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Research Article

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Individual Identification and Forensic Applications with Microsatellites in a Non-Invasive Monitoring of Antilophia Bokermanni

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ABSTRACT

The applicability of a panel of microsatellites in studies focused on conservation can be decisive in obtaining information on endangered species, such as the Soldadinho-do-Araripe. In the present work, it was tested a set of 12 microsatellite loci in order to evaluate the potential of carrying out the individual identification process and the establishment of family relationships necessary for future conservation monitoring of the species. This set of microsatellites proved capable of carrying out the potential identification of exclusive genotypes, in addition to revealing the family pedigree between individuals, determining possible parents/offspring, full siblings and half-siblings. This research will provide support in obtaining predictions of one or more possible sites (springs that have family groups) that can serve as a source of rare alleles and from that translocation programs are implemented and consequently ecological stations function as in situ germplasm banks, enabling the current and future monitoring of this species whose maintenance and conservation will help as a model in the preservation of biodiversity in Chapada do Araripe, Ceará, Brazil.

Keywords: Antilophia bokermanni, Chapada do Araripe, conservation, genetic diversity, microsatellites

Highlights

- The 12 loci were capable of individually identifying 82 specimens of Antilophia bokermanni with the potential to differentiate exclusive genotypes;
- The microsatellite panel was able to establish Family relationships by identifying possible parents/offsprings and siblings;
- Future actions of direct interventions, such as translocations and assembling of breeding pairs, can have their efficiency evaluated with the microsatellite panel presented here.

Introduction

Antilophia bokermanni (Soldadinho-do-Araripe) is a bird endemic to Brazil and globally threatened with extinction, whose main cause is the loss of its habitat, as a direct result of deforestation of hillside forests and degradation of water sources [1]. This species is among the 190 birds classified as critically endangered in the world [1]. Several works have been carried out in order to better understand the biology and ecology of this species [1-4], and thus provide of information that can guide more objectively the implementation of conservation actions. More recently, Souza et al. provided a panel of species-specific microsatellite markers, enabling a more accurate assessment of its variability and population genetic structure.

In recent decades, microsatellite markers have been widely used in population studies due to their polymorphic and informative nature, featuring a more effective variability detection pattern than most other molecular markers [5]. Its applicability goes beyond measuring the existing diversity in the population, making it possible to carry out individual identification studies, paternity tests and family kinship, both in natural and captive populations, thus enabling more direct intervention actions, such

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as events of translocations of individuals and setting new family groups [6,7].

In the current context of the Soldadinho-do-Araripe, the National Action Plan (PAN) for this species has suggested that carrying out actions to monitor its diversity on a temporal and regional scale is essential for the adequate preservation process [1]. In this process, the possibility of characterizing existing family groups, monitoring the flow of individuals within the area of occurrence and understanding their reproductive dynamics become information of high conservation value, with microsatellite markers being a tool capable of generating such knowledge. However, it is worth mentioning that the low genetic diversity already reported in A. bokermanni compared to its sister species (A. galeata) puts in check whether the microsatellites available so far are capable of enabling the obtainment of such information [8].

In this regard, the main objective of this study is to evaluate the applicability of a panel of microsatellite markers in measuring individual identification and defining the degree of kinship by a parental relationship matrix, enabling its future use in taking more effective conservation actions to guarantee the perpetuation of the Soldadinho-do-Araripe.

Methods

DNA Sampling and Extraction

Samples of 82 individuals were collected from 2003 to 2016 under the authorization of environmental licenses (IBAMA License 027/2005; n. 40116-1/CEMAVE n. 3731/2), from the northeast portion of the downhill of Chapada do Araripe, state of Ceará - Brazil. The choice of collection sites occurred cohesively to the identification and visual confirmation of the presence of the species through observations or vocalization records. Specimens were captured using mist-nets set up beside river springs and on nearby trails. Blood and/or feather samples were collected and stored in alcohol.

Total genomic DNA was isolated using the Wizard® Genomic DNA Purification kit (Promega), according to the manufacturer's instructions. Then, the concentration (ng/μL) of the samples was measured using the NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific®), for later obtaining dilutions in concentrations of ~15 ng/μL of DNA.

Microsatellites Amplification

The setting of the primer bank, already described in the scientific literature, occurred through the selection of 12 microsatellite loci, as described by Francisco et al. Duval and by Souza et al. (Table 1). PCRs were performed using a thermal cycler for a final volume of 13μL. Each PCR reaction had 5 to 10 ng of genomic DNA, 1 x PCR buffer, 1.5 mM magnesium chloride, 1.2 mM dNTP, 8pM M13 tail with fluorescence FAM or HEX (MWG Biotech Inc.) of the reverse primer, 2 pM of forward primer and 1 U of Taq polymerase Schuelke. The samples were genotyped using the GeneScan-500 Rox TM molecular size standard, through a run on an ABI 3500 automatic sequencer (Applied Biosystems). The microsatellite fragments were analyzed using the GeneMapper v4.1 program (Applied Biosystems). It was also used the loci set database described by Souza.

