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ORIGINAL ARTICLE

Genetic diversity among genotypes of *Uncaria guianensis* (Aubl.) J.F. Gmel. maintained in an in vitro germplasm bank

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Abstract
Phytotherapeutic preparations from *Uncaria guianensis* (Aubl.) J.F. Gmel. (Rubiaceae) are marketed worldwide and are mainly used for their anti-inflammatory activity. The species has not yet been domesticated and is threatened by deforestation and overexploitation. It is, therefore, important to preserve and manage this genetic resource in germplasm banks, so that the extractive provision of plant material can be replaced by cultivated production. The aim of this study was to evaluate the genetic diversity among 20 genotypes maintained under in vitro conditions using 9 primers start codon targeted (SCoT) polymorphism, and to determine the concentrations of the pentacyclic oxindole alkaloids (POAs); mitraphylline and isomitraphylline in methanolic extracts by high-performance liquid chromatography (HPLC). Plantlets were cultivated on woody plant medium supplemented with 20 $g.L^{-1}$ sucrose and 4.4 µM benzylaminopurine and incubated under a 16 h photoperiod for 45 days. SCoT analysis separated the genotypes into four divergent clusters and confirmed significant genetic diversity with up to 70% dissimilarity. Moreover, HPLC revealed considerable chemical variability and allowed the separation of the tested genotypes into high, medium and low producers of mitraphylline/isomitraphylline. Genotypes with the highest concentrations of POAs originated from the state of Acre and Amapá, while those with the lowest levels were from the state of Pará. The results demonstrate that the genetic diversity within the in vitro germplasm bank is sufficient to support breeding studies, selection of elite genotypes and the large-scale multiplication of plants that could serve as feedstock for the industrial-scale production of phytomedicines.

Keywords Cat's claw · Herbal medicine isomitraphylline · Mitraphylline · Molecular marker · Rubiaceae · SCoT

Introduction

Uncaria guianensis (Aubl.) J.F.Gmel. (Rubiaceae), commonly known as cat's claw (*unha-de-gato*), is a perennial liana (Fig. 1A) that grows in the forests of South and Central America, particularly in the Amazon region (Zevallos-Pollito and Tomazello Filho 2010). The species possesses various pharmacological properties, including anti-inflammatory, anticancer and immunostimulant activities (Heitzman et al. 2005), many of which have been attributed to the presence of pentacyclic oxindole alkaloids (POAs). The major alkaloidal constituents of *U. guianensis* include mitraphylline and isomitraphylline, and these POAs are recognized as chemical markers of the species (Falkiewicz and Lukasiak 2001; Zhang et al. 2015).

U. guianensis is a forest-derived commodity that is traded worldwide by over 39 distribution companies, with much of the market in the USA, Europe and Latin America (De Jong et al. 2000). However, deforestation and indiscriminate extraction of the bark for phytotherapeutic preparations have led to a decline in native populations of the species *Uncaria tomentosa* and *Uncaria guianensis* (Honório et al. 2016). For this reason, unique conservation strategies, based mainly

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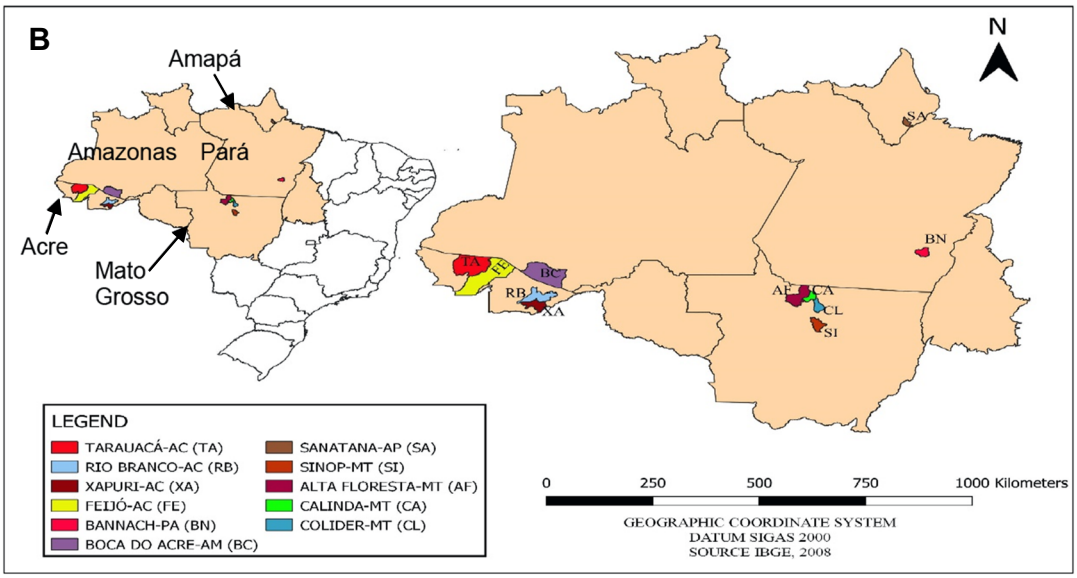

