Diversity and genetic structure of *Carapichea ipecacuanha* and the implications for its conservation

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ABSTRACT

Carapichea ipecacuanha (Rubiaceae), known as ipecac, is a medicinal plant native of America. The state of Mato Grosso (MT) is its area of greatest occurrence in Brazil. Therefore, the aim of the present study is to analyze the diversity and genetic structure of natural ipecac populations in Cáceres (CAC), Diamantino (DIA), Mirassol D'Oeste (MDO) and Vila Bela da Santíssima Trindade (VBS) counties, Mato Grosso State, Brazil, using ISSR molecular markers. It supports the elaboration of management and conservation strategies by the species. Ten (10) primers were used to amplify the DNA fragments of 59 individuals, which generated 110 bands, with 58.2% polymorphism at species level, in total. The MDO and DIA populations have presented greater NEI´s genetic diversity (H= 0.1846 and H = 0.1817), higher Shannon´s index (I = 0.2649 and I = 0.2622) and higher polymorphism (P = 34.55% and P = 35.45%). The AMOVA test has revealed 53.05% of the total genetic variation within the populations; and 46.95%, among them. The estimated gene flow (Nm) 0.5925 corroborates the population differentiation (FST = 0.4577). The populations' geographic structure was confirmed through cluster analysis. Results highlighted the need of protecting areas of natural population and of providing trainings on sustainable extraction, among other conservation strategies.

Keywords: Phytotherapy, Molecular markers, Ipecac, Rubiaceae.

INTRODUCTION

Carapichea ipecacuanha (Brot.) L. Andersson, popularly known as "poaia" or "ipecac", is a medicinal species belonging to family Rubiaceae, native of the American continent. It occurs in shaded and humid regions of tropical forests, with disaggregated distribution in Panama, Costa Rica, Colombia and Brazil (Atlantic forest and Amazon rainforest) (Assis; Giulietti 1999; Coelho et al. 2013).

C. ipecacuanha plants are perennial and develop in aggregates called clusters, wherein they receive low photoirradiation levels. The species is classified by the CNC Flora (National Center for Plant Conservation 2012) as vulnerable. Its propagation occurs through vegetative multiplication and ornithocoria, besides the sexed mechanism (heterodilic monoecious flowers favoring allogamy) (Veloso 1947; Souza et al. 2008; CNC Flora 2012). The species is worldwide acknowledged for its pharmacological potential, which is linked to the presence of the alkaloids 'emetin' and 'cefelin'. These alkaloids are found in ipecac's roots and it gives emetic, amebicide, expectorant and antifebrile power to the plant (Assis; Giulietti 1999; Carrinconde et al. 1996).

The medicinal potential of the ipecac root has been known in Europe since 1762 (Coelho et al. 2013). The species has been exploited in Brazil since the XVIII century (Oliveira et al. 2010); however, it was intensified by the imperialist powers from 1870 on as a medicine to combat diseases. Ipecac valorization and its uncontrolled extraction resulted in the devastation of forests where the plant is found. Fires caused by *poaieiros* (poaia

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extractors) compromised the availability of proper habitats and accelerated the demographic decline of wild populations (Figueiredo et al. 2009). Thus, the intense ipecac extractive process in the last two centuries, the opening of new agricultural frontiers and the reduction of the plant's natural occurrence areas have threatened the species and promoted genetic erosion (Lameira 2002).

Knowing the genetic diversity is fundamental for the elaboration of conservation practices and for the management of natural populations (Renau-Morata et al. 2005). The molecular characterization is a tool used in studies on genetic diversity at any plant development stage, which is not influenced by the environment. It is also used in experiments focused on identifying the differences along the genome, because it is more precise than the characterization through morphological, biochemical and cytological markers (Caixeta et al. 2009; Kamada et al. 2009).

