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Nuclear and chloroplast molecular markers for a tropical tree in dry forests

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ABSTRACT: The seasonal dry tropical forests (SDTFs) on limestone outcrops are peculiar formations subject to degradation by the removal of timber and exploitation of limestone for cement production. The species *Ceiba pubiflora* (Malvaceae) is conspicuous in limestone outcrops in Brazil. This work aimed to select nuclear primers Inter Simple Sequence Repeat (ISSR) and chloroplast DNA (cpDNA) for application in studies aimed at genetic diversity and phylogeographic inferences in natural populations of *C. pubiflora*. After DNA extraction, the samples went through the polymerase chain reaction (PCR), electrophoresis and sequencing. We utilized a total of eight ISSR primers that exhibited the most optimal amplification profiles. This led to the identification of a combined total of 95 loci, along with the successful sequencing of five cpDNA regions, all achieving a satisfactory level of resolution. Thus, the selected molecular markers are suitable to study of the genetic diversity of populations and phylogeography of *C. pubiflora* and other species of the genus.

Key words: *Ceiba pubiflora*; conservation genetics; DNA sequencing; Inter Simple Sequence Repeat

Marcadores moleculares nucleares e de cloroplasto para uma árvore tropical em florestas secas

RESUMO: As florestas tropicais sazonalmente secas (FTSS) em afloramentos de calcário são formações peculiares sujeitas à degradação devido a extração de madeira e de calcário para produção de cimento. A espécie *Ceiba pubiflora* (Malvaceae) tem ocorrência comum em afloramentos calcários no Brasil. Este trabalho teve como objetivo selecionar primers do genoma nuclear Entre Sequencias Simples Repetidas (ISSR) e do DNA de cloroplasto (cpDNA) para aplicação em estudos de diversidade genética e inferências filogeográficas em populações naturais de *C. pubiflora*. Após a extração do DNA, as amostras foram submetidas à reação em cadeia da polimerase (PCR), eletroforese e sequenciamento. Ao todo, foram obtidos oito primers ISSR que apresentaram melhor perfil de amplificação, resultando em um total de 95 locus e cinco regiões de cpDNA sequenciadas com resolução satisfatória. Assim, os marcadores moleculares selecionados são adequados para subsidiar estudos de diversidade genética de populações e filogeografia de *C. pubiflora* e outras espécies do gênero.

Palavras-chave: *Ceiba pubiflora*; conservação genética; sequenciamento de DNA; Entre Sequências Simples Repetidas

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Introduction

The limestone outcrops in Brazil in areas with seasonal climates favour the occurrence of forest formations on these rock structures. In these environments, tree species are naturally selected for their resistance to drought, some of which are restricted to rocky environments and mesic conditions. This process also occurs in other phytogeographic domains, such as the Cerrado and Atlantic Forest (Aguiar-Campos et al., 2020). In these domains, limestone outcrops are perceived to be covered by seasonally dry tropical forests (SDTFs), having a peculiar structure and floristic composition. However, it is similar to the surrounding areas and shares characteristic and persistent species of SDTFs (Fagundes et al., 2020).

Among the tree species in SDTFs on limestone outcrops in central Brazil *Ceiba pubiflora* (A.St.-Hil.) K. Schum (Malvaceae) stands out in the landscape (Figure 1). The species has a significant ecological value in terms of conservation because of its unique capability of growing on rocky and seasonally water-deficient soils (Fagundes et al., 2020). Trees that inhabit adverse sites, such as *C. pubiflora*, usually store water in the trunk and commonly flower and fruit in the dry season with individuals devoid of foliage. These leaves only appear during the rainy season's beginning (Lima & Damasceno-Junior, 2020). In the dry season (June to August), the flowering of *C. pubiflora* is massive and synchronous (Lima & Damasceno-Junior, 2020), and its anthesis is diurnal. These characteristics attract many visitors, especially bees and hummingbirds (e.g., *Hylocharis chrysura* and *Heliomaster furcifer*) (Previatto et al., 2016), becoming a primary source of resources also for other species that feed on flowers that fall to the ground after being fertilized. The seeds are light and surrounded by hairy arils that help in anemochory dispersion (Gibbs & Semir, 2003). Besides ornamental beauty, the species is a candidate for afforestation and can favour the development of wildlife in the urban environment, providing food and promoting city biodiversity (Silva, 2018).