State	Municipality	Genotype code	Latitude (S)	Longitude (W)	Voucher
Acre	Rio Branco	RB	9º 54' 44.2"	67º 26' 46.8"	2506
	Feijó	FE	8º 11' 8.10"	70º 22' 26.3"	2850
	Tarauacá	TA	7º 56' 55.19"	71º 28' 51.3"	3239
	Xapuri	XA	10º 36' 2.50"	68º 00' 32.8"	2907
Mato Grosso	Alta Floresta	AF	9º 66' 0.95"	56º 01' 84.9"	3240
	Carlinda	CA	9º 97' 76.9"	55º 80' 14.1"	3130
	Colider	CL	10º 77' 49.7"	55º 45' 57.8"	3131
	Sinop	SI	11º 98' 7.30"	55º 51' 71.0"	3127
Amazonas	Boca do Acre	BC	8º 47' 52.0"	67º 17' 21.0"	2510
Pará	Bannach	BN	7º 33' 24.0"	50º 37' 12.4"	3122
Amapá	Santana	SA	0º 03' 47.5"	51º 14' 86.0"	2828

Fig. 1 **A** Aerial parts of field *Uncaria guianensis* and in vitro plantlets; and **B** locations and geographical distribution within the Brazilian Amazon of the original sampled populations of *U. guianensis*

on ex situ living collections, are warranted for this species. In this context, studies of the phenotypic, genetic and chemical variation of different genotypes are important because they not only enable the selection of elite individuals that would be of interest to the pharmaceutical industry but also provide the required germplasm to support plant breeding programs for crop improvement (Greene et al. 2014).

DNA-based markers have become very important in evaluating genetic diversity in medicinal plants, in plant breeding and provide an efficient link between phenotypic and genotypic variation (Joshi et al. 2004; Nadeem et al. 2018). Start codon targeted (SCoT) polymorphism markers, which are based on the short conserved region flanking the ATG translation initiation codon in plant genes, have proven to be particularly useful for the identification of genetically distinct individuals with economic potential (Collard and Mackill 2009; Etminan et al. 2016; Satya et al. 2015), including endangered medicinal species such as *Taxus media*, *Dendrobium nobile* and *Rauwolfia tetraphylla* (Bhattacharyya et al. 2013; Hao et al. 2018; Rohela et al. 2019).

Considering the ethnopharmacological and pharmaceutical importance of *U. guianensis*, as well the need to preserve and manage genetic diversity within the species, the aim of the present study was to analyze 20 genotypes of this species maintained under in vitro conditions in the germplasm bank of Universidade de Ribeirão Preto (UNAERP) in Brazil using SCoT markers, and to determine the concentrations of mitraphylline and isomitraphylline in plant extracts using chromatographic techniques. The practical significance of our study lies in its implications for: (i) the conservation of a medicinal Amazonian species that is threatened by habitat loss and overexploitation, and (ii) the possibility of providing feedstock to the phytomedicine industry in a sustainable and non-extractive manner.

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. A1772CE) through the offices of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Specimens voucher samples were deposited at the Herbarium of the Universidade de Ribeirão Preto (UNAERP).

The in vitro germplasm bank at UNAERP was established from seeds of *U. guianensis* obtained from 11 populations originating from the Brazilian States of Acre (AC), Mato Grosso (MT), Amazonas (AM), Para (PA) and Amapá (AP) (Fig. 1B). Each population contributed with five individuals, each of which provided 30 seeds. The seeds were soaked in 0.5% (w/v) Cercobin® solution (Iharabras, Sorocaba, SP, Brazil) under constant shaking for 24 h, followed by 1% (w/v) sodium hypochlorite solution for 20 min. Seeds were subsequently rinsed in sterile distilled water and transferred to test tubes containing woody plant medium (Lloyd and McCown 1981) supplemented with 20 $g.L^{-1}$ of sucrose (WP) and 2.5 $g.L^{-1}$ of Gellan Gum (Gellex®) (BidScientific, Lakeway, TX, USA) pH 6.0. Incubation was carried out in the growth room at 22 ± 3 ºC and 16 h photoperiod (25 µmol/m^2 /s) provided by white fluorescent lights. A total of 200 single seed-derived genotypes were selected on the basis of their vigor to compose the germplasm bank and were further analyzed by high-performance liquid chromatography (HPLC) to determine the levels of POAs, including mitraphylline, isomitraphylline, speciophylline, uncarine F, pteropodine and rhynchophylline (data not shown). To establish a germplasm bank, the genotypes were grown under photoautotrophic growth conditions (µmol*m^{-2}*s^{-1}) in woody plant medium without sucrose and growth regulator at 20 ± 2 ºC. Under these conditions, the plantlets could be maintained for approximately 2 years without subculturing.

For the experiments described in this paper, 20 different genotypes (BN1, BN2, BN3, BN4, BN5, BN6, CA1, CL1, RB1, XA1, XA2, XA3, SA1, SI1, SI2, TA1, AF1, AF2, BC1, FE1) representing the 11 populations were further selected on the basis of their vigor and diversity of alkaloidal constituents.