The ISSRs (*Inter Simple Sequence Repeat*) are dominant markers that analyze the multiple loci per reaction and generate high polymorphism degree and quality bands. The method has been frequently used, since it presents advantages in comparison to other markers. Actually, it does not demand prior knowledge about the genome to be amplified, but requires small DNA amounts and is fast and of low cost. These markers are mainly used to characterize the levels and organization of the genetic variability in and among related species, subpopulations, breeding groups and progenies. Thus, the ISSR is an effective method to study the genetics of natural or cultivated populations (Faleiro 2007; Rodrigues et al. 2010; Rivas et al. 2013; Rossi et al. 2014; Giustina et al. 2014; Silva et al. 2015; Ramalho et al. 2016; Tiago et al. 2016).

The lack of genetic variation, the small size of the populations and the great risk of genetic "erosion" indicate that *C. ipecacuanha* is very susceptible to extinction; therefore, conservation measures are demanding (Oliveira et al. 2010). Accordingly, the aim of the present study is to analyze the diversity and genetic structure of natural Ipecac populations occurring in Mato Grosso State, Brazil, in order to subsidize the elaboration of species management and conservation strategies.

MATERIALS E METHODS

Ipecac leaves (*C. ipecacuanha* (Brot.) L. Andersson – Rubiaceae) were collected from 59 individuals distributed in four natural populations in Mato Grosso State (Table 1, Figure 1). Young and fully expanded leaves from a single rod were collected in each cluster (aggregate) in the field. The samples were conditioned in silica gel and stored at -80 ºC in the laboratory. Specimen vouchers from each assessed population were collected and deposited at VIC Herbarium (n. 26818) in the Plant Biology Department of Viçosa Federal University.

The extraction of the genomic DNA was followed by the application of the 2% CTAB protocol described by Doyle & Doyle (1987), which underwent some modifications to be used in *C. ipecacuanha*. The 2% polyvinylpyrrolidone (PVP) and β-mercaptoethanol concentration in the extraction buffer was increased. Approximately 100mg of young leaves from the same stem in each cluster were used. DNA quantification was carried out using 1% agarose gel electrophoresis with the λ DNA marker. The dilutions were performed in autoclaved ultrapure water to prepare the stock solutions, and it resulted in final concentration of approximately 10 ng/µl of genomic DNA. The total of 100 primers (synthesized by the University of British Columbia) were tested and 10 of them were selected for final analyses because they have produced more reliable bands and reproducible polymorphism.

The polymerase chain reaction (PCR) was performed in final volume 25 µL, it was composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% tween 20, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM of each dNTP, 1.25 U of Taq DNA polymerase, 1.25 µl dedimethylsulfoxide (DMSO), approximately 60 ng of DNA and ultrapure water. The amplifications were conducted in GeneAmp PCR System 9700 thermocycler (Applied Biosystems) at the following conditions: 94 °C for 5 minutes followed by 35 cycles at 94 °C for 45 seconds, at 45-53 °C (depending on the primer in use) for 45 seconds and at 72 °C for 1.5 minutes, plus a final extension cycle at 72 ºC for 7 minutes. The amplification products were separated using 1.5% agarose gel electrophoresis in 1x TBE run buffer, at constant power 110 V for

TABLE 1. Location of the 4 *Carapichea ipecacuanha* populations and sample size (N).

Population	Population Code	N	Latitude (S)	Longitude (W)
Mirassol D'Oeste	MDO	15	15º31'34"	58º00'42"
Cáceres	CAC	15	15 40'41''	57 32'03''
Diamantino	DIA	18	14º04'38"	57º03'45"
Vila Bela da Santíssima Trindade	VBS	11	15º00'29"	59º57'02"