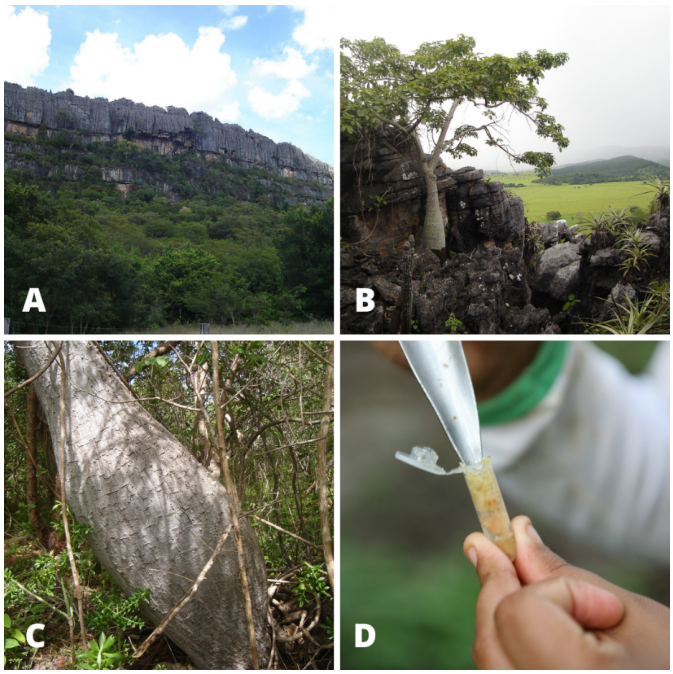

Figure 1. Limestone outcrop (A), *Ceiba pubiflora* (B), trunk detail (C) and vegetal tissue for DNA extraction.

The analysis of genetic variability is necessary to define ecosystem conservation strategies, mostly for ecologically essential species that occur in economically relevant environments, such as limestone outcrops. Genetic diversity is measured using molecular markers, such as isoenzymes, RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphisms), ISSR (inter simple sequence repeat), microsatellite (SSR), besides sequencing of conserved regions of molecules such as chloroplast DNA (cpDNA), which allow historical inferences about gene flow and dispersion pattern for the species under study. Markers between simple sequence repeat (ISSR) use a primer composed of di-, tri-, tetra- or pentanucleotide repetitions, with or without an anchoring sequence of 1 to 3 nucleotides that have microsatellites as a target sequence (Adhikari et al., 2017). The ISSR marker has been frequently used in tree population genetics studies (Pádua et al., 2021; Santos et al., 2021).

Studies on the evolution of the chloroplast genome have revealed a high degree of conservation in size, structure, gene content and linear order of genes between related species (Dobrogojski et al., 2020), making it suitable for phylogeography analyses (Vieira et al., 2015). The conservative mode of cpDNA evolution suggests that any change in genome structure, arrangement, or content can have significant phylogenetic implications. Unlike the nuclear genome evaluated by alloenzymatic or DNA markers, cpDNA is usually of uniparental inheritance; in angiosperms, it is often maternal but not exclusive (Vinson et al., 2018).

Thus, the tree species *C. pubiflora* was chosen for this study to determine molecular markers of cpDNA and ISSR for application in species genetic diversity studies. It is hypothesized that both markers provide an adequate visualization pattern of loci and polymorphism sufficient for population genetics analysis.

Materials and Methods

Sampling

For the study of the selection of ISSR and cpDNA primers, leaf samples of *C. pubiflora* were collected from nine populations in the Brazilian States of Minas Gerais, Bahia, and Goiás (Table 1, Figure 2). The selection of these sites aimed to accentuate the potential genetic polymorphism present within our sample. Through trails in the evaluated limestone outcrops, all individuals found of *C. pubiflora* were sampled. Therefore, the sample size reflected the species' population size at the sites. The leaf samples were placed in identified plastic bags containing silica gel for tissue dehydration. At the end of the sampling, the samples were transported and

Table 1. Locations and sample size in the *C. pubiflora* populations. n = Sample size.

