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Abstract

*Uncaria guianensis* is native to the Amazon and is used traditionally as an anti-inflammatory. Natural populations of the species have declined markedly in recent times because of strong anthropic pressure brought about by deforestation and indiscriminate collection. The aim of the present study was to assess the genetic and chemical diversity among eight natural populations of *U. guianensis* located in the Brazilian states of Acre, Amapá and Amazonas. A set of four primer combinations was employed in sequence-related amplified polymorphism (SRAP) amplifications of leaf DNA, and the fragments were analyzed in an LI-COR model 4300 DNA Analyzer. Genetic variability within the populations (81%) was substantially greater than that detected between them (19%). The highest percentage of polymorphic loci (90.21%) and the largest genetic variability were observed in the population located in Mazagão, Amapá. Genetic differentiation between populations was high ($F_{st} = 0.188$) and the studied populations formed three distinct genetic groups ($K = 3$). The population located in Assis Brasil, Acre, presented the highest average content of the mitraphylline (0.60 mg/g dry weight,). However, mitraphylline and isomitraphylline not detected in most individuals in the studied populations, and it is questionable whether they should be considered as chemical markers of the species. The genetic data confirm the urgent need for conservation programs for *U. guianensis*, and for further studies aimed at ascertaining the genetic basis and heritability of alkaloid accumulation.
Introduction

Uncaria guianensis (Aubl.) J.F.Gmel. (Rubiaceae), commonly known as *uña de gato*, is endemic to the Amazonian regions of Bolivia, Brazil, Colombia, Ecuador, Guiana, French Guiana, Peru, Suriname and Venezuela [1]. The plant, which grows as a woody vine that can attain a height of 5–10 m, is used by indigenous populations to treat asthma, arthritis, dermatitis, diabetes, gastritis, inflammation of the genitourinary tract, tumors and ulcers [2]. Pharmacological studies have verified the anticancer, antidiabetic, antimicrobial, anti-inflammatory, antioxidant, anti-Parkinson and immunostimulant effects of the extracts [3]. These properties have been attributed to the presence of pentacyclic oxindole alkaloids (POA), mainly mitraphylline and isomitraphylline, which are recognized as chemical markers of *U. guianensis* [4–6].

Natural populations of *U. guianensis* have declined dramatically in recent times because of strong anthropic pressure brought about mainly by deforestation and indiscriminate extraction of the bark for the commercial production of phytotherapeutic preparations [7,8]. In this context, studies on the genetic and chemical variability of medicinal plants are particularly important since they enable the selection of elite individuals that would be of interest to the pharmaceutical industry [9]. Furthermore, in the field of species conservation, molecular markers such as sequence-related amplified polymorphism (SRAP) are very useful for the identification of genetically distinct individuals with biotechnological potential [10,11]. The SRAP technique is based on five forward and six reverse primers that can be combined randomly for the amplification of a large number of open reading frames. Moreover, the SRAP method is reliable, reproducible and does not require prior knowledge of the genome [12].

Considering the ethnopharmacological and industrial importance of *U. guianensis*, investigations on the genetic and chemical diversity of the species would be of significant interest. Thus, the objectives of this study were to analyze the genetic diversity of natural populations of *U. guianensis* using SRAP markers and to determine the concentrations of mitraphylline and isomitraphylline in leaf extracts using high performance liquid chromatography (HPLC). The results of our study will contribute to the selection of individuals that could be considered elite in respect of POA concentrations and to ascertain the need for immediate conservation strategies.

Materials and methods

Plant material

The collection of specimens of *U. guianensis* for use in the study was authorized by the Conselho de Gestão do Patrimônio Genético/Ministério do Meio Ambiente (CGEN/MMA; protocol no. 010102/2015-9) through the offices of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). A total of 157 specimens of *U. guianensis* were collected from eight populations located at different sites in the Amazonian region of Brazil, namely Boca do Acre in Amazonas state, Assis Brasil, Cruzeiro do Sul, Feijó, Rio Branco and Xapuri in Acre state, and Mazagão and Oiapoque in Amapá state. The identification codes of the samples are presented in Table 1 together with the geographical coordinates and altitudes of the sampling sites, while the locations of the sites are mapped in Fig 1. Specimens were identified by one us (PGD; Herbier de Guyane, Institut de Recherche pour le Développement, Cayenne, French Guiana) and voucher specimens deposited at the Herbarium of the Universidade de Ribeirão Preto (UNAERP) with identification codes HPMU2844 to 2926.