Effects of culture medium and growth regulator on explant development

To assess the influence of culture medium on the growth of *U. guianensis*, nodal segments of in vitro plantlets were cultivated on WP medium, or Murashige and Skoog (1962) medium supplemented with 30 $g.L^{-1}$ sucrose (MS) or Schenk and Hildebrandt (1972) (Sigma®) medium supplemented with 20 $g.L^{-1}$ sucrose (SH) (Sigma®). The pH of each medium was adjusted to 6.0 before the addition of addition of 2.5 $g.L^{-1}$ of Gellan Gum (Gellex®) . Explants remained in this medium for 45 days.

In a further experiment performed to evaluate the influence of benzylaminopurine (BAP) (Sigma®) on the growth of explants, nodal segments from in vitro plantlets were transferred to WP medium supplemented with 0, 2.2, 4.4 and 11 µM BAP. Explants remained in this media for 45 days, stem heights and the numbers of buds were determined. The experiments were of randomized design and were performed in triplicate with ten repetitions each.

DNA extraction and SCoT analysis

Genomic DNA was extracted from the aerial parts of in vitro plantlets using the cetyltrimethylammonium bromide (CTAB) (Sigma®) method (Doyle and Doyle 1987). The integrity of the extracted DNA was confirmed by electrophoresis on 1% agarose gel in 1X Tris/Borate/EDTA buffer at 100 V for 60 min followed by staining with ethidium bromide and analysis under a UV transilluminator. The concentration of DNA was determined spectrophotometrically using a NanoPhotometer® P-360 (Implen, Munich, Germany). All of the 36 SCoTprimers suggested by Collard and Mackill (2009) were used initially for DNA amplification and nine that generated clear and unequivocal amplicons were selected for the subsequent analyses. The polymerase chain reaction (PCR) mixture contained 1 µL of 10X Taq buffer, 0.3 µL of $MgCl_2$ (25 mM), 1 µL of dNTPs (2.5 mM), 1 µL of each primer (5 µM), 0.1 µL of Taq DNA polymerase (5 U/µL), 1 µL of DNA template (10 ng/µL) and ultrapure distilled water to a final volume of 10 µL. Amplification reactions were carried out in Thermal Cycler (Jingle Scientific, Shanghai, China) under the following conditions: initial denaturation at 94 ºC for 3 min, followed by 35 cycles each comprising denaturation at 94 ºC for 1 min, annealing at 50 ºC for 1 min, extension at 72 ºC for 2 min, and a final extension for 5 min at 72 ºC. The PCR products and DNA size markers 100 bp ladder, were separated by electrophoresis on 1.2% agarose gel for 6 h at 60 V, stained with 1% ethidium bromide, visualized under a Gel Logic 112 digital imaging system (Bruker BioSpin, Billerica, MA, USA) and analyzed using Carestream Molecular Imaging (Rochester, NY, USA) software.

Analysis of POAs by HPLC

The aerial parts of 3-month-old in vitro-grown plantlets (WP medium containing 4.4 µM BAP; 4 cm high with 3 leaf pairs) of the 20 selected *U. guianensis* genotypes were dried separately in a forced-air oven at 45 ºC for 48 h and powdered in a ceramic pestle and mortar. For each genotype, 10 mg of the dried powder was extracted with 1 mL of pure methanol (Labsynth, Diadema, SP, Brazil) under sonication for 5 min in an Eco-sonics model Q5.9/37 (Tecnal, Piracicaba, SP, Brazil) ultrasonic bath. The suspensions were maintained at room temperature (22 ± 1 °C) for 24 h, filtered and were dried in a fume cupboard. Extractions were performed in triplicate for each of the studied specimens. The dried extracts were redissolved in pure methanol and applied to Supelco LC-18 solid-phase extraction tubes (Sigma, St. Louis, MO, USA) as previously described (Azevedo et al. 2018).

Calibration curves were constructed using solutions containing mitraphylline and isomitraphylline reference standards (CromaDex, Los Angeles, CA, USA; product numbers ASB 00,013,955–005 and ASB 00,009,417–005, respectively) at concentrations of 125, 62.5, 31.25, 15.625 and 7.8125 µg/mL, each of which was analyzed by HPLC in triplicate. The respective limits of quantification (LoQ) and detection (LoD) of mitraphylline were 0.40 and 0.13 µg/mL while those of isomitraphylline were 1.03 and 0.34 µg/mL.

Analysis of POAs by ultra-performance liquid chromatography–mass spectrometry (UPLC–MS)

The POAs present in *U. guianensis* extracts were identified by UPLC–MS using a Waters (Milford, MA, USA) Acquity UPLC H-Class system equipped with a PDA detector and a Waters Xevo TQ-S tandem quadrupole mass spectrophotometer with the electrospray source operated in the positive ion mode as previously described (Azevedo et al. 2018).

Data analysis

Data relating to the influence of genotype, growth medium and BAP concentration on the accumulation of mitraphylline and isomitraphylline were subjected to analysis of variance (ANOVA) and multiple regression analysis. Genotypes were classified according to the production of mitraphylline as high- [> 3.5 mg/g dry weight (DW)], medium- or low- (< 2.0 mg/g DW), and of isomitraphylline as high- (> 5.0 mg/g DW), medium- or low- (< 3.5 mg/g DW). A matrix of quantitative variables (mitraphylline and isomitraphylline) was generated based on the Euclidean distance and from this matrix grouping was performed using the unweighted pair group method with arithmetic mean (UPGMA) method. All analyses were performed using SISVAR version 5.1 software (Ferreira 2011). When significant differences were detected, mean values were compared using the Scott–Knott test at 5% probability.

