reproducible in order to assess genetic diversity, mating
system and gene flow in this tree species. We also included an analysis of repeats and GO classification of these reads.

**Results**

**Identification and classification of SSR markers**

In this work, the sequence run produced 189 million of paired-end reads, of which more than 95 million successfully joined reads. The trinucleotides motifs the most abundant followed by di-, hexa-, penta, and tetranucleotide motifs (approximately 41.8, 37.5, 9.8, 7.6, and 3.4%, respectively, Table 1). From these, on average, 32% of the sequences had enough flanking sequence for designing of the primers, except for the tetra- and trinucleotides loci where this was 40.9 and 36.9%, respectively. From an initial screening, more than 125 thousand sequences (redundant sequences; available upon request) were identified with tandem repeats and then we designed twenty primer pairs for amplification and test in a population study.

![Table 1](image)

**Summary of Illumina paired-end sequence data; it includes non- and perfect motifs di-, tri-, tetra-, penta- and hexa-nucleotides for *Astronium fraxinifolium***

| Motif                              | Di-   | Tri-   | Tetra- | Penta-  | Hexa-  |
|------------------------------------|-------|--------|--------|---------|--------|
| Number of contigs containing microsatellites | 146,182 | 163,062 | 13,274 | 29,540  | 38,160 |
| Number of contigs with flanking sequence | 37,389  | 60,331 | 5,434  | 8,852   | 13,202 |

Total number of reads = 189,057,492; All contigs = 95,768,009

The SSR functional annotation was classified under the three major categories: cellular component, molecular function and biological process (Fig. 1). The GO classification related to cellular component showed that the most abundant GO are intracellular (GO:0005622) or intracellular part (GO:0044424). Considering molecular function, the most representative GOs were related to organic cyclic compound binding (GO:0097159) and heterocyclic compound binding (GO:1901363). The biological process category are mainly represented by genes involved in organic substance metabolic process (GO:0071704) and cellular metabolic process (GO:0044237). Considering the tissue analyzed in the transcriptome analysis, the GO classification also show a great number of genes related to cell communication (GO:0007154), anatomical structure development (GO:0048856) and multicellular organism development (GO:0007275) which are consistent with the initial steps of the development, showing a great potential for future studies in these regions.

![Insert Fig. 1](image)

**Figure 1** Functional annotation of SSRs in coding regions for *A. fraxinifolium* transcriptome, including the number of genes putatively involved in different subcellular functions in GO classification.

**Genetic diversity of natural populations**

The use of SSR-derived from RNA-Seq studies increases the success of amplification, including related species, due to its conservation of transcribed regions flanking sites. All microsatellite primer pairs designed were successfully
amplified and polymorphic in the studied populations, being detected between 4 to 11 alleles per locus and ranging from 0.346 to 0.857 (Table 2), which indicate useful markers for population studies.