Populations	Code	State	n	Coordinates (S/W)		Biome
Bom Jesus da Lapa	BJL	BA	03	13º03'13.9"S	43º17'28.6"W	Caatinga
Montalvânia	MON	MG	16	14º28'21.0"S	44º22'14.8"W	Caatinga
Januária	JAN	MG	16	15º08'03.1"S	44º14'58.4"W	Caatinga
Nova Roma	NOR	GO	10	13º42'25.7"S	46º51'14.5"W	Cerrado
Vila Propício	VIP	GO	19	15º29'14.0"S	48º51'52.7"W	Cerrado
Santo Hipólito	SAH	MG	16	18º17'23.7"S	44º11'13.9"W	Cerrado
Matozinhos	MAT	MG	13	19º33'08.9"S	44º04'14.4"W	Atlantic
Arcos	ARC	MG	03	20º19'52.5"S	45º34'43.8"W	Atlantic
Doresópolis	DOR	MG	03	20º18'25.2"S	45º55'08.5"W	Atlantic
Total			101			

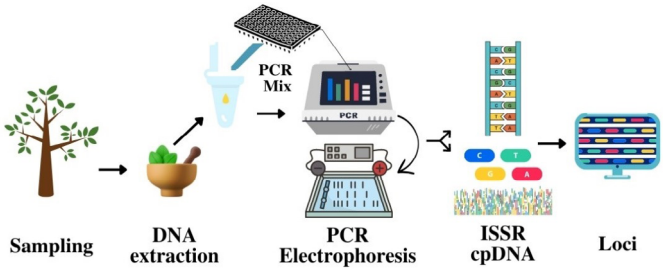

Figure 2. Flowchart showing the procedures for selection of ISSR and cpDNA primers for *Ceiba pubiflora*.

stored in the laboratory until DNA extraction. The access to the genetic heritage of this research was registered in Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen) and identified by the code A77014D.

DNA extraction

Genomic DNA was extracted from the samples of the 106 individuals of *C. pubiflora* by the method described in Doyle and Doyle (1987), with some modifications, where: 100 mM of Tris pH 8.0; 1.4 M NaCl; 20 mM EDTA pH 8.0; 2% (w/v) CTAB; 1% (w/v) PVP-40 and 0.2% (v/v) of β-mercaptoethanol preheated at 65 ºC. After extraction, the DNA was solubilized in TE (Tris mM; EDTA mM) and stored at -20 °C until the PCR-ISSR reaction. The concentrated DNA was diluted in TE at a final concentration of approximately 5 ng μL^{-1} for PCR-ISSR and cpDNA reactions.

PCR-ISSR

The DNA amplification was made using the polymerase chain reaction (PCR-ISSR), consisting of 10 ng μL^{-1} of each DNA sample, reaction buffer 1x (Tris-HCl 500 mM pH 8,0, KCl 200 mM, BSA 2,5 mg mL^{-1}, Tartrazine 200 mM and 1% Ficoll), $MgCl_2$ 2.6 mM, dNTPs 0,25 mM, Taq DNA Polymerase 0.125 U μL^{-1} and primer ISSR 0.4 µM, in a total volume of 12 µL. The PCR amplification program consisted of an initial denaturation at 94 ºC for 2 minutes and 37 cycles with denaturation at 94 ºC for 15 s, annealing at 42 ºC for 30 s and extension at 72 ºC for 1 minute, with a final extension at 72 ºC for 7 minutes. We tested fifteen ISSR primers. The amplified DNA fragments were separated by electrophoresis in 1.5% agarose gel in 0.5X TBE solution (Tris 90 mM, boric acid 92 mM, and EDTA 2.5 mM), stained with ethidium bromide, photographed under ultraviolet light, and analysed using the UVP Doc-It-LS software.

PCR-cpDNA

We tested thirteen universal primers pairs of cpDNA (forward and reverse) for *C. pubiflora* (Table 3). We used the final volume of 15 µL for the PCR-cpDNA amplification reaction, consisting of 10X IB reaction buffer (Phoneutria – KCl 500 mM; Tris-HCl 100 mM pH 8.4; Triton X-100 1%; $MgCl_2$ 15 mM); BSA (2.5 ng mL^{-1}); dNTPs at 2.5 mM, and each primer at 1 µM; Taq polymerase 3.4 U μL^{-1} (Phoneutria); DNA at approximately 5 ng μL^{-1}.

