Genetics Analysis of the Biggest Cashew Tree in the World

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ABSTRACT. This study performed a genetic study of several canopies of *Anacardium occidentale* L. specimen called "Cashew King" (Cajueiro da Praia-PI, Brazil) in order to assess whether all canopies belong to the same plant. Leaves were collected from

different distant points, and preserved for analysis of DNA. The analysis was made from automated sequencing by capillary electrophoresis equipment ABI3500 Genetic Analyzer (Applied Biosystems) and alignment of nucleotide sequences generated with reference sequences deposited in GenBank. According to the analysis, it was shown that all canopies belong to the same plant. Thus, the "Cashew King" can be considered as a single specimen *A. occidentale* occupying an area of 8,834 m^2. The length of the specimen indicates that the "Cashew King" is the largest cashew registered according to the literature and therefore should be preserved.

Key words: *Anacardium occidentale*; DNA fingerprinting; ITS region; PCR; extraction DNA; BLAST; Cashew

SHORT COMMUNICATION

The cashew tree belongs to the Anacardiaceae family, genus *Anacardium* L., *Anacardium occidentale* L. species (common cashew) (Aliyu 2007; Almeida et al. 2010; Ramírez-Malagón et al. 2014; Campos et al. 2015; Hiwale 2015). The Anacardiaceae family has about 80 genera and over 800 species, distributed predominantly in tropical, subtropical and temperate regions, consisting of trees and shrubs, with whole or compound leaves, small flowers, and with the presence of fruit type walnut in addition to a pseudo fruit (Santos et al. 2007; Aguilar-Barajas et al. 2014).

A. occidentale is an evergreen tree native to the Northeast region of Brazil, which expanded itself spontaneously in countries in South America. During the 16th century, the Portuguese introduced specimens in India and Africa (Rico et al. 2016). Currently, the plant is found in South America, Central America, Africa and Asia (Santos et al. 2007).

The use of cashew is associated with one of the main agronomic activities in the Northeast of Brazil, which stands for 70% of production of cashew nuts in the world (Santos et al. 2007). In 2002, agribusiness cashew plantation amounted to 700,000 hectares, employing over 100,000 people, and providing an annual amount of 200 million dollars (Freire et al. 2002). Although cashew is quite consumed, the branches of *A. occidentale* are used as anti-asthmatic, anti-diarrheal, astringent, tonic and anti-diabetic in the popular medicine (Leitão et al. 2013).

In fact, different studies have focused attention to the pharmaceutical and biotechnology properties of compounds derived from *A. occidentale*. However, there are no studies in the literature involving questions related to preservation, knowledge and sustainable development of cashew. At the same time, there is a poor knowledge about the development and growth of anomalous specimens of *A. occidentale*, as those who are termed as the biggest specimens of *A. occidentale*.

The occurrence of a cashew tree, located in Cajueiro da Praia-PI, Brazil, of great economic and cultural importance was recorded. This cashew apparently has 8,834 m^2 extensions, which proves to be the biggest cashew tree of extensions in the world. In the face of its importance to the local community and knowing the importance of preserving this plant, a genetic analysis study was performed to determine whether all existing canopy belongs to the same cashew. Leaf samples of different canopy of "Cashew King", were extracted and collected at five different points using GPS and Google Maps in order to obtain an area of about 9,000 m^2 (Figures 1 and 2). Before the analysis of DNA "Cashew King" was conducted to assess the extent of the tree and distant treetops. The length was calculated based on the layout of the image obtained via Google Maps and with the support of a GPS location to the farthest points. According to analyze, the "Cashew King" has a central structure with a size of 8,834 m^2. This structure exceeds in size the cashew tree located in Pirangi do Sul, region of Rio Grande do Norte has 8,500 m^2 in extent. The measurement suggests that the Cashew King is the highest recorded in the literature.