For comparing the loci patters of primers, various genetic parameters such as polymorphism information content (PIC) (Roldán-Ruiz et al., 2000), resolving power (RP) (Prevost and Wilkinson 1999), marker index (MI) and effective multiplex ratio (EMR) (Varshney et al., 2007) were calculated. Genetic similarities between the 20 genotypes were determined using the Jaccard similarity coefficient, while genotype clustering was based on the UPGMA and principal coordinate analysis (PCoA). The support values for the degree of confidence at the nodes of the dendrogram were analyzed using the bootstrap re-sampling with 1000 permutation and Free Tree software version v.9.1.50 (Pavlícek et al., 1999). Population diversity was established with the aid of the Genalex 6 package running within Microsoft Excel (Peakall and Smouse 2006). Bayesian analysis (admixture model) was employed to discriminate gene groups within the *U. guianensis* populations using STRUCTURE software

version 2.1 (Prithard et al. 2000). Analyses were performed 3 times for each hypothetical number of subpopulations ($K = 1$ to 10) with 500,000 iterations in Markov chain Monte Carlo (MCMC) and 1,000,000 burn-in phases for each value of K. The most likely number of groups (K) was estimated using the delta K method (Evanno et al. 2005).

Results and discussion

Effects of growth media and BAP on the development of *U. guianensis*

Explants of *U. guianensis* that had been incubated for 45 days on MS medium showed mean stem heights of 0.85 cm with an average of 1.5 buds per explant, values that were similar to those recorded for explants grown on WP medium (0.76 cm stems; 1.43 buds) but significantly higher ($P < 0.05$) than those established for explants grown on SH medium (0.57 cm stems; 1.03 buds) (Table 1).

The effects on growth and development of explants incubated on WP medium supplemented with different concentrations of BAP are shown in Fig. 2. Optimal stem height (1.5 cm) and bud formation (4.7 per explant) were obtained with WP medium containing 4.4 µM BAP, and this medium was employed in the propagation of plantlets required for the analysis of POA content. Under these conditions, the propagules produced roots that favored acclimatization within three months. In previous studies, the micropropagation of *U. guianensis* was performed using basal MS medium, conditions that were less favorable than those described in the present study (Pereira et al. 2006, 2008).

Genetic diversity of *U. guianensis* genotypes

DNA could be extracted efficiently from each of the 20 genotypes of *U. guianensis* maintained in vitro. The 9 primers selected for the SCoT analysis amplified between 16 and 30 bands per primer, totaling 201 amplified bands and presenting an average polymorphism of 92.9% (Table 2, Supplementary file Fig. S1).

Table 1 Effects of culture media MS (Murashige and Skoog), WPM (Wood Plant Medium) and SH (Schenk and Hildebrant) on number of buds and height of *Uncaria guianensis*

Culture media	Height (cm)	Number of buds
MS	0.85 a	1.50 a
WP	0.76 a	1.43 a
SH	0.57 b	1.03 b

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

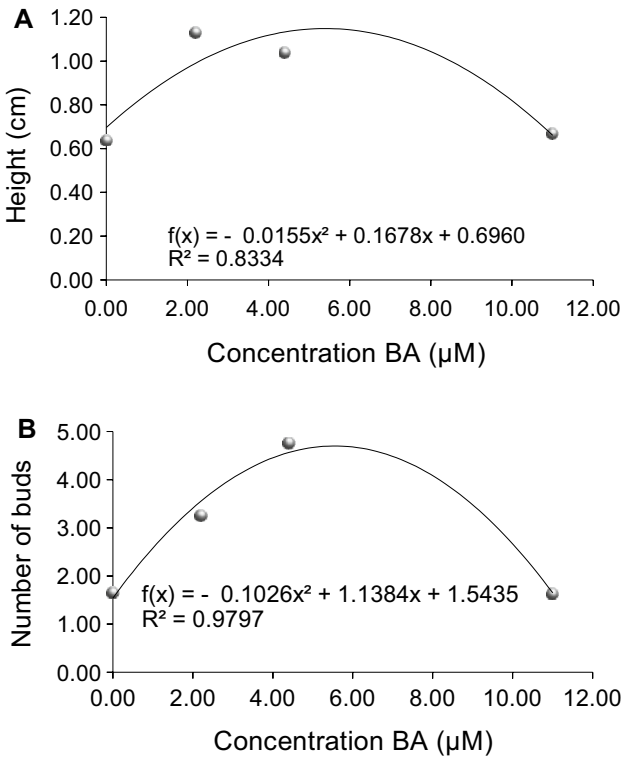

Fig. 2 Effects of supplementing woody plant medium with different concentrations of benzylaminopurine (BAP) on the growth and development of *Uncaria guianensis* showing: **A** height of stems and **B** numbers of buds per explant after 45 days in culture

The polymorphic information content (PIC) ranged from 0.27 (SCoT 18) to 0.39 (SCoT 21) with a mean of 0.34. In dominant molecular markers, the maximum expected value of PIC is 0.5 due to its biallelic character, so the closer to this value, the greater the efficiency of the marker in determining genetic variability (Roldán-Ruiz et al., 2000, Tatikonda et al., 2009).