Table 1: Locus approximation of genetic diversity by locus calculated from the data of 82 samples of Antilophia bokermanni. Na: Number of Alleles; Ho: Observed Heterozygosity; He; Expected Heterozygosity; HWE: Hardy-Weinber equilibrium; Fis: Wrigh's Fis Frequency Followed by mean and standard deviation.

Locus	Na	Ho	He	HWE	Fis
Locus1	4.000	0.512	0.568	0.222	0.099
Locus2	5.000	0.744	0.743	0.823	-0.002
Locus3	5.000	0.585	0.629	0.651	0.069
Locus4	4.000	0.731	0.690	0.794	-0.059
Locus5	3.000	0.372	0.327	0.505	-0.137
Locus6	7.000	0.732	0.665	0.003**	-0.100
Locus7	2.000	0.415	0.442	0.579	0.061
Locus8	2.000	0.341	0.414	0.112	0.175
Locus9	6.000	0.659	0.729	0.345	0.096
Locus10	2.000	0.366	0.464	0.055	0.212
Locus11	2.000	0.463	0.457	0.902	-0.014
Locus12	5.000	0.634	0.700	0.566	0.094
Mean	3.917	0.546ns	0.569ns		0.041
SE	0.499	0.044	0.041		0.031

HWE - assays for the Hardy-Weinber equilibrium. ** $p<0.01$ He and Ho values do not differ statistically by Tukey's test.

Marker Analysis

For the microsatellite markers characterized for the genus Antilophia, totaling nine loci (Abo3; Abo6; Abo7: Abo8; Abo9; Abo10; Abo11; Abo12; Abo14), each marker received the letters corresponding to the name of the species under study, in this case A. bokermanni, being the number according to the assembly sequence of the standardized primers and characterized by it. As microsatellite markers have the possibility of interspecific transferability, it was also used six more markers characterized by Durval et al. and Francisco et al. for the species Chiroxiphia lanceolata and Chiroxiphia caudata, respectively (Chiro4; Chiro5; Chiro7;

Chiro10; Chiro12; Chir3-22), which also belong to the Pipridae family. By measuring sampling consensus, these 12 microsatellite markers were evaluated with different sample numbers (20,40 and 80 specimens) randomly chosen from the total sampling, and subsequently performed an ANOVA followed by Tukey's statistical test, using the BioEstat 5.0 software [9].

Genetic Diversity Indexes

The genetic diversity indexes for each locus, as well as the mean and standard deviation, which have been used in the research were: Number of Alleles (Na), Observed Heterozygosity (Ho), Expected Heterozygosity (He), Deviations from the Hardy-Weinberg equilibrium (HWE), Frequency F of Wrigh (Fis). All these indexes were estimated using the GenAlEx V6.5 software [10]. In the data that give a mean and a standard deviation (except for the HWE) an ANOVA was performed followed by the Tukey statistical test, using the BioEstat 5.0 software [9].

Sampling Identification (PI) and Polymorphic Information Content (PIC)

The probability of identity in 82 samples (PI) was calculated, which provides an approximation of the average probability of two unrelated individuals, drawn from random mating, having the same multilocus genotype. PIsibs (probability of sibling identity) and P2Exc (probability of deletion) were also obtained, the deletion also called population matching probability, widely used in forensics as an indication of the statistical power of a specific set of loci of markers. PI has also been used for genetic tagging in molecular ecology, an indication of the minimum number of loci needed for reliable genetic tagging. GenAlEx and Gimletv were the programs used to provide both PI and PIsibs approximations, with P2Exc being calculated in GenAlEx [10-14]. (Table 2).

Table 2: (PI) Identification probabilities by Loco; (PIsibs) Sibling Identification Probability by locus; Identification probabilities to increase the combination of loci (PI and PIsibs); (PIC) Polymorphic Informational Content and Rank according to PI. These indicators were calculated from the data set of 82 samples.