### Table 2
Results of screening in two populations of *Astronium fraxinifolium*

| Locus | Ilha Solteira \( n = 30 \) | Selvília \( n = 30 \) |
|-------|----------------|----------------|
| Ga01  | 8 0.760 0.933 0.799 -0.171 | - 9 0.754 0.654 0.792 0.177* - 0.757 |
| Ga02  | 11 0.551 0.867 0.626 -0.394 | - 7 0.784 0.808 0.824 0.105 - 0.513 |
| Ga03  | 8 0.346 0.000 0.533 0.094 | - 8 0.375 1.000 1.000 0.465* + 0.088 |
| Ga04  | 11 0.788 0.467 0.818 0.434* | + 10 0.804 0.533 0.836 0.366* + 0.044 |
| Ga05  | 8 0.656 0.367 0.718 0.494* | + 5 0.664 0.296 0.726 0.597* + 0.785 |
| Ga06  | 9 0.755 0.733 0.797 0.081* | - 8 0.712 0.769 0.754 -0.020 - 0.959 |
| Ga07  | 10 0.762 0.800 0.804 0.005 | - 8 0.693 0.630 0.746 0.158* - 0.484 |
| Ga08  | 8 0.840 0.933 0.871 -0.073 | - 8 0.719 0.821 0.769 -0.069 - 0.583 |
| Ga09  | 10 0.805 0.667 0.833 0.202* | + 11 0.857 0.733 0.885 0.174* + 0.337 |
| Ga10  | 7 0.569 0.308 0.654 0.475* | + 4 0.725 0.600 0.785 0.106 - 0.263 |
| Ga11  | 8 0.724 0.800 0.777 -0.030 | - 6 0.785 0.731 0.827 0.159* - 0.058 |
| Ga12  | 8 0.657 0.944 0.730 -0.175 | - 6 0.743 0.800 0.799 0.137* - 0.314 |
| Ga13  | 7 0.756 0.722 0.810 0.124* | - 8 0.725 0.762 0.779 0.089 - 0.194 |
| Ga14  | 9 0.734 0.722 0.794 0.069 | - 8 0.746 0.824 0.804 0.133* - 0.088 |
| Ga15  | 8 0.711 0.826 0.769 0.085 | - 8 0.757 0.500 0.817 0.377* + 0.013 |
| Ga16  | 9 0.580 0.600 0.623 0.038 | - 6 0.761 0.724 0.797 0.093 - 0.693 |
| Ga17  | 5 0.669 0.828 0.721 -0.129 | - 8 0.655 0.567 0.719 0.215* - 0.340 |
| Ga18  | 8 0.690 0.655 0.743 0.125 | - 11 0.815 0.733 0.849 0.138* - 0.149 |
| Ga19  | 4 0.558 0.552 0.634 0.140 | - 7 0.551 0.133 0.606 0.783* + 0.086 |
| Ga20  | 8 0.710 0.759 0.760 0.010 | - 9 0.781 0.733 0.821 0.109 - 0.072 |
| Mean  | 8.2 0.681 0.674 0.741 -0.029 | - 7.5 0.720 0.668 0.797 0.059 - 0.363 |

= number of alleles per locus; = polymorphism information content; = observed heterozygosity; = expected heterozygosity; = fixation index; = null alleles occurrence; = genetic differentiation between populations; P < 0.05

Insert Table 2

Therefore, despite the history of fragmentation of the *A. fraxinifolium* populations, the SSR loci showed a large amount of genetic variation: the observed heterozygosity \( H_o \) ranged from 0 to 0.944 (mean of 0.674) and expected heterozygosity \( H_e \) ranged from 0.533 to 0.871 (mean of 0.741) in Ilha Solteira, and in Selvília ranged from 0.133 to
1.0 (mean of 0.668) and ranged from 0.606 to 1.0 (mean of 0.797). The fixation index (\(F_{\text{IS}}\)) ranged from −0.394 to 0.494 (mean of 0.090) in Ilha Solteira, and in Selvíria ranged from −0.069 to 0.783 (mean of 0.162). Null alleles were observed in four and six loci in Ilha Solteira and Selvíria, respectively. After Bonferroni sequential correction, genotypic linkage disequilibrium (LD) was observed in four pairs of loci in Ilha Solteira and in three pairs in Selvíria (Table 3).

| Pairwise loci | Ilha Solteira | Selvíria |
|---------------|---------------|----------|
| Ga01xGa05     | 0.00005       | 1.00000  |
| Ga01xGa06     | 0.00005       | 1.00000  |
| Ga01xGa07     | 0.00005       | 1.00000  |
| Ga01xGa08     | 0.00011       | 1.00000  |
| Ga01xGa19     | 0.89137       | 0.00005  |
| Ga01xGa20     | 0.73405       | 0.00005  |
| Ga02xGa03     | 0.00047       | 0.00005  |

The values represent the probability of genotypic disequilibrium after 19,000 permutations of alleles among individuals. Probability after Bonferroni’s corrections: \(P = 0.000263 \ (\alpha = 0.05)\).

To test the genetic similarity between populations, we used DAPC and STRUCTURE analysis. The genetic differentiation (\(\theta\)) between populations (0.363) was high great part of genetic is distribute within than among populations. In fact, the high genetic differentiation among the populations was expected given their geographical distance (50 km). The species is pollinated by bees, which have limited distances reported [1], which can explain the high genetic differentiation between both. The PCA showed a clear differentiation of both populations, with some individuals mixed between them. Furthermore, DAPC and both assignment probability tests (from adegenet package and STRUCTURE) resulted in the similar results of population structure, with two distinct populations (Fig. 2).