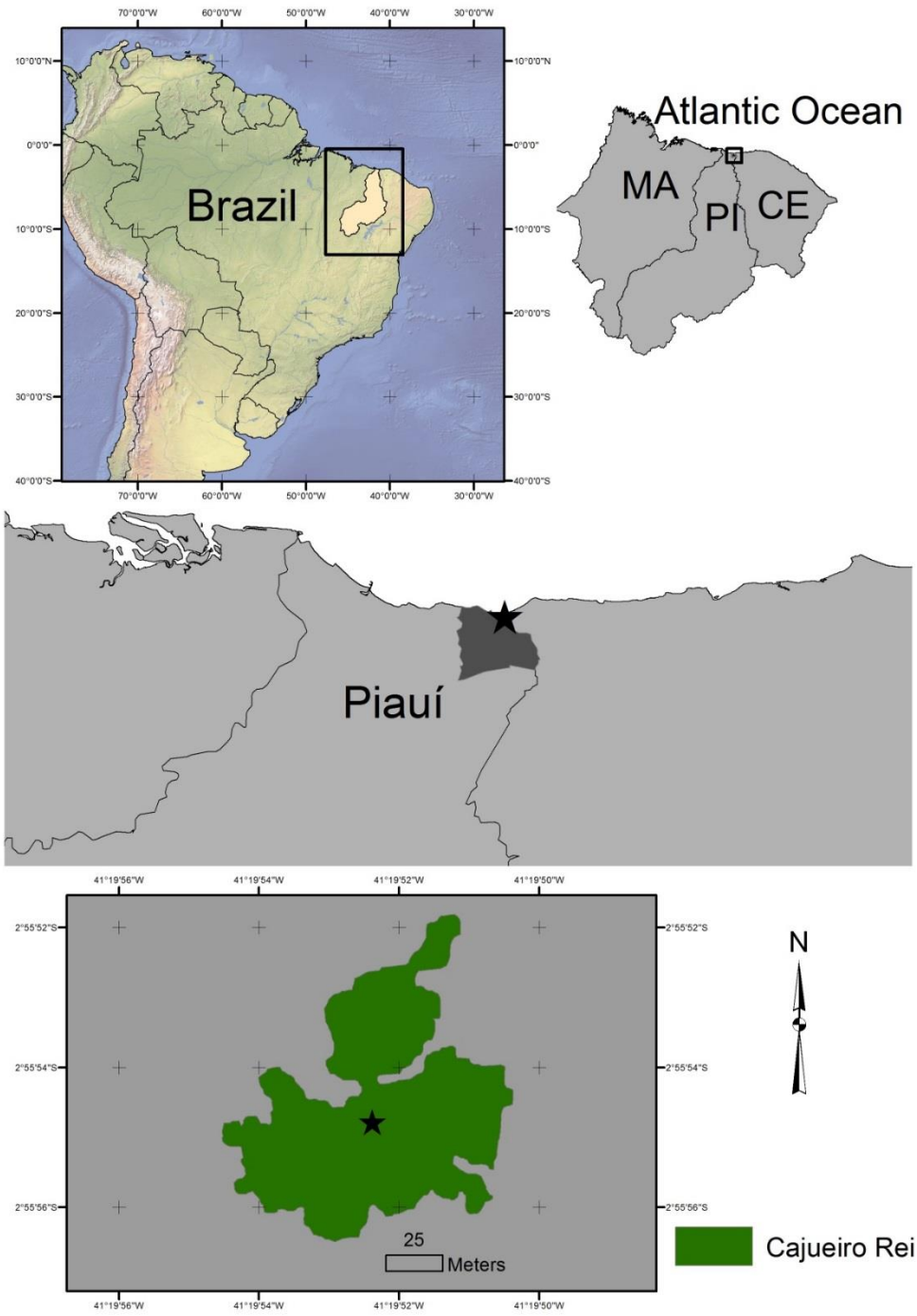

Figure 1. Location of Cashew Kingi, Cajueiro da Praia, Piauí, Brazil

To confirm this statement was made harvesting leaves of 5 neighbouring points (Figure 2 and Table 1) of the cup Cashew King for DNA sequencing and genetic analysis. DNA samples from leaves of five different regions of the cashew tree. The leaves were dried at room temperature, stored and transported in CTAB and NaCl solution and taken to the laboratory where extractions were performed. To extract DNA was used Doyle and Doyle modified protocol based on CTAB (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM TrisHCl, 0.2% β-mercaptoethanol, ultrapure H_2O) (Doyle 1987).

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CLUSTAL 2.1 multiple sequence alignment


469_trnL
AACCTACTAAGTGATAACTTTCAAATTCAGAGAAACCCTGGAATCAAAAATGGGCAATCC
471_trnL
AACCTACTAAGTGATAACTTTCAAATTCAGAGAAACCCTGGAATCAAAAATGGGCAATCC
468_trnL
AACCTACTAAGTGATAACTTTCAAATTCAGAGAAACCCTGGAATCAAAAATGGGCAATCC
P5_trnL
AACCTACTAAGTGATAACTTTCAAATTCAGAGAAACCCTGGAATCAAAAATGGGCAATCC
P3_trnL
AACCTACTAAGTGATAACTTTCAAATTCAGAGAAACCCTGGAATCAAAAATGGGCAATCC

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469_trnL
TGAGCCAAATCCTATTTTACGAGAACAAAAACAAACAGGGGGTCAGAACGGGAGAAAAAA
471_trnL
TGAGCCAAATCCTATTTTACGAGAACAAAAACAAACAGGGGGTCAGAACGGGAGAAAAAA
468_trnL
TGAGCCAAATCCTATTTTACGAGAACAAAAACAAACAGGGGGTCAGAACGGGAGAAAAAA
P5_trnL
TGAGCCAAATCCTATTTTACGAGAACAAAAACAAACAGGGGGTCAGAACGGGAGAAAAAA
P3_trnL
TGAGCCAAATCCTATTTTACGAGAACAAAAACAAACAGGGGGTCAGAACGGGAGAAAAAA