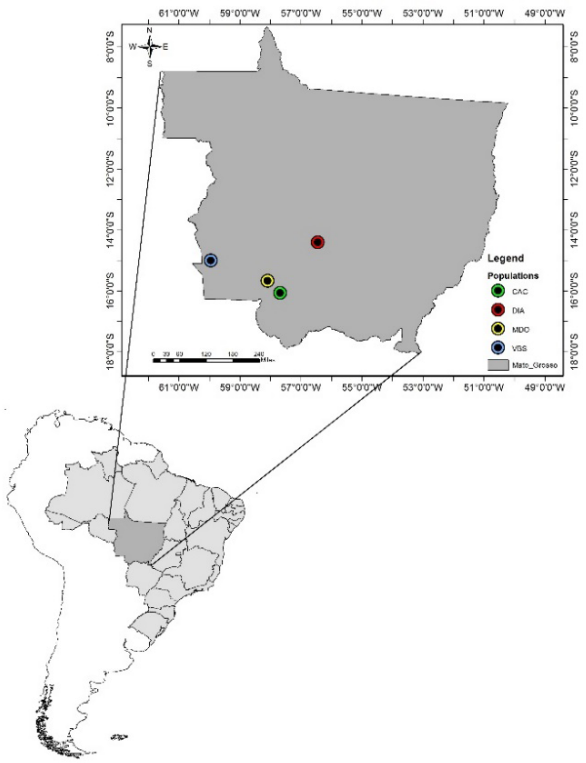

FIGURE 1. Geographic location of the *Carapichea ipecacuanha* populations sampled in the Southwestern region of Mato Grosso State, Cáceres (CAC); Diamantino (DIA); Mirassol D'Oeste (MDO) and Vila Bela da Santíssima Trindade (VBS) counties.

four hours. Gel staining was conducted in ethidium bromide (0.6 ng/mL). The gel was photographed under ultraviolet light.

The ISSR fragments were treated as dominant markers and judged as binary characters: (1) presence or absence (0) of bands. Robust and unambiguous bands only were evaluated. Bands with weak intensity or coalescent with other bands were excluded.

The genetic diversity was analyzed in the POPGENE 1.32 software (Yeh et al. 2000). The following parameters were estimated through the presence/absence matrix: polymorphism percentage (% P), number of observed alleles (A_O), effective number of alleles (A_E), Nei´s genetic diversity (h) (Nei 1973), Nei´s genetic distance and identity, and Shannon Index (I). The analyses were carried out at population and species level. The POPGENE 1.32 software (Yeh et al. 2000) was also used to generate a dendrogram through the UPGMA method, according to the Nei´s genetic distances (1978) in order to analyze the genetic relationships at population level. The genetic relationship among all assessed individuals was visualized through Principal Coordinate Analysis (PCoA), obtained by genetic distance, using the GeneAlEX6.5 software (Peakall; Smouse 2006). The genetic distribution variability within and between populations was found through Molecular Variance Analysis (AMOVA), according to Excoffier et al. (1992), in the Arlequim software, version 3.01 (Excoffier et al. 2006). The variation significance was tested in 1023 permutations, wherein P was the probability of, by chance, observing a value higher than or equal to the observed value.

The number of groups (K) was inferred in the STRUCTURE software (Pritchard et al. 2000) according to Bayesian statistics. Twenty (20) interactions were performed using 200000 "burn-ins" and 500000 Monte Carlo simulations by Markov Chains (MCMC) in each K value.

RESULTS AND DISCUSSION

Genomic DNA amplification

The 10 selected ISSR primers enabled finding 110 DNA fragments collected from 59 *C. ipecacuanha* individuals in the four studied populations. The number of fragments per primer ranged from five (UBC 824) to 16 (UBC 834), with 11 bands per primer, on average. The markers showed the total of 58.2% polymorphism, with 6.4 fragments per primer, on average (Table 2). It has indicated that the 10 *primers* herein used were satisfactory to detect polymorphism in the analyzed populations. The polymorphic content index (PCI) of each primer used in the current experiment varied between 0.03 (UBC 824) and 0.70 (UBC 873); 0.36 variation, on average. The PCI was used to estimate molecular marker efficiency in the detection of polymorphism between individuals. The information content was categorized as: satisfactory (PCI > 0.5), median (0.25 ≤ PCI ≤ 0.5) and low (PIC < 0.25) (Botstein et al.1980). Two *primers* of satisfactory content were obtained through this categorization system; six were median and two were low. Overall, the used primers were categorized as median (Table 2).