**Discussion**

In *A. fraxinifolium* transcriptome data, a predominance of -tri, followed by dinucleotides motifs, with more than 79% of all identified contigs, which could not affect the protein structure, with non-perturbation of the reading frame [9, 10]. When analyzing all repeats number, we identified that repeats number greater than 10 corresponds with less than 7.8% of all SSRs (Table 4). The SSR frequency decreased with an increase in motif length, as reported for
Magnolia wufengensis [11]. The frequency of motifs from AG/CT corresponds to more than 24.3%, being the most abundant motif in this species, followed by TCT/AGA repeats with less than 6%. (Fig. 3). These frequencies of AG/CT repeats are higher than found for other species such as bamboo (17.11%) [12], but less than for Magnolia (37.8%) [11]. High frequencies of AG repeats are also reported for other plant species being suggested that could be related to mutation mechanism of generation of SSRs or selective pressure to particular sequences [9, 10, 11, 13, 14].

### Table 4

Frequency distribution of SSRs identified in the A. fraxinifolium transcriptome.

| Repeat number | Di- | Tri- | Tetra- | Penta- | Hexa- | Total |
|---------------|-----|------|--------|--------|-------|-------|
| 4             | N/A | N/A  | N/A    | 6685   | 11008 | 17693 |
| 5             | N/A | N/A  | 3003   | 1740   | 1644  | 6387  |
| 6             | N/A | 33155| 2139   | 365    | 466   | 36125 |
| 7             | 13340| 15352| 217    | 56     | 55    | 29020 |
| 8             | 6167 | 7539 | 52     | 3      | 20    | 13781 |
| 9             | 5489 | 2413 | 10     | 2      | 4     | 7918  |
| 10            | 3511 | 1013 | 4      | 0      | 2     | 4530  |
| >10           | 8882 | 859  | 9      | 1      | 3     | 9754  |
| **Total**     | 37389| 60331| 5434   | 8852   | 13202 | 125208|

The microsatellite markers developed were efficient in the genetic differentiation among populations sampled. The average levels of heterozygosity observed and expected were above that reported for populations of Astronium graveolens [15] and in other tropical species, as in populations of Cedrela fissilis (Meliaceae) [16], Campomanesia xanthocarpa (Myrtaceae) [17], Myracrodruon urundeuva [18] and Eugenia uniflora L. (Myrtaceae) [19], which confirms the existence of high genetic variability in the populations studied here. Therefore, these genetic markers are reliable to be used in population genetics studies, as such in the investigation of the pollen and seeds dispersal patterns aid to understand the actual distribution of natural populations, with impacts in the evolutionary history of a species. Previous studies with other tree species show a great range of pollen dispersal, such in Hymenaea stignocarpa, showed long-distance pollen dispersal reaching values of more than 8 km between the populations analyzed [20] and even more in Ceiba pentandra, reaching 18 km [21, 22]. However, these long distances are mainly related to the dispersion by bats, which have a large feeding area. For A. fraxinifolium, which are pollinated by bees, distances of almost 6 km were reported of the insects feeding behavior [23, 24]. Such results indicate that further investigations of pollen/seed dispersion are necessary for the species. To date, few studies were conducted based in natural populations of A. fraxinifolium, focused on silvicultural traits [25]. Recently in the genus, A. graveolens SSR loci were described, but not tested in other Astronium species [15]. Given this, the microsatellite markers in this work developed may be useful in genetic studies such as diversity and genetic structure, gene flow and mating.
system, providing information for conservation, breeding and reforestation plans of the species. In addition, our study provides a database with more than 125 thousand of expressed SSR sequences in the genome that will serve as a basis for studies of consequences of forest fragmentation in tropical forest of Brazil, thus contributing to the development of adequate strategies for the conservation of A. fraxinifolium and related species from Anacardiaceae family.

Conclusions

This study reports the first SSR markers for A. fraxinifolium. The frequency and distribution of SSR motif types showed great diversity, with a predominance of trinucleotides motifs, as reported for other plant species. Functional annotation of SSRs can help future breeding programs in the selection of genes related to important characteristics of the development. Also, the use of transcriptome derived SSR can increase the rate of amplification in related species, due the conservative flanking sequence of these loci. At population level, these SSR markers showed enough levels of polymorphism in both populations analyzed. Therefore, the obtained results suggest that these markers can be used as tools for ecological population genetic studies, such as genetic diversity, spatial genetic structure, mating system and gene flow, besides improving the development of genetic conservation strategies and management of fragmented populations and related species.

