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Botanical validation of *Ecclinusa* (Chrysophylloideae: Sapotaceae) from forest remnants using molecular analysis as a complementary method to morphological identification

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ABSTRACT

In the context of a forest restoration program in a mining area in the eastern Brazilian Amazon, the objective of this study was to identify the species of trees of *abiu-seringarana* (*Ecclinusa* sp.) in forest remnants adjacent to the mine. As the identification of *Ecclinusa* based on external morphology alone is difficult, it was corroborated by molecular analysis. Leaf samples of three trees were compared with *Ecclinusa* in the Embrapa herbarium collection. DNA was extracted with a CTAB modified protocol for sequencing with ITS markers. Both the morphological identification and the alignment of the obtained sequences with material in GenBank indicated that the individuals belong to *Ecclinusa guianensis*, which is confirmed as the species occurring in the mining area.

KEYWORDS: Amazon, forest restoration, ITS marker, Pará, Brazil, abiu-seringarana

Validação botânica de *Ecclinusa* de remanescentes florestais utilizando análise molecular como método complementar à identificação morfológica

RESUMO

No contexto de um programa de restauração florestal em área de mineração na Amazônia oriental brasileira, o objetivo deste estudo foi identificar a espécie de abiu-seringarana (*Ecclinusa* sp.) em remanescentes florestais adjacentes à mina. Como a identificação de *Ecclinusa* com base apenas na morfologia externa é difícil, ela foi corroborada por análise molecular. Amostras de folhas de três árvores foram comparadas com *Ecclinusa* no acervo do herbário da Embrapa. O DNA extraído seguiu um protocolo CTAB modificado, e o sequenciamento foi com marcadores ITS. Tanto a identificação morfológica quanto o alinhamento das sequências obtidas com material do GenBank indicaram que os indivíduos pertencem a *Ecclinusa guianensis*, que é confirmada como a espécie que ocorre na área de mineração.

PALAVRAS-CHAVE: Amazônia, restauração florestal, marcador ITS, Pará, Brasil, abiu-seringarana

The genus *Ecclinusa* Martius (subfamily Chryshophylloideae, family Sapotaceae) has about 12 species in the Neotropics (Terra-Araujo *et al*. 2015), most of them distributed in the states of Amazonas, Amapá, Pará and Roraima, in the Amazon region of Brazil (Alves-Araújo 2022). Its representatives are medium to tall trees (8–30 m heigh), rarely shrubs, found in savanna, dry forest and rainforest (Alves-Araújo 2023).

In the eastern Brazilian Amazon, *Ecclinusa* is commonly known as *abiu-seringarana* (Reis *et al*. 2015) and is easily distinguished morphologically from other Sapotaceae genera by the combination of characters including the presence of stipules, sessile flowers and the absence of staminode (Pennington 1990; 2006). Within the genus, however, *Ecclinusa* species are difficult to distinguish due to the homogeneity of their external morphological characteristics (Pennington 1991; Swenson and Anderberg 2005), especially considering that frequently only vegetative structures are available (Gomes *et al*. 2013), wich has limited use for species identification (Camargo *et al*. 2008).

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In the context of a timber species survey for the forest restoration program of a mining enterprise in the eastern Brazilian Amazon, the aim of this study was to identify which species of *Ecclinusa* occur in forest remnants in the area using molecular analysis as a complimentary method to morphological identification of non-reproductive structures.

Leaf samples were obtained from three trees of *abiu-seringarana* in two forest remnants belonging to the Hydro S.A. mining enterprise, in the municipality of Paragominas, (2°59'42"S, 47°21'10"W), Pará state, Brazil (Figure 1). Collection procedures followed Ferreira (2006). The samples were labeled as HYD45, HYD52 and HYD264 and sent for analysis to the Laboratory of Arboreal Taxonomy of University Federal Rural da Amazônia – UFRA (Belém, Brazil). The material was oven-dried at 65 °C. After drying, the leaves were identified morphologically by comparison with the herbarium collection of the Instituto Agronômico do Norte (IAN) at Embrapa Amazônia Oriental (Belém, Brazil). The specimens were deposited at the Felisberto Camargo Herbarium at UFRA with the voucher codes FC-UFRA 5137 (HYD45), FC-UFRA 5138 (HYD52) and FC-UFRA 5143 (HYD264).

Molecular analyses were carried out at the Plant Protection Laboratory (LPP) at UFRA. For DNA extraction, the modified CTAB (Cetyl trimethylammonium bromide) protocol proposed by Gibbs and Mackenzie (1997) was used.