Young healthy leaves were collected from each specimen and either stored in labeled test tubes in the freezer at -20°C until required for DNA extraction and SRAP analysis, or dried at the desiccator.
45°C in a forced-air oven (Marconi, Piracicaba, SP, Brazil) for HPLC analysis. All experiments were performed in the Molecular Biology and Phytochemical Laboratories of the Biotechnology Department at UNAERP.

SRAP analysis

Genomic DNA was extracted from leaf samples (100 mg) using the cetyltrimethylammonium bromide (CTAB) method [13]. The integrity of extracted DNA was evaluated by electrophoresis on 1% agarose gels in 1 X Tris/Borate/EDTA (TBE) buffer, and quantitative evaluation was performed spectrophotometrically using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were subsequently diluted to 5 ng/μl and submitted to SRAP analysis as described by Li and Quiros [12]. Four combinations of published forward (me) and reverse (em) primers (Table 2) that produced sharp bands with a high percentage of polymorphism were selected after testing six initial primer pairs on samples from two individuals of each population.

For all four primer pairs, polymerase chain reactions (PCR) were performed with reaction mixtures containing 1 μl of 10 X reaction buffer, 0.8 μl of MgCl₂ (25 mM), 1 μl of dNTP mixture (2.5 mM), 0.4 μl of forward primer (5 μM), 0.4 μl of reverse primer (5 μM), 0.2 μl of Taq DNA polymerase (5 U/μl), 1 μl of DNA template (5 ng/μl) and deionized water to a final volume of 10 μl. Amplification procedures involved 5 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min.
Amplicons were denatured at 95˚C for 5 min and an aliquot (0.6 μl) of each sample was applied to a KB Plus 6.50% Gel Matrix (LI-COR Biosciences, Lincoln, NE, USA) together with a 50–700 bp DNA ladder (0.8 μl). Electrophoresis was performed at 1.500 V and 40 W for 2:30 h at a constant temperature of 45˚C in an LI-COR model 4300 DNA Analyzer. Primers were labeled with LI-COR 700 and 800 nm infrared dyes to allow collection of fluorescent images in real time during electrophoresis. Image data were viewed, analyzed and converted into numerical data files using LI-COR SAGA\textsuperscript{MX} automated analysis software version 3.3.

Extraction and quantification of mitraphylline and isomitraphylline

A modified version of the method of Bertol et al. [14] was employed to extract mitraphylline and isomitraphylline from dried leaves of \textit{U. guianensis} that had been reduced to a fine powder.
in a Marconi MA048 cutting mill fitted with a 40 mesh sieve. Powdered leaf material (100 mg) was mixed with 1 ml of methanol (J.T. Baker HPLC grade; Avantor Performance Materials, Center Valley, PA, USA) in an amber flask and submitted to static maceration at room temperature (22 ± 1˚C) for 24 h, following which the mixture was filtered and the filtrate reduced to dryness in a fume cupboard. Triplicate extractions were performed for each of the studied specimens.

Samples (15 mg) of dried extracts were redissolved in 1 ml of an 80: 20 (v/v) mixture of methanol (J.T. Baker HPLC grade) and Milli-Q Ultrapure water (Merck Millipore, Darmstadt, Germany) and applied to Supelco LC-18 solid-phase extraction (SPE) tubes (Sigma, St. Louis, MO, USA) that had been previously eluted with 1 ml of methanol followed by 1 ml of 80:20 (v/v) methanol:water mixture. Tubes were subsequently eluted with 3 ml of 80:20 (v/v) methanol:water mixture and 20 μl aliquots of the eluents (5 mg/ml) were analyzed by HPLC on a Shimadzu (Kyoto, Japan) model LC-10ADvp instrument coupled to an SPD-M10Avp diode array detector (DAD). Separations were carried out at room temperature (22 ± 1˚C) on a Zorbax Eclipse XDB-C18 column (150 x 4.6 mm i.d., 5 μm; Agilent, Santa Clara, CA, USA) protected by a Zorbax Eclipse XDB-C18 pre-column (4.6 x 12.5 mm i.d., 5 μm). The mobile phase comprised 10 mM aqueous ammonium acetate (pH adjusted to 6.9 with triethanolamine) (solvent A; Neon Comercial, São Paulo, Brazil) and acetonitrile (solvent B; J.T. Baker HPLC grade) and was supplied at a continuous flow rate of 0.8 ml/min according to the program: 35% B between 0.01 and 18.00 min, 50% B between 18.01 and 25.00 min, 35 to 100% B from 25.01 to 40 min, and 35% B between 40.01 and 45 min. The detection wavelength was set at 245 nm and the acquired data were processed using Shimadzu LabSolutions Multi LC-PDA software.