Methods

Sample collection and DNA extraction for validation analysis

For the validation step, a total of 60 A. fraxinifolium samples from two natural populations were collected: 30 individuals from along the SP-595 highway in the municipality of Ilha Solteira, in the State of São Paulo (20° 21′ 36.46″ S, 51 °01 ′15.52″ W), Brazil, characteristic of the Semideciduous Seasonal Forest. The second population is composed of 30 adult trees from along the BR-158 highway in the municipality of Selvíria, in the state of Mato Grosso do Sul (20°12′ 02.30″ S, 51° 14′ 56.81″ W), Brazil, characteristic of the Cerrado biome. Collection of leaf tissues was authorized by the Institute for Biodiversity Conservation (ICMBio), linked to the Ministry of the Environment (MMA) under the number 73998-1. The identification of trees in field was performed by PhD. José Cambuim, São Paulo State University (UNESP), based on the testimony plants of the areas that were sampled and are deposed at the Herbarium of Ilha Solteira of the São Paulo State University according to the following vouchers: HISA 1566, HISA 1570, HISA 4213, HISA 4214, HISA 4215, HISA 4216, HISA 4217, HISA 4218, HISA 4219, HISA 4220, HISA 4221, HISA 4222, HISA 4223, HISA 4224, HISA 6041, HISA 10299, HISA 10300, HISA 10301, HISA 10302, HISA 10303, HISA 10304, HISA 10305, HISA 10308, HISA 10531.

SSR loci identification and characterization

Species-specific microsatellite primers were generated from cDNA genomic library of the species [26] (data not published) and submitted to new generation sequencing (HiSeq Sequencing 2500 System, Illumina). Briefly, the total RNA was extracted from fresh embryonic axis of seeds collected from Alto Paraíso, Cavalcante, Colinas do Sul and Niquelândia cities of Goiás state, and Montes Claros, Mirabela and Lontra municipalities of Minas Gerais state, using NucleoSpin RNA Plant Kit (Macherey-Nagel, Düren, Germany. Seed collection was authorized by the Institute for Biodiversity Conservation (ICMBio) under the number 41166-1. The library was constructed using the TrueSeq RNA Library Prep Kit V2 (Illumina) and sequenced in a 2 × 100 bp paired-end run. For development of useful microsatellite markers for population genetics, we considered a subset of four samples from this run and the software SSR_pipeline [27] was used for search of SSRs by the following parameters: di-, tri-, tetra-, penta- and
hexanucleotides by the minimum of seven, six, five, four and four repeats respectively, with 40 bp flanking sequence. This software used as input the raw data reads, processed in a module of quality sort, followed by joining reads and completed the search for the SSRs. Primer design was conducted in BatchPrimer3 v1.0 [28]. Functional annotation of SSR-containing coding sequences were analyzed in Blast2GO software, using EnsemblePlants database from UniProt [29].

For the population analysis, genomic DNA was isolated from fresh leaves using the cetyltrimethylammonium bromide (CTAB) protocol [30], quantified in NanoDrop ND-1000 Spectrophotometer (NanoDrop Products, DE, USA) and its integrity was verified in 1% agarose gels running with TBE (1X), at constant voltage of 5V/cm. We selected 20 SSR loci for population validation. Polymerase chain reactions were performed in a Mastercyler thermocycler (Eppendorf, Hamburg, Germany) with an addition of a M13 tail for fluorescent labeling [31] in a reaction mixture containing 5.0 µL GoTaq Colorless Master Mix, 0.3 µL of forward primer (2 pmol), 0.3 µL of reverse primer (8 pmol) 0.3 µL of fluorescent primer with M13 (8 pmol; 6-FAM, VIC, PET or NED, Applied Biosystems), 0.5 µL of BSA (Bovine Serum Albumin), 1.0 µL genomic DNA (approximately 50 ng) and nuclease-free water for 10 ul of final reaction. The PCR program was as follows: 2 min at 96 °C, 35 cycles of 30 s at 96 °C, a primer-specific annealing temperature (Table 5) for 1 min 30 s, 72 °C for 1 min 30 s, followed by 12 cycles of 96 °C for 30 s, 53 °C for 1 min 30 s, 72 °C for 1 min 30 s, with final extension at 72 °C for 20 min. The PCR products were genotyped in ABI3130xl Genetic Analyzer (Applied Biosystems) with GeneScan 500 LIZ (Applied Biosystems). Genotypes were assigned in GeneMapper v.5.0 software (Applied Biosystems).
### Table 5
Characteristics of the 20 microsatellite markers isolated for *Astronium fraxinifolium*