We used the ABI GeneAmp Thermal Cycler 9700 to make the PCR. The programme consisted of an initial denaturation at 94 ºC for 5 minutes, followed by 35 cycles with denaturation at 94 ºC for 1 minute, annealing at 53 ºC for 1 minute and extension at 72 ºC for 1 minute and 30 s, final extension at 72 ºC for 10 minutes. The amplification product was put through electrophoresis on a 1% agarose gel in 0.5 X TBE solution to verify the success of the PCR reaction, stained with ethidium bromide, and photographed under ultraviolet light using the UVP Doc-It-LS photo documentation system (Figure 3). The amplified fragments were sequenced in an ABI 3500 automated sequencer to verify the size of the fragment (base pairs, bp).

Results and Discussion

The DNA extraction method proposed by Doyle & Doyle (1990) proved efficient for the species. Eight of the 15 ISSR primers tested presented a clear profile for identification in all individuals evaluated (Table 2, Figure 3). These primers generated a total of 95 loci, ranging from 6 to 19 loci per primer, with an average of 12 loci.

The number of ISSR loci used in studies of the genetic diversity of tree species is variable (Fajardo et al., 2018; Freire et al., 2010). A review of studies on the genetic diversity of forest species, analysed using ISSR markers, found a median of 10 primers and 102 loci (Chagas et al., 2023), numbers close to the present study (8 and 95, respectively). The ISSR markers in this study proved efficient for quantifying the genetic diversity of *C. pubiflora* and identifying polymorphism

Table 2. Primers used for amplification of genomic DNA of *C. pubiflora*, their sequences and the number of loci amplified.

Primer ISSR	Sequence (5'-3')	Number of loci
UBC 825 (AC)8T	ACA CAC ACA CAC ACA CT	14
UBC 835 (AG)8-YC	AGA GAG AGA GAG AGA GYC	8
UBC 842 (GA)8-YG	GAG AGA GAG AGA GAG AYG	11
UBC 857 (AC)8YG	ACA CAC ACA CAC ACA CYG	13
UBC 888 BDB(CA)7	BDB CAC ACA CAC ACA CA	19
OMAR (GAG)4-RC	GAG GAG GAG GAG RC	17
CHRIS (CA)7-YG	CAC ACA CAC ACA CA**Y G**	7
UBC 841 (GA)8-YC	GAG AGA GAG AGA GAG A**YC**	6
Total/Average		95/12

R = purine (A or G); Y = pyrimidine (C or T); B = (C, G, T, i.e., not A); D = (A, G, T, i.e., not C).

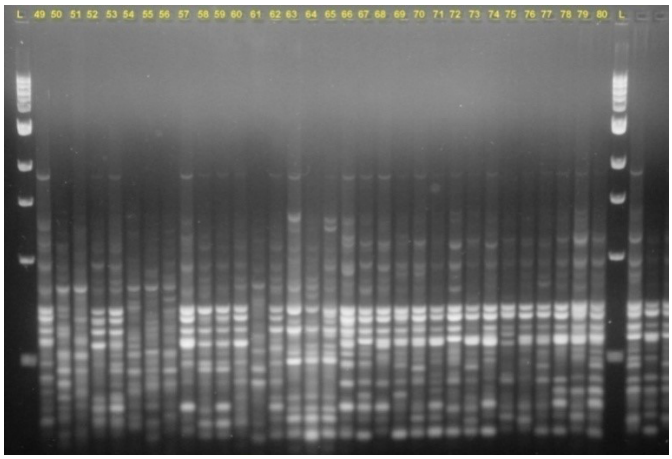

Figure 3. Amplified DNA of *C. pubiflora* (individuals number 49-80) for the primer ISSR Omar. L = molecular weight marker 1 Kb.

between individuals in populations, considering the pattern obtained from the loci (Figure 3).

The best-performing primers for PCR-cpDNA amplification for *C. pubiflora* were EF, CD, HA, CY6, CS3, BF, QS2 and 20-12 (Figure 4, Table 3). The intergenic regions of the chloroplast are valuable sources of information for studies in population genetics, biogeography, and conservation biology (Vieira et al., 2015; Buzatti et al., 2017). These regions offer insights into the genetic variability and evolutionary processes that shape populations and species. It is important to select, among the universal primers of cpDNA, those that can reveal the information necessary, i.e., optimal-performing and polymorphic markers. This selection is vital for quantifying genetic diversity and establishing centers of refuge or species diversity, thereby contributing to cost-effectiveness in the methodologies implementation (Vieira et al., 2015). Similar studies have carried out investigations in this direction, with the objective of selecting optimal DNA extraction methods, primers, and polymorphic regions (Vieira et al., 2010; Felix et al., 2020).