The highest values of resolving power (RP), marker index (MI) and effective multiplex ratio (EMR) were obtained with primers SCoT 13 and SCoT 21 (Table 2). Positive correlation values were found between MI and PIC ($r = 0.63$, $p < 0.005$) and between RP and MI ($r = 0.96$, $p < 0.005$). Therefore, due to the strong positive correlation between RP and MI, either of the two parameters can be used to select the most informative primers. These results indicate that the molecular marker was efficient in the evaluation of genetic variability among genotypes of *U. guianensis* cultivated in vitro. While previous studies have described the efficiency of SCoT analysis in studies of genetic diversity in *Prosopis cineraria* (Fabaceae), *Dendrobium nobile* (Orchidaceae), *Silybum marianum* (Asteraceae) and *Lonicera macranthoides* (Caprifoliaceae) (Chen et al. 2015; Rafizadeh et al. 2018; Shekhawat et al. 2018), this report is the first to describe the use of the molecular marker with *U. guianensis*.

Table 2 SCoT markers used in the amplification of genomic DNA from *Uncaria guianensis* genotypes

Marker	Sequences (5'-3')	Total loci	*n*	% Polymorphic loci	PIC	EMR	MI	RP
SCoT13	CGACATGGCGACCATCG	30	28	93.3	0.38	27.9	10.6	17.7
SCoT 14	CGACATGGCGACCACGC	27	26	96.3	0.33	20.2	6.6	13.0
SCoT 18	CCATGGCTACCACCGCC	23	20	86.9	0.27	19.9	5.3	8.80
SCoT 19	CCATGGCTACCACCGGC	22	21	95.4	0.35	19.9	6.9	11.9
SCoT 21	CGACATGGCGACCCACA	24	22	91.6	0.39	21.9	8.5	14.6
SCoT 28	CATGGCTACCACCGCCA	21	21	100.0	0.35	21.0	7.35	11.7
SCoT 30	CATGGCTACCACCGGCG	16	15	93.7	0.32	14.8	4.7	8.5
SCoT 31	CATGGCTACCACCGCCT	17	15	88.2	0.38	14.9	5.7	10.8
SCoT 35	ATGGCTACCACCGGCCC	21	19	90.4	0.37	18.9	7.0	12.9
Total		201	187	–	–	–	–	–
Average		22.3	20.7	92.9	0.34	19.9	7.0	12.2

n Number of polymorphic loci, *PIC* polymorphic information content, *EMR* effective multiplex ratio, *MI* Marker index, *RP* resolving power

Examination of the UPGMA dendrogram (Fig. 3) and the PCoA plot (Fig. 4) revealed that the 20 genotypes were clustered into four groups based on genetic similarity. Group 1 comprised four BN genotypes from Bannach (PA), with BN2 and BN3 being the most similar, and the SA1 genotype from Santana (AP). Group 2 comprised ten genotypes from four different States, with four individuals from Acre (XA2 and XA3 from Xapuri, TA1 from Tarauacá, and FE1 from Feijó), four from Mato Grosso (SI1 and SI2 from Sinop, and AF1 and AF2 from Alta Floresta), and one each from Amazonas (BC1 from Boca do Acre) and Pará (BN5 from Bannach). This finding reveals that there is high genetic