| Locus | Primer sequences 5'- 3' | Repeat motif | Size range (bp) | Tm (°C) | Genbank accession |
|-------|--------------------------|--------------|-----------------|---------|------------------|
| Ga01  | F<sup>a</sup>: CATTCCACATTGCCCTTG  
R: GCTTTCTGTTCCTCAAATCC | (GA)<sub>13</sub> | 151–170 | 54 | MN129762 |
| Ga02  | F<sup>a</sup>: TCTCCCCCTCTACCTGCACTC  
R: ATCACCTTCTCCACGAAGA | (CT)<sub>12</sub> | 92–118 | 54 | MN129763 |
| Ga03  | F<sup>a</sup>: CAAGAACGAAAGAAGATCAACC  
R: ATGCTGATGGATCGATTGTG | (TA)<sub>24</sub> | 84–100 | 54 | MN129764 |
| Ga04  | F<sup>a</sup>: AAACAAACATGATGCAGGA  
R: CACTTCAATCCAGGAAAGA | (AG)<sub>20</sub> | 120–142 | 54 | MN129765 |
| Ga05  | F<sup>a</sup>: CATCGATGATGAACCCAGAA  
R: CTCTACGGAAGCGGAGTCAC | (TC)<sub>12</sub> | 230–260 | 54 | MN129766 |
| Ga06  | F: CCGCGCAATAGTTAATAGGA  
R<sup>a</sup>: TGATCTACAGCCCATCCTGA | (AG)<sub>13</sub> | 104–138 | 54 | MN129767 |
| Ga07  | F: GATGGTAGAGGCTGTTTGTG  
R<sup>a</sup>: CCAAGCTCATTTCCCGTCATC | (AG)<sub>10</sub> | 123–139 | 54 | MN129768 |
| Ga08  | F<sup>a</sup>: GAAAAGAAGAGAGATCGGAACG  
R: CATCACCCACCCACAAACTC | (GA)<sub>37</sub> | 105–121 | 54 | MN129769 |
| Ga09  | F<sup>a</sup>: GCCCAGATTTCTCTCAATCTCC  
R: CGCGTTCTTTGTAGCAAGACA | (CT)<sub>24</sub> | 73–123 | 54 | MN129770 |
| Ga10  | F<sup>a</sup>: TCGCACAACCTCATCTACAGC  
R: AACGACAAGTCGCTTTGACA | (AG)<sub>14</sub> | 97–111 | 54 | MN129771 |
| Ga11  | F<sup>a</sup>: GACCTTCAAAGCCAACCAAC  
R: TTAGCTTGGAGTGCGATGTG | (GGT)<sub>11</sub> | 93–120 | 54 | MN129772 |