POA content was determined by a previously validated HPLC-DAD method [15] using mitraphylline (LGC Standards, Teddington Middlesex, UK; # CDX 00013955–005) and isomitraphylline (Chromadex, Irvine, CA, USA; # ASB-00009417-005) as external standards. Analytical data were validated with respect to linearity, precision and accuracy according to the guidelines issued by the Agência Nacional de Vigilância Sanitária [16], and limits of detection (LoD) and quantitation (LoQ) were determined to be 0.02 and 0.07 μg/ml, respectively, for mitraphylline and 0.01 and 0.02 μg/ml for isomitraphylline. Standard solutions containing POAs at concentrations of 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9 μg/ml were prepared and calibration curves constructed by subjecting each solution to HPLC analysis in triplicate. The ratio of peak areas of mitraphylline and isomitraphylline standards were calculated and plotted against the corresponding standard concentrations using linear regression of the standard curves.

Statistical analyses

The sequence data were used to perform analysis of molecular variance (AMOVA) in order to decompose the total genetic variance to within and between population components. Descriptive analysis of total variability was obtained by calculating the percentage of polymorphic loci, the observed number of alleles (N_a), the effective number of alleles (N_e), Nei’s genetic diversity index (H) and Shannon’s diversity index (I). The unweighted pair group method with arithmetic mean (UPGMA) was used to group populations according to genetic divergence estimated from Nei’s genetic distances [17]. Geographical distances were calculated with the help of TrackMaker software version 13.8 (Geo Studio Tecnologia, Belo Horizonte, MG, Brazil).

Variability and genetic structure of populations were investigated through principal coordinate analysis (PCoA) using the software packages GenAlEx version 6.5 [18] and STRUCTURE version 2.2.4 [19,20]. The most likely number of population groups was established using the Bayesian model-based clustering algorithm (in which individuals are assigned to K population
genetic clusters based on their nuclear multilocus genotypes) and the admixed ancestry model. For each run, the initial burn-in was 200,000 iterations followed by a run-length of 500,000 iterations for \( K = 1 \) to 10 population genetic clusters.

Data relating to the accumulation of mitraphylline and isomitraphylline within and between populations were submitted to analysis of variance (ANOVA) and, when significant differences were detected, mean values were compared using the Scott-Knott test at 5% probability. A dendrogram was constructed using the UPGMA clustering method to establish the organization of chemical variables among the evaluated populations. The matrix-based cophenetic correlations produced by the UPGMA dendrogram were calculated using the \textit{vegan} and \textit{ecodist} R packages \[21\]. The Euclidian distance matrix of chemical data (POA content) was correlated with geographical distance, altitude and Nei’s genetic distance matrices. Pairwise relationships between populations were evaluated using simple Mantel tests with 10,000 permutations performed with the aid of \textit{vegan}, \textit{fields} and \textit{ecodist} R packages.

**Results and discussion**

**Genetic diversity within and between \textit{U. guianensis} populations**

Of the four primer pairs studied, me1/em3 generated the highest number of bands, while me3/me3 produced the smallest number, yielding 235 amplified bands with 100% polymorphism (Table 2). The highest percentage variability (90.21%) was observed within the Mazagão population (MZ), which was collected from an igarapé (a narrow, shallow tributary) located in a non-anthropized area (Table 3 and S1 Table). In contrast, the lowest percentage variability (39.57%) was observed within the Assis Brazil population (AB), which is located on the margins of the BR317 highway (Table 3 and S1 Table).

Mantel tests revealed a positive but not significant \((r = 0.33; P > 0.0308)\) correlation between geographical and genetic distances among the eight \textit{U. guianensis} populations (Table 4), indicating that the geographical distribution of the populations did not influence the organization of their genetic variability. In general, geographic distribution and evolutionary history play important roles in the genetic structure of a population \[22\].