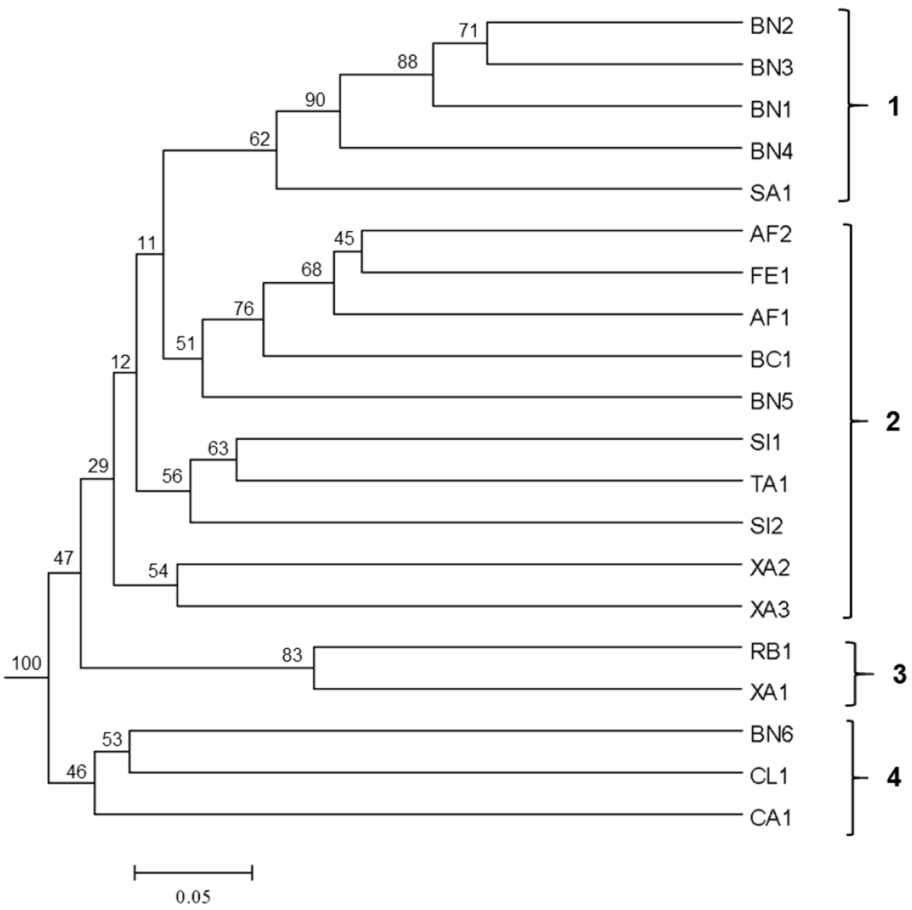

Fig. 3 Dendrogram constructed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm showing 20 genotypes of *Uncaria guianensis* separated into four clusters on the basis of start codon targeted (SCoT) markers. Genotypes derived from populations sampled at locations: BN1—BN6 (Bannach, Pará), CA1 (Carlinda, Mato Grosso), CL1 (Colider, Mato Grosso), RB1 (Rio Branco, Acre), XA1—XA3 (Xapuri, Acre), SA1 (Santana, Amapá), SI1 and SI2 (Sinop, Mato Grosso), TA1 (Tarauacá, Acre), AF1 and AF2 (Alta Floresta, Mato Grosso), BC1 (Boca do Acre, Amazonas), and FE1 (Feijó, Acre)

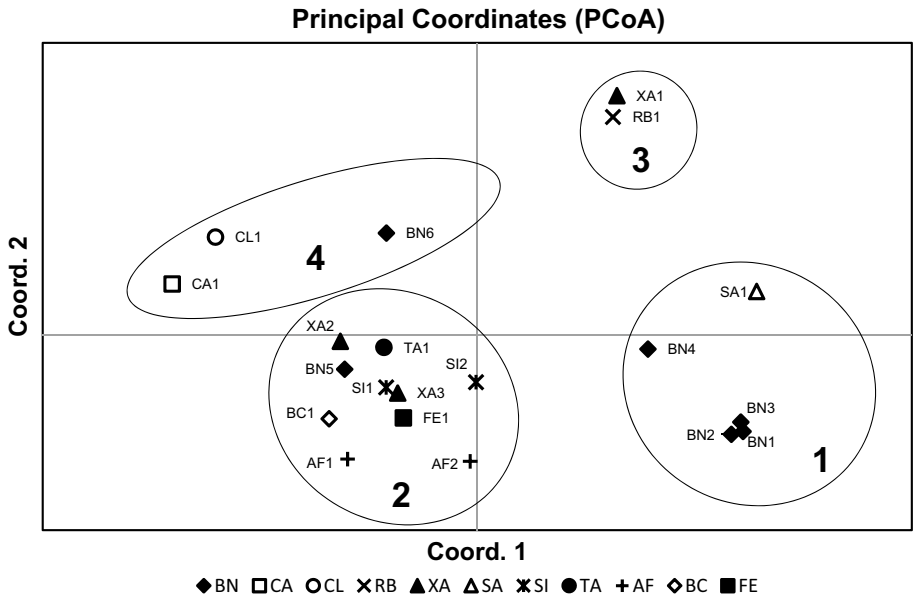

Fig. 4 Graphical representation of principal coordinate analysis of 20 genotypes of *Uncaria guianensis* accessions grown under in vitro conditions. Accessions derived from populations sampled at locations: BN1—BN6 (Filled Diamond; Bannach, Pará), CA1 (Unfilled square; Carlinda, Mato Grosso), CL1 (Unfilled circle; Colider, Mato Grosso), RB1 (×; Rio Branco, Acre), XA1—XA3 (Filled Triangle; Xapuri, Acre), SA1 (Triangle; Santana, Amapá), SI1 and SI2 (*; Sinop, Mato Grosso), TA1 (Filled Circle; Tarauacá, Acre), AF1 and AF2 (+; Alta Floresta, Mato Grosso), BC1 (Diamond, Boca do Acre, Amazonas), and FE1 (Filled square; Feijó, Acre)

similarity among some of the original populations despite the large geographical distances between them. For example, the pairs AF2/FE1 and TA1/SI1 were genetically very similar despite a straight-line distance of some 1600 to1700 km between the paired locations (Fig. 3). Group 3 comprised two individuals from neighboring locations in Acre, namely XA1 from Xapuri and RB1 from Rio Branco. Finally, group 4 comprised two genotypes from Mato Grosso (CL1 from Colider and CA1 from Carlinda) and one from Pará (BN6 from Bannach). The pair BN6/CL1 presented the highest genetic similarity of the group despite the large distance (1160 km) between the parental populations, whereas CA1 was the most genetically divergent from all of the 19 genotypes tested.