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469_trnL
AAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTGATTGCCTTTTT
471_trnL
AAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTGATTGCCTTTTT
468_trnL
AAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTGATTGCCTTTTT
P5_trnL
AAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTGATTGCCTTTTT
P3_trnL
AAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTGATTGCCTTTTT

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469_trnL
GGGGGAAAGAAGGGAATTCTTCTATCGAATATCGAAAGGTTATAAAGGATGAAGGATAAG
471_trnL
GGGGGAAAGAAGGGAATTCTTCTATCGAATATCGAAAGGTTATAAAGGATGAAGGATAAG
468_trnL
GGGGGAAAGAAGGGAATTCTTCTATCGAATATCGAAAGGTTATAAAGGATGAAGGATAAG
P5_trnL
GGGGGAAAGAAGGGAATTCTTCTATCGAATATCGAAAGGTTATAAAGGATGAAGGATAAG
P3_trnL
GGGGGAAAGAAGGGAATTCTTCTATCGAATATCGAAAGGTTATAAAGGATGAAGGATAAG

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469_trnL
CCTATATTACACTATGTATAAGTAATGAAAAAATACACTATGTATACGTAATGAAAAAGG
471_trnL
CCTATATTACACTATGTATAAGTAATGAAAAAATACACTATGTATACGTAATGAAAAAGG
468_trnL
CCTATATTACACTATGTATAAGTAATGAAAAAATACACTATGTATACGTAATGAAAAAGG
P5_trnL
CCTATATTACACTATGTATAAGTAATGAAAAAATACACTATGTATACGTAATGAAAAAGG
P3_trnL
CCTATATTACACTATGTATAAGTAATGAAAAAATACACTATGTATACGTAATGAAAAAGG

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469_trnL
ATCTCAAAAATGATGACCCGAATCCTTTTTGTTTTTCTTTTGAAGAACTAATTAATCGGA
471_trnL
ATCTCAAAAATGATGACCCGAATCCTTTTTGTTTTTCTTTTGAAGAACTAATTAATCGGA
468_trnL
ATCTCAAAAATGATGACCCGAATCCTTTTTGTTTTTCTTTTGAAGAACTAATTAATCGGA
P5_trnL
ATCTCAAAAATGATGACCCGAATCCTTTTTGTTTTTCTTTTGAAGAACTAATTAATCGGA
P3_trnL
ATCTCAAAAATGATGACCCGAATCCTTTTTGTTTTTCTTTTGAAGAACTAATTAATCGGA

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469_trnL
CGAGAATAAAGATAGAGTCCCATTCTACATGCCAATATCAATACTGGCAACAATGAAATT
471_trnL
CGAGAATAAAGATAGAGTCCCATTCTACATGCCAATATCAATACTGGCAACAATGAAATT
468_trnL
CGAGAATAAAGATAGAGTCCCATTCTACATGCCAATATCAATACTGGCAACAATGAAATT
P5_trnL
CGAGAATAAAGATAGAGTCCCATTCTACATGCCAATATCAATACTGGCAACAATGAAATT
P3_trnL
CGAGAATAAAGATAGAGTCCCATTCTACATGCCAATATCAATACTGGCAACAATGAAATT

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469_trnL        TATAGTAAGAGGAAA
471_trnL        TATAGTAAGAGGAAA
468_trnL        TATAGTAAGAGGAAA
P5_trnL         TATAGTAAGAGGAAA
P3_trnL         TATAGTAAGAGGAAA
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Figure 2. Image obtained from the Google Maps system of Cashew King. The table shows the coordinates of the points of collection of the leaves of the treetops.

Table 1. Samples received codes that are described in the table.

Species	Voucher no.	Geographic coordinates
Anacardium occidentale L.	P1 (469_trnL)	(2°55'55.9"S 41°19'53.6"W)
Anacardium occidentale L.	P2 (471_trnL)	(2°55'55.7"S 41°19'51.8"W)
Anacardium occidentale L.	P3 (P3_trnL)	(2°55'54.0"S 41°19'50.8"W)
Anacardium occidentale L.	P4 (468_trnL)	(2°55'54.3"S 41°19'53.6"W)
Anacardium occidentale L.	P5 (P5_trnL)	(2°55'52.7"S 41°19'52.5"W)

The electrophoretic profile of DNA samples was visualized on 1% agarose gel and DNA concentration was determined by Nanodrop spectrophotometer 2000. The polymerase chain reaction (PCR) amplifications were performed with a final volume of 20 µL containing 10 µL of GoTaq® Colourless 2x Master Mix (Promega, USL), 0.7 mM of oligonucleotide, 0.7 mM of oligonucleotide reverse, 40 ng of genomic DNA and ultra-pure enough sterile water to 20 µL.