The results from AMOVA (Table 5) revealed that 19% of the observed genetic variability was associated with the between population component, as shown by the \(F_{ST}\) value of 0.188 \((P > 0.001)\). On this basis, the conservation of \textit{U. tomentosa} should prioritize the collection of as many individuals as possible within populations that presented the highest genetic diversity.
variability. For allogamous species, the within population genetic variability is expected to be high [23], hence it is likely that *U. guianensis* is allogenic, although the mechanism of fertilization of this species has yet to be described. It is important to emphasize that during the collection of specimens of *U. guianensis* for the present study, we observed extensive areas of fragmented forest, a situation that will certainly lead to the diminution of species diversity in the medium term.

The genetic differentiation between populations of *U. guianensis* (F$_{ST}$ = 0.188) was high but slightly lower than that observed for *U. tomentosa* (F$_{ST}$ = 0.246) [8]. However, this index can be very variable among species of the same family and/or genus that inhabit a common biome, as has been described for populations of the Amazonian medicinal plant *Psychotria ipecacuanha* (Rubiaceae), which presented high F$_{ST}$ values ranging from 0.355 to 0.457 [24].

The UPGMA dendrogram (Fig 2), the PCoA plot (Fig 3) and Bayesian analysis performed using STRUCTURE software (Fig 4) revealed that the eight populations of *U. guianensis* tended to form three genetic clusters (K = 3). The formation of three genetic clusters clearly demonstrates that the environment was fragmented by human interference, rendering the species vulnerable to genetic erosion and indicating the need to implement conservation strategies.

**Variation of POA content within and between *U. guianensis* populations**

Although mitraphylline and isomitraphylline are considered to be chemical markers of *U. guianensis*, the concentrations of these POAs varied considerably within the populations studied (S1 Fig). Some individuals (7.6%) accumulated only isomitraphylline, others (11.5%) accumulated only mitraphylline, a few (12.7%) accumulated both alkaloids, while most (68.1%) accumulated neither, as for example the members of the MZ and OI populations (Table 6).

Table 4. Geographical and genetic distances between populations of *Uncaria guianensis* collected in the Amazonian region of Brazil.

|       | RB   | BC   | MZ   | OI   | CS   | FJ   | XA   | AB   |
|-------|------|------|------|------|------|------|------|------|
| RB    | 111  | 2088 | 2274 | 644  | 359  | 112  | 242  |
| BC    | 0.0191 | 2018 | 2183 | 623  | 349  | 217  | 316  |
| MZ    | 0.0493 | 0.0460 | 398  | 2534 | 2296 | 2186 | 2341 |
| OI    | 0.0784 | 0.0558 | 0.0375 | 2624 | 2409 | 2353 | 2487 |
| CS    | 0.0519 | 0.0475 | 0.0478 | 0.0455 | 279 | 622  | 525  |
| FJ    | 0.0441 | 0.0452 | 0.0514 | 0.0519 | 0.0123 | 378  | 321  |
| XA    | 0.0693 | 0.0640 | 0.0760 | 0.0713 | 0.0354 | 0.0460 | 146  |
| AB    | 0.0888 | 0.0732 | 0.0818 | 0.0730 | 0.0442 | 0.0636 | 0.0187 |

Geographic distances (km) are shown above the diagonal line while genetic distances are shown below the line. Population codes are defined in Table 1.

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Table 5. Genetic variability within and between populations of *Uncaria guianensis* collected in the Amazonian region of Brazil.

| Source                  | Degrees of freedom | Mean square error | Variance components | Percentage variability | P       | Fixation index F$_{ST}$ |
|-------------------------|--------------------|-------------------|---------------------|------------------------|---------|-------------------------|
| Within populations      | 149                | 25.747            | 25.747              | 81                     | > 0.001 | 0.188                   |
| Between populations     | 7                  | 143.047           | 5.980               | 19                     |         |                         |
| Total                   | 156                | 31.728            | 100                 |                         |         |                         |

Data estimated by analysis of molecular variance (AMOVA).

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The AB population accumulated the highest amounts of mitraphylline (0.60 mg/g dw) of all eight populations studied (Table 7). Interestingly, one individual (no. 17) from population BC presented five times more mitraphylline (2.69 mg/g dw) than the average of all other mitraphylline-producing individuals (0.5 mg/g dw). The discovery of POA-producing elite specimens is important because such individuals can become targets for large scale multiplication and conservation with the aim of providing quality raw material for the pharmaceutical industry.

The OI and MZ populations exhibited genetic and chemical similarity, and this profile may be related to the low altitude of these two populations (4–12 m). An association between altitude and POA content has also been observed for *U. tomentosa* in which the accumulation of these alkaloids was maximal in plants growing at altitudes above 230 m [15].