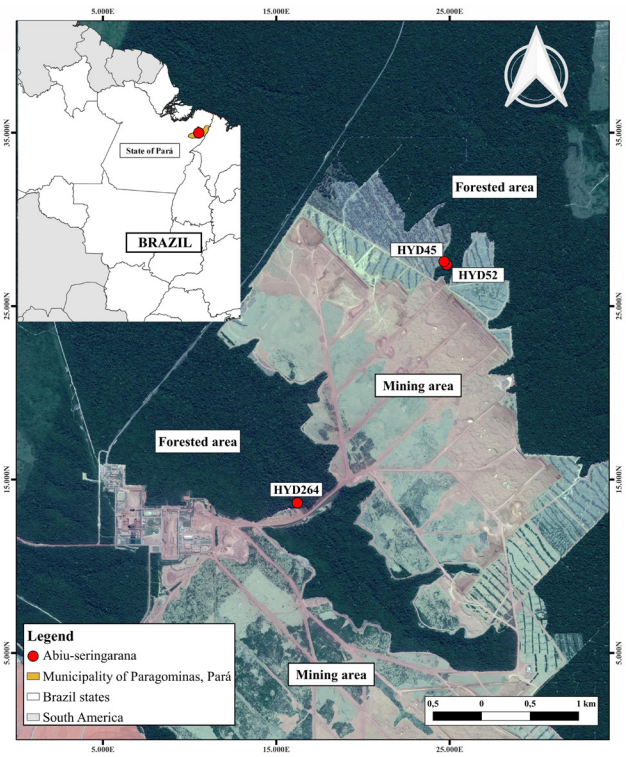

Figure 1. Location of the three trees of *Ecclinusa* sp. (locally known as *abiu-seringarana*) sampled for this study in forest remnants in a mining area in the municipality of Paragominas, state of Pará, eastern Amazon region of Brazil. This figure is in color in the electronic version.

ITS primers were used, as they show high specific resolution in the sequencing reactions for the discrimination of Sapotaceae species (Yoccoz *et al*. 2012; Vivas *et al*. 2014).

Changes were made to the volume of the following reagents: recommended CTAB (800 µl), Chloroform and isoamyl alcohol (24:1) (800 µl; 600 µl), ice-cold isopropanol (700 µl) and ammonium acetate (60 µl), 70% ethanol (1000 µl). In addition, changes were also made to the rotation speed and time (13,200 rpm for 10 minutes). Before addition of CTAB, there was a first wash with 1600 µl of wash advance and 2 µl of β-mercaptoethanol. For DNA amplification, the polymerase chain reaction was performed using primers for the Forward ITS-u1 (GGAAGKARAAGTCGTAACAAGG) and reverse ITS-u4 (RGTTTCTTTTCCTCCGCGCTTA) regions based on Cheng *et al*. (2016), using a GoTaq® qPCR Master Mix kit. The thermocycler program was 4 minutes at 94°C, 34 cycles of 30 seconds at 94°C (denaturation), 40 seconds at 55°C (annealing), 60 seconds at 72°C (extension), and 10 minutes at 72°C after cycles.After the purification reaction, sequencing was conducted in an ABI3730 automated sequencer at the Laboratory of Bioinformatics and Evolutionary Biology of University Federal de Pernambuco (LABBE-UFPE).

Analysis of DNA sequences and assembly of the *contigs* were performed with the Staden Package (Staden *et al*. 1998). The obtained nucleotide sequences were compared with the sequences of isolates of *Ecclinusa* available in the GenBank database (National Center for Biotechnology Information - NCBI), using the BLAST Link software (https://blast.ncbi.nlm.nih.gov**).** Sequences from Ecclinusa isolates were included in the alignment in all analyses. Alignments were performed using MAFFT, version 7 (Katoh and Toh 2013). The phylogeny of the nucleotide sequences was inferred using the GAMMA AUTO model. Maximum likelihood (ML) inference analyses were performed with 1000 bootstrap replications. The obtained tree was visualized with the Fig Tree 1.4.2 program (http://tree.bio.ed.ac.uk/software/figtree/). The generated sequences were deposited in Genbank with the accession codes SUB11711347: ON862915, ON862916 and ON862917.

The leaves of the sampled trees were generally elliptical with flat surface, acute base and acuminate apex, eucamptodromous to brochidodromous venation, midrib sunken on the upper side or prominent and with secondary veins, oblique parallel tertiary veins, channeled petiole, covered with appressed minute hairs. These characteristics corresponded to those described by Pennington (1990) and Ribeiro and Pennington (1999) for *Ecclinusa guianensis* Eyma. The comparison with the IAN collection also indicated that the samples belonged to *E. guianensis*.