Some techniques can be employed to access the variation in the chloroplast DNA, such as restriction enzyme digestion or the digestion of specific regions amplified by PCR, delimited by cpDNA primers (Ngondya et al., 2013). The development of universal primers for specific regions of cpDNA was an important advance to improve the techniques of studies in phylogeography based on variations in the sequence of these molecules. Yang et al. (2015) mention the main contributions to developing these primers and present other perspectives on the design of new primers. Specifically, the selected ISSR and cpDNA primers subsidize other genetic population approaches for species of the *Ceiba* Miller genus, which has about seventeen species with Neotropical distribution and 11 species of *Ceiba* registered in Brazil (Gibbs & Semir, 2003).

In this study, of the eight amplified cpDNA regions, five were successful in the automatic sequencing: EF, with an approximate size of 470 pb; CD, with 600 pb; CY6, with 610 pb; CS3, with 940 bp; and BF, with 800 pb. We selected the region trnQ–5'rps16, which presented a considerable length (960 bp) and the highest number of polymorphic sites. Then, there are several possibilities for using these cpDNA regions together, increasing the chance of obtaining polymorphism for the species of interest. For example, Vieira et al. (2015) selected the region trnQ-5'rps16, which presented a considerable length (960 bp) and the highest number of polymorphic sites. Recent studies indicated that using at least

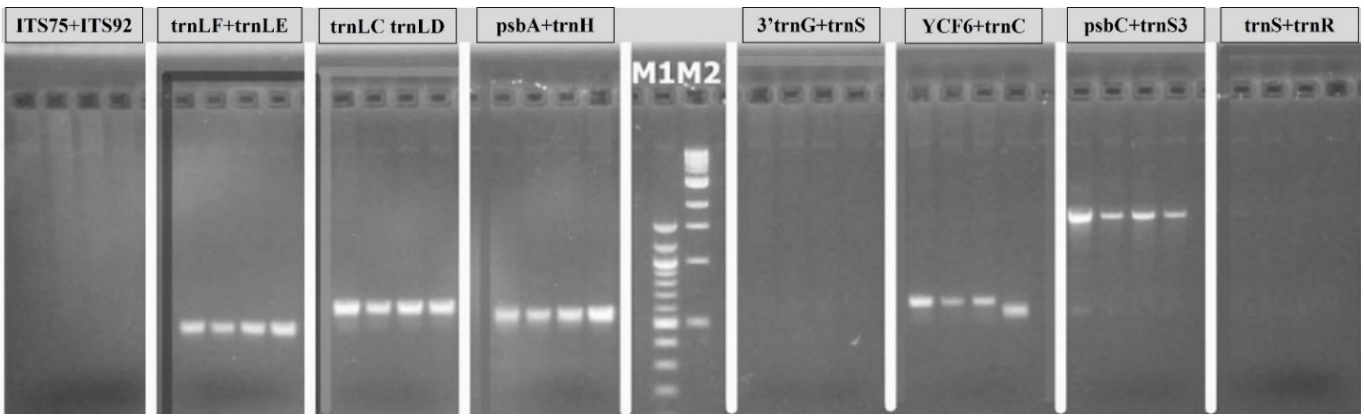

Figure 4. Fragments of cpDNA amplified in four individuals of *C. pubiflora*. The cpDNA primers representation is the pairs ITS75 + ITS92, trnLF + trnLE, trnLC + trnLD, psbA + trnH, 3'trnG + trnS, YCF6 + trnC, psbC + trnS3 and trnS + trnR. Molecular weight marker M1= 100 bp and M2 = 1 Kb.

Table 3. Name and sequence of universal primers of cpDNA, amplification status (Amplif.), and size (bp) observed in samples of *C. pubiflora*. NS = Not Sequenced.