The polymerase chain reaction (PCR) amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems®). For the amplification of the ITS region were used ITS-5 forward primer (5'GGAAGTAAAAGTCGTAACAAGG3') and ITS-4 reverse primer (5'TCCTCCGCTTATTGATATGC 3'). The amplification program for ITS region consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 40s; extension at 72°C for 1 min and a final extension at 72°C for 5 min. As for amplifying the intron region trnL, Caju_trnl forward primer were used (5'CGAAATCGGTAGACGCTACG3') and Caju_trnlreverse primer (5'GGGGATAGAGGGACTTGAAC3') following the initial denaturation programming 97°C for 1 min, followed by 30 cycles of denaturation at 94°C for 1 min, 2 min at 48°C (annealing); and 2 min at 72°C; and a final extension of 16 min at 72°C.

The amplification products were separated on a 1% agarose gel with 0.5X TBE buffer and and purified with AgencourtAMPure XP (Beckman Coulter, Inc.). The sizes of the amplified fragments were determined based on a 100-pb DNA ladder (Invitrogen), and the products were visualized on an ultraviolet transilluminator and documented. The PCR samples were evaluated and 1% agarose gel and purified with AgencourtAMPure XP (Beckman Coulter, Inc.) and quantification of the purified products was performed using a Nanodrop (ThermoFhischer).

The sequencing reaction utilized the Big Dye® Terminator v3.1 Cycle Sequencing Kit reagent (Applied Biosystems) and by precipitation reaction Ethanol/EDTA/sodium acetate. The analysis was made from automated sequencing by capillary electrophoresis equipment ABI3500 Genetic Analyzer (Applied Biosystems) and alignment of nucleotide sequences generated with reference sequences deposited in GenBank. For alignment of sequences the NCBI programs were used (National Center for Biotechnology Information) feature: Standard Nucleotide BLAST: Used to compare the result with the highest identity body in NCBI database, EMBOSS/Merger used to join sequences and form contigs (consensus sequences), Chromas Late used to view the chromatogram of the sequences generated and MultAlin used to align the sequences.

Lastly, the cashew plant was measured by field data survey and geoprocessing measure tools. Geographical points were taken in the perimeter of the individual and then marked on satellite images (Digital Globe 2016), next the area occupied by “Cashew King” was measured using geographic information system tools from ArcMap 10.3.1.4959 software (ESRI 2015). In the 90s, the analysis of rbcL gene of Anacardiaceae chloroplasts was held in conjunction with the morphological anatomy to interpretation of the phylogeny (Terrazas 1993). The advancement of molecular biology, other genes as matK, rps16 and intron trnL is used for phylogenetic analysis of Anacardiaceae (Pell et al. 2010).

For sequencing tests, the regions of intron trnL and intergenic spacer trnL-trnF chloroplast were chosen in order to compare the samples (Figure 3).

Figure 3. DNA sequencing samples of leaves taken from five different points Cashew King.

The genetic sequencing analysis showed that the genetic sequences are identical to each other, thus proving that these parts belong to the same individual. According to the genetic analysis, the Cashew King has different branches that make up a structure with almost 9000 $m^{2.}$ Thus, this work shows that the Cashew King, can be considered the largest cashew tree recorded in the world.

CONCLUSION

One hypothesis to explain the great length of the cashew tree is the formation of several cashew clones from the original. This type of propagation can occur several ways, one of which comes from a so-called soil air layering technique or air layers, a technique that is already applied in dwarf type Cashew Trees consisting of burying parts of the plant still attached to the mother plant, targeting the roots of region covered by soil, thus creating a new individual as the same genetic constitution (Aliyu 2007; Almeida et al. 2010; Ramírez-Malagón et al. 2014; Campos et al. 2015; Hiwale 2015). In the case of Cashew King, it is suspected that this process would have occurred naturally through a process of somatic embryogenesis. The somatic embryogenesis process produces non-chimeric plants and show greater genetic uniformity and fidelity clonal (Martin 2003). Some reports in the literature indicate that cashewgrafts do not easily form roots, suggesting failure or difficulty of cuttings (Cardoza and D'Souza 2002; Martin 2003; Saranga and Cameron 2007). Such reports suggest that this process would have occurred naturally. According to the population reports cashew existed for over 100 years in the region. Despite the little knowledge about the "Cashew King" records, it should be preserved for further studies in relation to its development. According to the genetic analysis, the "Cashew King" has different branches that make up a structure with almost 9000 m^2. Thus, this work shows that "Cashew King" can be considered the largest cashew tree recorded in the world and should be protected for its environmental and genetic patrimony.

CONTRIBUTIONS

All authors contributed to the study and to the writing of this brief communication arising.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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