According to BLAST, the samples HYD45 (583 bp), HYD52 (745 bp) and HYD264 (698 bp) showed 97%

similarity with the aligned sequences obtained from GenBank, corresponding to *Ecclinusa guianensis* recorded by Brasil Assunção et al. 162 (INPA) with access code KJ399358 (Table 1). The test sequences presented satisfactory values for the parameters of maximum score of 994 bits, total score 994, query coverage 100%, E-value was zero, accession length 795, *indetities* 568/583 (97%) and *Gaps* 1/583 (0%) to back up the reliable identification of the species as *E. guianensis*. The phylogenetic tree showed three well defined clades, grouping our DNA sequences with *E. guianensis*, with a bootstrap of 96%, confirming the botanical diagnosis (Figure 2).

Even though the botanical identification was based only on the morphological characteristics of leaves, without using reproductive structures (flower, fruit and seed), it was accurate as confirmed by the ITS analysis. The phylogenetic tree was consistent with the current delimitation of species in *Ecclinusa, E. guianensis* being closer to *E. lanceolata* (Swenson and Andeberg 2005) forming a monophyletic group supported by molecular and morphological data (Swenson and Anderberg 2005; Terra-Araujo *et al*. 2015; Faria *et al*. 2017).

The molecular results of this study corroborate the morphological identification of *Ecclinusa guianensis* as the species that occurs in the mining area. This information will assure the correct management of the species in the forest restoration program after ore exploitation, reducing the loss in quality of seeds. Therefore, we suggest the use of ITS as a supporting tool for fast and reliable identification of *Ecclinusa* species in the Amazon region.

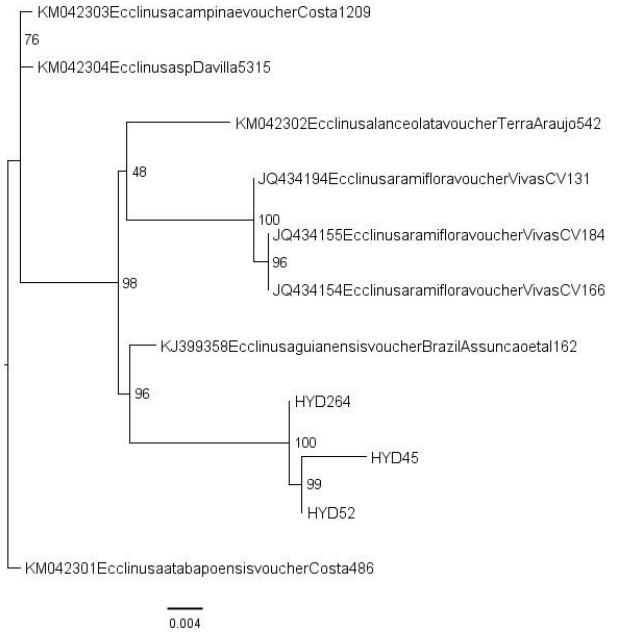

Figure 2. Phylogenetic tree of the genus *Ecclinusa* constructed by means of maximum likelihood comparing the ITS region of samples of three trees (HYD45, HYD52 and HYD264) from forest remnants in a mining area in the eastern Brazilian Amazon with the sequences producing significant alignments for *Ecclinusa* registered in GenBank (NCBI). Values of the branches indicate percentage of bootstrap of 1000 replications.

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Table 1. Sequences that produced significant alignments of the *Ecclinusa* species registered in GenBank (NCBI), compared with samples of *Ecclinusa* trees from remnant forests in a mining area in Paragominas, Pará state, eastern Brazilian Amazon. NCBI description = description of the GenBank sequence; Identification = percentage of identification of the GenBank sequence with the test sequences; Length = length of the GenBank accession; Accession code = code of the sequence in GenBank.

Species	NCBI description	Identification (%)	Length (pb)	Accession code
Ecclinusa guianensis Eyma	Brazil Assunção *et. al*. 162	97.43	795	KJ399358
Ecclinusa lanceolata (Mart. & Eichler) Pierre	Terra-Araújo 542 (INPA)	96.57	786	KM042302
Ecclinusa campinae Terra-Araujo & Costa	Costa 1209 (INPA)	96.06	765	KM042303
Ecclinusa sp. Mart.	Davilla 5315 (INPA)	95.90	742	KM042304
Ecclinusa atabapoensis (Aubrév.) T.D.Penn.	Costa 486 (INPA)	95.89	782	KM042301
Ecclinusa ramiflora Mart.	Vivas, C.V. 131 (CEPEC)	95.73	697	JQ434194
Ecclinusa ramiflora Mart.	Vivas, C.V. 166 (CEPEC)	95.56	713	JQ434154
Ecclinusa ramiflora Mart.	Vivas, C.V. 184 (CEPEC)	95.56	713	JQ434155

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DATA AVAILABILITY

The nucleotide sequences that support the results of this study are available at the National Center for Biotechnology Information - NCBI and can be accessed at https://blast.ncbi.nlm.nih.gov. The botanical specimens were deposited at the Felisberto Camargo Herbarium at Universidade Federal Rural da Amazônia (Belém, Pará state, Brazil).