Primer	Name	Sequence 5'-3'	Amplif.	Size (bp)
ITS 92	ITS 92-75*	AAG GTT TCC GTA GGT GAA	No	–
ITS 75		TAT GCT TAA ACT CAG CGG G		
trnLE	EF	GG TTC AAG TCC CTC TAT CCC	Yes	470
trnLF		ATT TGA ACT GGT GAC ACG AG		
trnC	CD	CCA GTT CAA ATC TGG GTG TC	Yes	600
trnD		GGG ATT GTA GTT CAA TTG GT		
trnH	HA	GTT ATG CAT GAA CGT AAT GCT C	Yes	NS
psbA		CGC GCA TGG TGG ATT CAC AAA TC		
trnS	SG	GCC GCT TTA GTC CAC TCA GC	No	–
trnG		GAA CGA ATC ACA CTT TTA CCA C		
ycf6	CY6	GCC CAA GCR AGA CTT ACT ATA TCC AT	Yes	610
trnC		CCA GTT CRA ATC YGG GTG		
psbC	CS3	GGT CGT GAC CAA GAA ACC AC	Yes	940
trnS3		GGT TCG AAT CCC TCT CTC TC		
trnS	SR	CGC CGC TTT AGT CCA CTC A	No	–
trnR		ATT GCG TCC AAT AGG ATT TGA A		
psbB	BF	GTT TAC TTT TGG GCA TGC TTC G	Yes	800
psbF		CGC AGT TCG TCT TGG ACC AG		
trnQ	QS2	GGG ACG GAA GGA TTC GAA CC	Yes	NS
trnS2		ATT GCG TCC AAT AGG ATT TGA A		
rpl 20	20-12	CGY YAY CGA GCT ATA TAT CC	Yes	NS
rps 12		ATT AGA AAN RCA AGA CAG CCA AT		
trnV	VL	CGA ACC GTA GAC CTT CTC GG	No	–
rbcL		GCT TTA GTC TCT GTT TGT GG		
trnF	FV	CTC GTG TCA CCA GTT CAA AT	No	–
trnV		CCG AGA AGG TCT ACG GTT CG		

* Nuclear DNA region fragment.

two cpDNA regions is essential to obtain satisfactory results in phylogeographic investigations (Morris & Shaw, 2018).

The ease of using universal primers in the polymerase chain reaction is associated with the primer pairs being in conserved regions of cpDNA, being a molecule that presents a low rate of molecular evolution and generally well-conserved structure (Dobrogojski et al., 2020). On the other hand, alterations such as deletion, insertion, or inversion of nucleotide bases in the annealing sites of these primers can interfere with the amplification of the fragment. These events can explain the non-amplification of some regions delimited by them (Table 3). These changes along the cpDNA molecule are significant for phylogeographic studies because they have allowed inferences about species evolutionary history, favouring the definition of genetic conservation strategies (Rico et al., 2021; Barrandeguy et al., 2022). The ISSR and cpDNA primers selected within our study are potentially useful for genetic analyses within other species of the genus *Ceiba*. Nevertheless, a transferability analysis must be conducted to substantiate this hypothesis and establish the applicability of these primers in other related species.

Conclusions

The protocol proposed for the PCR-ISSR and cpDNA reactions showed a good amplification pattern. The ISSR primers that offered the best profile for genotyping were UBC 825, 835, 841, 842, 857, 888, OMAR, and CHRIS. The selected primers of cpDNA were EF, CD, CT6, CS3, and BF. These results will support studies on the genetic and phylogeographic diversity of *C. pubiflora*, a tool for indicating conservation units of the species.

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Compliance with Ethical Standards

Author contributions: Conceptualization: MMB, FAV, RMS, DC; Data curation: MMB, FAV, PAM; Formal analysis: MMB, FAV, CGF; Funding acquisition: AFML, RMS, DC; Investigation: MMB, FAV, DC; Methodology: MMB, PAM, DC; Project administration: MMB, DC; Resources: MMB, FAV, DC; Resources: MMB, FAV, AFML, DC; Software: MMB, FAV, CGF; Supervision: RMS, DC; Validation: MMB, FAV, PAM, CGF, AFML, RMS, DC; Visualization: MMB, FAV, CGF; Writing - original draft: MMB, FAV, CGF; Writing - review & editing: MMB, FAV, PAM, CGF, AFML, RMS, DC.

Conflict of interest: The authors have declared that there is no possible conflict of interest (professional or financial) that could influence the article.

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