Genetic Diversity of the Population

The MDO and DIA have presented the greatest genetic diversity according to Nei (h) and Shannon (I) among the studied populations, whereas VBS had the lowest genetic diversity (H = 0.0758 and I = 0.1115); in level of species, the diversity showed the following results: (H = 0.1749 and I = 0.2700 (Table 3). The polymorphism percentage ranged from 20.00% to 35.45% among populations; 28.41%, on average (Table 3). Different results were found in the study by Silva (2007) about the forest fragmentation effect on the reproductive success and genetic diversity of *Psychotria hastisepala* Müll.

Arg. (Rubiaceae) using the ISSR markers. The markers presented 60.99% polymorphism between fragments.

The mean value of the observed alleles (A_o) was 1.2841; the effective alleles (EA), 1.1692; and the Nei´s index diversity (h), 0.0976. The mean Shannon´s index diversity (I) was 0.1459. According to Padua (2011), the value of this index varies from 0 to 1, and the closer it gets to zero, the lower the genotype diversity of the population. Accordingly, the herein studied populations presented different diversity levels. The Nei´s and Shannon´s indices at species level have presented mean values 0.1749 and 0.2700, respectively. The Nei´s genetic identity applied to pairs of populations ranged from 0.8378, between MDO and VBS, to 0.9336, between MDO and CAC. The Nei´s genetic distance conducted to infer divergences between populations has ranged from 0.0687, between CAC and MDO, to 0.1811, between VBS and CAC (Table 4). Therefore, the MDO and CAC populations presented the highest Nei´s identity values and the lowest genetic distance. It can be explained by the geographic distance between population locations (54 km straight and 79.4 km through BR-174).

Genetic structure and population differentiation

The dendrogram of the *C. ipecacuanha* populations was constructed through the UPGMA method by taking the Nei´s genitic distance matrix (1978) of the sampled population into consideration (Figure 2a). By analyzing the dendrogram, we could observe the formation of two main groups. The VBS population showed genetic dissimilarity greater than that of other analyzed populations and, thus, it formed an exclusive group. The second group comprised a subpopulation (MDO, CAC, DIA, respectively). The Bayesian analysis carried out in the "Structure" software corroborated the results found through the UPGMA method after the formation of two distinct groups (k = 2) (Figure 2b).

The Principal Coordinate Analysis (PCoA) was consistent with the UPGMA clustering method and the Bayesian analysis. It was applied to allocate populations into two distinct groups and to explain 35.62% of the total variation. The grouping can be explained as the result of the geographical distance between counties; wherein VBS, which forms a group apart from other populations, is

TABLE 2. ISSR *primers* used to amplify the DNA fragments of 59 *Carapichea ipecacuanha* individuals sampled in 04 populations. Ring temperature (Tm), Total number of amplified fragments (TNF), Total number of amplified fragments (NAF), Polymorphism percentage (%P) and Polymorphic Content Index (PCI).

Primer			TNF	NAF	%P	PCI
Code	Sequence (5' – 3')*	Tm (º C)				
UBC 814	$(CT)_8$[a]	46	12	10	83.3	0.45
UBC 824	$(TC)_8G$	48	5	2	40.0	0.03
UBC 834	$(AG)_8YT$	45	16	10	62.5	0.35
UBC 835	$(AG)_8YC$	52	12	7	58.3	0.51
UBC 848	$(CA)_8RG$	52	8	4	50.0	0.26
UBC 855	$(AC)_8YT$	53	12	7	58.3	0.37
UBC 866	$(CTC)_6$	52	10	7	70.0	0.47
UBC 873	$(GACA)_4$	48	8	6	75.0	0.70
UBC 880	$(GGAGA)_3$	45	13	5	38.5	0.22
UBC 891	$HVH(TG)_7$	53	14	6	42.9	0.28
Total			110	64	58.2	-
Mean			11	6.4	57.9	0.36

T = C or T; R = A or G; H = A, C or T; V = A, C or G

TABLE 3. Genetic diversity parameters of four *Carapichea ipecacuanha* populations. %P = polymorphism; A_o = Observed Alleles; A_E = Effective number of alleles; h = Diversity of Nei´s genetic diversity (1973); I = Shannon Index [Lewontin (1972)].