According to supplementary file TableS2 the lowest Jaccard similarity coefficient (0.32) was found between genotypes CA1 and BN1 (0.32) while the highest (0.78) was between BN2 and BN3. The finding of up to 70% dissimilarity among the *U. guianensis* genotypes implies that there is significant genetic diversity within the UNAERP germplasm bank. The SCoT-based similarity coefficient values of *U. guianensis* were comparable with those obtained for 23 accessions of coconut (*Cocos nucifera* L.) from different Asian regions, which ranged from 0.37 to 0.91 (Rajesh et al. 2015).

The results of Bayesian analysis performed using STRU CTURE software (Fig. 5) classified the 20 *U. guianensis* genotypes into two clear genetic clusters ($K=2$), one comprising individuals BN1 to BN4 (shown in green) and the other comprising all the other genotypes (shown in red). In an earlier study by our group of the genetic structure and diversity of natural populations of *U. guianensis* using sequence-related amplified polymorphism (SRAP) markers (Honório et al. 2018), Bayesian analysis revealed three clusters ($K=3$). This discrepancy can be explained by the small size of the populations studied and by the specific amplifying loci of the SRAP and SCoT marker systems.

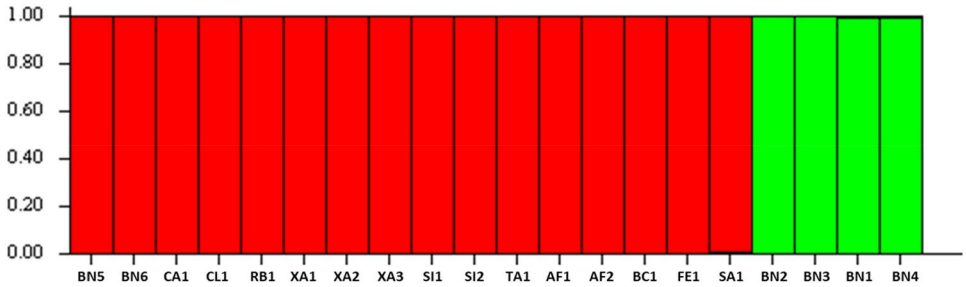

Fig. 5 Bayesian analysis of 20 genotypes of *Uncaria guianensis* grown under in vitro conditions showing the formation of two clear clusters: group I (red/dark grey) contained 19 individuals originating from Acre, Amazonas, Amapá and Mato Grosso, while group II (green/light grey) encompassed specimens BN1—BN6 originating from Pará

Table 3 Mean concentrations of mitraphylline and isomitraphylline in genotypes of *Uncaria guianensis* cultivated in vitro

Genotypes[#]	Concentration (mg/g dry extract)[†]	
	Mitraphylline	Isomitraphylline
BN1	0.98[q]	1.33s
BN2	1.29[o]	1.65[q]
BN3	0.74[r]	1.21[t]
BN4	1.72m	2.64[n]
BN5	3.15[f]	4.72[d]
BN6	1.47[n]	2.41[o]
CA1	4.13[c]	5.58[c]
CL1	2.06l	3.15[j]
RB1	2.03l	2.64[n]
XA1	2.81h	4.01g
XA2	3.70[e]	4.52[e]
XA3	5.43[a]	6.42[a]
SA1	4.79[b]	6.35[b]
SI1	1.74m	2.85m
SI2	2.20[j]	2.97l
TA1	2.96g	3.73[i]
AF1	1.15[p]	1.50[r]
AF2	1.70m	2.32[p]
BC1	2.58[i]	3.85h
FE1	3.86[d]	4.12[f]

[#]Genotypes derived from populatios sampled at the locations shown in the legend to Table 3

[†]In each column, mean values bearing dissimilar superscript letters are significantly different according to Scott–Knott test at 5% probability

According to Franco-Duran et al. (2019), sample sizes equal to or greater than 10% of the whole collection fully satisfy the prerequisites of representativeness and diversity. Thus, the SCoT markers employed in this study were able to detect the genetic diversity of 10% (20/200 genotypes) of the in vitro *U. guianensis* collection of UNAERP, and the information gathered will be useful for the improvement of the species in the future.

Variation in POA content among *U. guianensis* genotypes

The identities of mitraphylline and isomitraphylline in methanolic extracts of *U. guianensis* were confirmed unambiguously by UPLC–MS (Supplementary file Fig. S3). The genotypes with the highest concentrations of POAs were XA3 (Xapuri, AC), SA1 (Santana, AP) and CA1 (Carlinda, MT) (Table 3), all of which were classified in different genetic groups (Figs. 3 and 4) and were, therefore, genetically dissimilar. In contrast, the lowest concentrations of POAs were observed in BN1 and BN3 (Table 3), both of which came from Bannach (PA) and were classified in the same genetic group. A strong and positive correlation ($r = 0.97$) between the levels of mitraphylline and isomitraphylline was detected among all genotypes, implying that individuals containing high concentrations of one POA also contain high concentrations of the other (Supplementary file Fig. S3).