**Conclusions**

Analyses employing polymorphic SRAP markers enabled the genetic variability within and between eight natural populations of *U. guianensis* to be characterized. It was possible to detect
Fig 3. Analysis of the dispersion of the principal coordinates obtained from the Jaccard similarity matrix created with SRAP molecular markers among individuals from eight natural populations of *Uncaria guianensis*. 3.

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Fig 4. Bayesian analysis, performed using STRUCTURE software, of eight populations of *Uncaria guianensis* from the Amazon region of Brazil showing the tendency to form three clusters. Group 1 (predominantly red): Rio Branco (RB) and Boca do Acre (BC); Group 2 (predominantly green): Mazagão (MZ) and Oiapoque (OI); Group 3 (predominantly blue): Cruzeiro do Sul (CS), Feijo (FJ), Xapuri (XA) and Assis Brasil (AB).

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the presence of three genetic groups, indicating the need to develop conservation strategies in order to preserve such variability. Geographic distance did not explain the distribution of genetic variability between the studied populations. Since genetic variability was higher within populations, we suggest that the best conservation strategy would involve the collection of large numbers of individuals from populations presenting the highest variability. Although a few POA-rich individuals were detected, the majority of specimens did not accumulate mitraphylline and/or isomitraphylline. This finding suggests that these two POAs are not the best chemical markers for *U. guianensis* and that further studies are required in order to understand the heritability of chemical traits in this species for domestication and breeding purposes. The scarceness of POA-rich specimens of *U. guianensis* means that commercial production of phytotherapeutics based on this species may be problematic since it depends on the collection of specimens from natural environments without previous selection. We

**Table 6. Mean concentrations of mitraphylline (Mit) and isomitraphylline (Iso) in populations of *Uncaria guianensis* collected in the Amazonian region of Brazil.**

| Population code | Mitraphylline (mg/g dw) | Isomitraphylline (mg/g dw) |
|-----------------|-------------------------|---------------------------|
| AB              | 0.60a                   | 0.06a                     |
| BC              | 0.25b                   | 0.09a                     |
| CS              | 0.00c                   | 0.01a                     |
| FJ              | 0.03c                   | 0.03a                     |
| MZ              | 0.00c                   | 0.00a                     |
| OI              | 0.37a                   | 0.02c                     |
| RB              | 0.17b                   | 0.03a                     |

In each column, mean values bearing dissimilar letters are significantly different according to Scott-Knott test at 5% probability.

*Population codes are defined in Table 1.*

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**Table 7. Mean concentrations of mitraphylline and isomitraphylline in populations of *Uncaria guianensis* collected in the Amazonian region of Brazil.**

| Population code | Mitraphylline (mg/g dw) | Isomitraphylline (mg/g dw) |
|-----------------|-------------------------|---------------------------|
| AB              | 0.60a                   | 0.06a                     |
| BC              | 0.25b                   | 0.09a                     |
| CS              | 0.00c                   | 0.01a                     |
| FJ              | 0.03c                   | 0.03a                     |
| MZ              | 0.00c                   | 0.00a                     |
| OI              | 0.37a                   | 0.02c                     |
| RB              | 0.17b                   | 0.03a                     |

In each column, mean values bearing dissimilar letters are significantly different according to Scott-Knott test at 5% probability.

*Population codes are defined in Table 1.*

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conclude that there is an urgent need for conservation projects involving *U. guianensis*, with particular emphasis on the creation of germplasm banks.

**Supporting information**

**S1 Fig.** High performance liquid chromatographic (HPLC) analyses of leaf extracts of three specimens of *Uncaria guianensis* populations from the Amazonian region of Brazil. The chromatograms show the standards (A) mitraphylline and (B) isomitraphylline, along with extracts from specimens collected in (C) Boca do Acre, AM (BC) in which peaks labeled a correspond to mitraphylline, (D) Assis Brasil, AC (AB) in which the peak labeled b corresponds to isomitraphylline, (E) Xapuri, AC (XA) in which peaks labeled a and b correspond to mitraphylline and isomitraphylline, respectively, and (F) Rio Branco, AC (RB) in which peaks a and b are absent.

**S1 Table.** Population parameters for all four primer pairs employed in SRAP analysis of *Uncaria guianensis* populations from the Amazonian region of Brazil.

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