Population	%P	A_o	A_E	h	I
MDO	34.55	1.3455	1.2004	0.1153	0.1726
DIA	35.45	1.3545	1.2002	0.1170	0.1765
CAC	23.64	1.2364	1.1427	0.0822	0.1228
VBS	20.00	1.2000	1.1335	0.0758	0.1115
Mean	28.41	1.2841	1.1692	0.0976	0.1459
Species	58.2	1.6182	1.2867	0.1749	0.2700

TABLE 4. Nei´s identity and genetic distance of four *Carapichea ipecacuanha* populations.

Populations	MDO	DIA	CAC	VBS
MDO	------	0.9114	0.9336	0.8378
DIA	0.0928	------	0.9207	0.8551
CAC	0.0687	0.0826	------	0.8344
VBS	0.1770	0.1565	0.1811	------

Genetic identity (above the diagonal) and genetic distance (below the diagonal).

approximately 209.50 Km distant from MDO and CAC; and 384 Km, from DIA.

The Molecular Variance Analysis (AMOVA) results of the 4 *C. ipecacuanha* populations (Table 5) made it possible identifying the genetic partition within and between populations. Such partition showed intra-population genetic variability (53.05%) greater than the interpopulation one (46.95%). It can be explained through alogamy, since species that have a mixed breeding system (vegetative and cross propagation), and efficient pollen and seed dispersal mechanisms often present greater genetic diversity within populations than between populations (Loveless; Hamrick 1987).

The value of genetic differentiation of populations (F_{ST}) was 0.46954 in 1.023 random permutations. The populations ranged from 0.45935 (DIA) to 0.477915 (VBS). The FST measures the heterozygosity reduction with values ranging from zero to one. High FST values correspond to a considerable genetic differentiation between populations (Tavares, 2010). The differentiation can be categorized as low ($0.00 \leq F_{ST} < 0.05$), moderate ($0.05 \leq F_{ST} < 0.15$), high ($0.15 \leq F_{ST} < 0.25$) or higher ($F_{ST} > 0.25$) through the Wright's definition (1978). It can originate from natural selection, gene drift and/or migration. The FST values for the populations were high in the current study and it suggested a moderate gene flow level (Nm = 0.5925). It may have resulted from a contemporary process intermediated by anthropogenic actions (Souza et al. 2008) or by a past fragmentation process prior to the time when populations were contiguous (Kageyama et al. 2013).

Implications for conservation

The ipecac natural populations assessed in the study areas were affected by fragmentation and loss of habitat. It resulted from the advance of agricultural frontiers and cattle ranches, as well as from uncontrolled extraction. Therefore, species management and conservation plans are necessary in order to preserve the herein identified genetic diversity. Based on the results, we suggest the creation of conservation units *in situ* and *ex situ* as a strategy to protect natural population areas and introduce individuals from different populations, i.e., genetically different, and to train *poaieiros* on sustainable extraction in order to assure the species' survival and evolutionary potential.