The 20 *U. guianensis* genotypes were categorized according to the levels of POAs detected in dried aerial parts and classified as low, medium or high alkaloid producers (Table 3, Fig. 6). Genotypes XA2, FE1, CA1, SA1 and XA3 were grouped (in increasing order of content) as high producers of mitraphylline, with concentrations varying from 3.70 to 5.43 mg g^{-1} DW, whereas CA1, SA1 and XA3 were

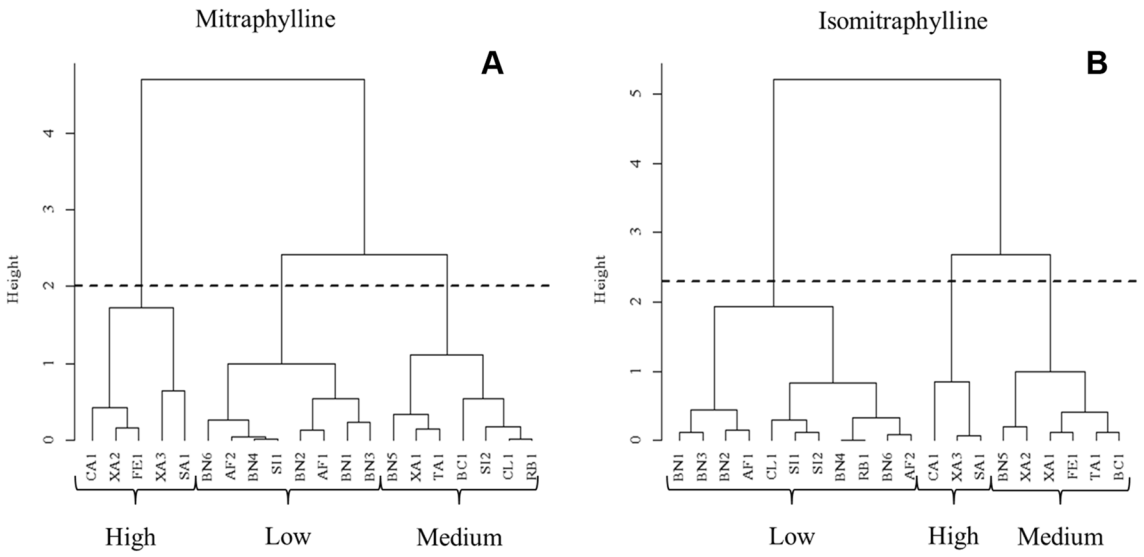

Fig. 6 Classification of 20 *Uncaria guianensis* genotypes grown under in vitro conditions according to the levels of pentacyclic oxidole alkaloids, comprising low, medium and high producers

classified as high producers of isomitraphylline with concentrations ranging between 5.58 and 6.42 mg g^{-1} DW. The medium producers of mitraphylline were RB1, CI1, SI2, BC1, XA1, TA1 and BN5 (2.03–3.15 mg g^{-1} DW), whereas those of isomitraphylline were TA1, BC1, XA1, FE1, XA2 and BN5 (3.73–4.72 mg g^{-1} DW). Genotypes BN3, BN1, AF1, BN2, BN6, AF2, BN4 and SI1 were categorized as low producers of mitraphylline (0.74–1.74 mg g^{-1} DW), while BN3, BN1, AF1, BN2, AF2, BN6, BN4, RB1, SI1, SI2 and CL1 were classified as low producers of isomitraphylline (1.21–3.15 mg g^{-1} DW). It is worth noting that genotypes originating from Xapuri (AC) accumulated the highest levels of POAs whilst those from Bannach (PA) presented the lowest. Considering that there is controversy regarding the relationship between the anti-inflammatory activity of *Uncaria* species and the presence of mitraphylline/isomitraphylline, the separation of genotypes according to the accumulation of POA is important because the pharmaceutical industry may demand either high or low producers (Allen-Hall et al. 2007; Sandoval et al. 2002).

A previous study by our research group (Honório et al. 2018) showed that the majority of native populations of *U. guianensis* sampled in Rio Branco, Feijó and Boca do Acre lacked both mitraphylline and isomitraphylline. However, the in vitro genotypes RB1, FE1 and BC1 that originated from the same areas typically produced medium amounts of these alkaloids, thereby confirming that growth conditions can influence the biosynthesis and accumulation of secondary metabolites (Yang et al. 2018). A specific example of environmental influence on the production of POAs has been reported by Luna-Palencia et al. (2013), who showed that micropropagated plantlets of *U. tomentosa* produced four times more alkaloids (mitraphylline, isomitraphylline and others) than greenhouse-grown plants.

Conclusions

Twenty genotypes selected from the in vitro germplasm bank at UNAERP showed significant genetic diversity (70% dissimilarity) according to SCoT analysis, while HPLC analysis demonstrated that these genotypes also diverged significantly regarding the levels of mitraphylline and isomitraphylline.

The germplasm bank of *U. guiansensis* can provide plant material for large-scale multiplication of plants with high- and low-alkaloid contents, to meet the demand of the herbal medicine industry, and in addition, the conserved genotypes present intra-specific variability that may be used in the genetic improvement of the species.

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