Tm = melting temperature; <sup>a</sup>M13-tailed primer fluorescent labeled, following Schuelke (2000)
| Locus | Primer sequences 5'- 3' | Repeat motif | Size range (bp) | Tm (°C) | Genbank accession |
|-------|--------------------------|--------------|-----------------|---------|------------------|
| Ga12  | F<sup>a</sup>: TGACCATCCCCAACACATTCT R: TGCGGTTAGATAGGGAATGC | (ACC)<sub>09</sub> | 92–115 | 54 | MN129773 |
| Ga13  | F<sup>a</sup>: TGCGGTTAGATAGGGAATGC R: TGACCATCCCCAACACATTCT | (GGT)<sub>11</sub> | 91–112 | 54 | MN129774 |
| Ga14  | F<sup>a</sup>: TGCGGTTAGATAGGGAATGC R: TGACCATCCCCAACACATTCT | (GGT)<sub>09</sub> | 92–113 | 54 | MN129775 |
| Ga15  | F<sup>a</sup>: TCAGGACACTCTCCACCGGTAG R: TACACGTTTTTCACCGCCAAC | (ATC)<sub>10</sub> | 92–113 | 54 | MN129776 |
| Ga16  | F<sup>a</sup>: CCACCACACCTCAGGAACCT R: GAGGTTGGAGTGTCAGTGT | (CCT)<sub>10</sub> | 115–133 | 54 | MN129777 |
| Ga17  | F<sup>a</sup>: GCGAAAGTGATGCTGTATTGG R: CATCGACACCCAACCTCATGT | (AAG)<sub>11</sub> | 108–120 | 54 | MN129778 |
| Ga18  | F<sup>a</sup>: TTCTAACACAGCAAACAAAACG R: TGGTGTTTTTCAGTTGTGA | (TCA)<sub>12</sub> | 108–126 | 54 | MN129779 |
| Ga19  | F<sup>a</sup>: GGCACGAAAAAGAAGGAGG AG R: CATGGGACTGCAAGAATTTT | (GAG)<sub>11</sub> | 107–123 | 54 | MN129780 |
| Ga20  | F<sup>a</sup>: TTCTAACACAGCAAACAAAACG R: TGGTGTTTTTCAGTTGTGA | (TCA)<sub>12</sub> | 111–129 | 54 | MN129781 |

Tm = melting temperature; <sup>a</sup>M13-tailed primer fluorescent labeled, following Schuelke (2000)

**Insert Table 5**

**Statistical population analysis**

The number of alleles per locus (), observed () and expected () heterozygosity, polymorphism information content () was estimated using CERVUS 3.03 software [32]. The fixation index () and genotypic linkage disequilibrium (LD) were estimated for each population using FSTAT software [33]. To test if the values and to the LD were significantly different from zero, we used Monte Carlo permutations and a Bonferroni correction (95%, α = 0.05). Micro-Checker v.2.2.3 [34] was used to detect the possibility of occurrence of null alleles () and estimated the genetic differentiation () with base in Hedrick's statistics [35]. The adegenet package [36] in R environment was used to conduct the
principal component analysis (PCA), discriminant analysis of principal components (DAPC) and the assignment probability of each individual. Additionally, a bayesian analysis was performed in STRUCTURE [37] assuming admixture model, correlated allele frequencies, testing each K (1–4) in 10 independent runs with 100,000 'burn-in' period and 1,000,000 generations. The search of optimal K was inferred using Evanno method [38] in Structure Harvester [39].

**Abbreviations**

GO: Gene Ontology; BSA:Bovine Serum Albumin; CTAB:Cetyltrimethylammonium bromide; DAPC:Discriminant analysis of principal components; F:Fixation index; GST:Genetic differentiation; H_e:Expected heterozygosity; H_o:Observed heterozygosity; LD:linkage disequilibrium; PCA:Principal component analysis; PIC:Polymorphism information content; SSR:Simple sequence repeats

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analysed during this study are included in this published article.

**Competing Interests**

The authors declare that they have no competing interests.

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**Author Contributions**

MRC, MAA and ROM carried out the field and laboratory steps of the research. EAAS and LGPN developed the cDNA genomic library. BCR and ROM performed analysis and wrote the manuscript. MLTM, BCR and CLM designed the experiment. BCR and CLM supervised the research and with MLTM and AMS reviewed the manuscript. All of the authors read and approved the final manuscript.
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**Figures**

![Graph showing functional annotation of SSRs in coding regions for A. fraxinifolium transcriptome](image)

**Figure 1**

Functional annotation of SSRs in coding regions for A. fraxinifolium transcriptome, including the number of genes putatively involved in different subcellular functions in GO classification.
Figure 2

PCA, DAPC and assignment tests for the two populations of Astronium fraxinifolium analyzed. a PCA showing the distribution of genotypes. b DAPC clearly showing the differences between populations. a, b Colors reflect each population: Ilha Solteira (red) and Selviria (blue). c assignment test from adegenet package. d STRUCTURE results from analysis at optimal K=2. c, d Each column and colors reflect the genetic assignment of individuals: in c Ilha Solteira (brown) and Selviria (blue); d Ilha Solteira (green) and Selviria (red).
Figure 3

Frequency distribution of the most representative SSR motifs types in the A. fraxinifolium transcriptome.