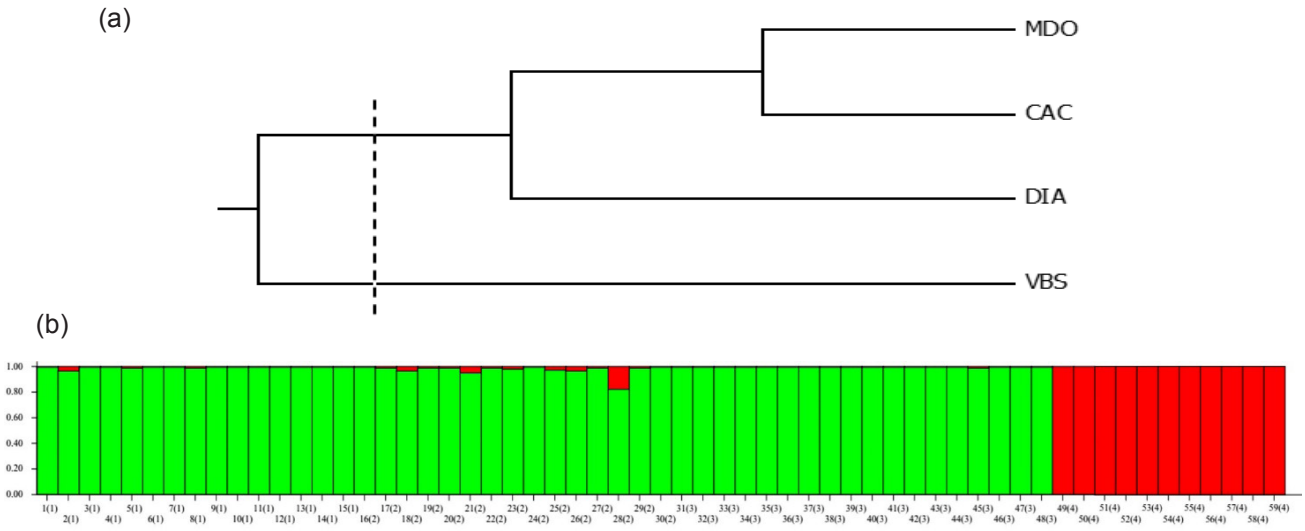

FIGURE 2. (a) Dendrogram obtained through the UPGMA method, based on the Nei genetic distances (1978), using 10 ISSR fragments detected in 04 *Carapichea ipecacuanha* populations in Mato Grosso State. (b) Fifty-nine (59) individuals from four *C. ipecacuanha* populations were grouped based on 10 ISSR markers in the "Structure" software. The individuals were represented by colorful vertical bars, according to the group they belonged to (2 groups, K = 2); Group 01 – Mirassol D'Oeste (MDO), Cáceres (CAC) and Diamantino (DIA); Group 02 – Vila Bela da Santíssima Trindade (VBS).

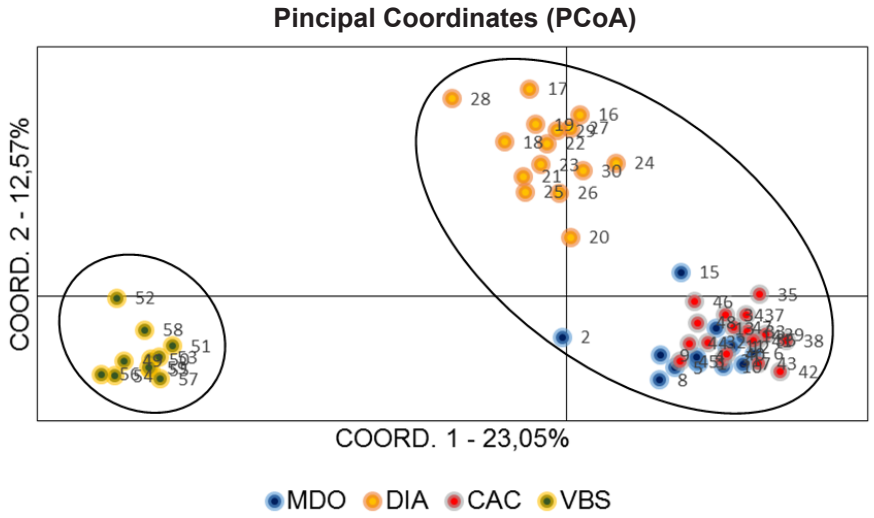

FIGURE 3. Distribution of individuals from the four *Carapichea ipecacuanha* populations, according to the first (Coord.1) and the second coordinates (Coord.2). It represents 23.05% and 12.5% of the total variation, respectively.

TABLE 5. Molecular Variance Analysis (AMOVA) of the four natural *Carapichea ipecacuanha* populations using 10 ISSR markers. Component of Variance (CV), Total Variance (VT) and Probabilities of having a component of variance greater than the values observed at random (P)

Source of Variation	GL	SQ	CV	VT (%)	P
Among populations	3	221.731	4.69571	46.95	<0.000
Within populations	55	291.778	5.30505	53.05	<0.000
Total	58	513.508	10.00076		
Fixing Index		F_{ST}: 0.46954			

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