Locus	PI/loci	PIsibs/loci	PI With Combination	PIsibs With Combination	Polymorphic information contente PIC	Sorting locus by PI value:
Locus1	2.4e-01	5.25e-01	2.4e-01	5.3e-01	0.723	7 º
Locus2	1.0e-01	4.0e-01	2.4e-02	2.1e-01	0.831	1 º
Locus3	1.8e-01	4.8e-01	4.3e-03	1.0e-01	0.760	6 º
Locus4	1.5e-01	4.4e-01	6.3e-04	4.5e-02	0.783	4 º
Locus5	4.8e-01	7.1e-01	3.0e-04	3.2e-02	0.607	12 º
Locus6	1.5e-01	4.6e-01	4.6e-05	1.5e-02	0.801	5 º
Locus7	4.1e-01	6.3e-01	1.9e-05	9.1e-03	0.619	10 º
Locus8	4.3e-01	6.5e-01	8.0e-06	6.0e-03	0.619	11 º
Locus9	1.2e-01	4.1e-01	9.5e-07	2.5e-03	0.827	2 º
Locus10	4.0e-01	6.2e-01	3.7e-07	1.5e-03	0.643	8 º
Locus11	4.0e-01	6.2e-01	1.5e-07	9.5e-04	0.777	9 º
Locus12	1.4e-01	4.4e-01	2.1e-08	4.1e-04	0.821	3º

PI: Identification probabilities; PIsibs: Sibling Identification Probability; e: Exponent.

Polymorphic information content (PIC) or PIC parameter refers to the value of a loci to detect polymorphism within a population, depending on the number of detectable alleles and their frequency of distribution [15]. This parameter is the general measure of how informative a marker is, and the higher the PIC value, the more informative a marker is. To calculate the PIC we used the MolKin_V3.0 program [16]. (Table 2).

Parental Relationship Matrix

In the construction of a relationship matrix that demonstrates the degrees of kinship between individuals, it was used the ML-Relate software, based on a report that shows the relationship between each pair of individuals that has the highest probability between the following four relationships: U: unrelated; HS: half brothers; FS: full siblings; PO: Father or Mother/offspring [17]. In this projection, a sample of 15 individuals were plotted, all originated from the same source, to increase the chances of finding individuals who were related and to test the resolution capacity of the microsatellite panel. The specimens came from two different collection dates, with the Abo1 to Abo7 samples collected between 2003 and 2005, and the Abo8 to Abo15 samples collected in the period between 2014 and 2016.

Statistical Analysis

To plot the distribution of individuals in a formed matrix, in wich it was possible to evaluate potential overlapping of individuals and the formation of clusters, a Principal Component Analysis (PCA) was performed in the R program, with the objective of complementing the simulation study that evaluated the performance overview of this study markers. It was illustrated its practical application by reproducing a typical analysis of microsatellite marker data starting with the visual identification of the most likely number of clusters, followed by the assignment of individuals to groups and the description of relationships between the groups, done by the "Factoshiny" dataset.

Results

In table 3 it was ilustrated the values of the indices of Allelic Wealth (Na), Observed Heterozygosity (Ho), Expected Heterozygosity (He) and Wrigh's Fis Frequency (Fis) and Hardy-

Weinber balance (HWE). Wright's Fis frequency showed positive results for the loci, on average. In the 82 samples it was obtained an average value of 3.917 in number of alleles (Na) and the average number of alleles per locus varied a lot, from two (Locus 7; Locus 10 and Locus 11) to seven (Locus 6) alleles. Values for heterozygosity (Ho; He) ranged from 0.546; 0.569, respectively, evidencing a high heterozygosity, which may provide greater power for kinship analysis. For the inbreeding index (Fis), seven loci are described with positive values ranging from 0.061 (Locus 07) to 0.212 (Locus 10), and five loci with negative values, ranging from -0.137 (Locus 5) to -0.002 (Locus two).

Table 3: Matrix of relationships with a representation of the population of A. bokermanni in 15 samples from two different breeding seasons for the same locality.

	Abo 01	**Abo 02**	**Abo 03**	**Abo 04**	**Abo 05**	**Abo 06**	**Abo 07**	**Abo 08**	**Abo 09**	**Abo 10**	**Abo 11**	**Abo 12**	**Abo 13**	**Abo 14**	**Abo 15**
Abo 01	-														
Abo 02	U	-													
Abo 03	U	HS	-												
Abo 04	U	U	U	-											
Abo 05	U	U	U	U	-										
Abo 06	U	U	U	U	U	-									
Abo 07	U	U	U	U	U	U	-								
Abo 08	U	U	U	U	U	PO	U	-							
Abo 09	U	U	U	U	U	PO	HS	U	-						
Abo 10	U	U	U	U	U	U	FS	U	PO	-					
Abo 11	U	U	U	PO	U	U	U	U	U	HS	-				
Abo 12	U	U	U	U	PO	U	U	U	U	U	U	-			
Abo 13	U	U	U	U	U	FS	U	PO	PO	HS	U	U	-		
Abo 14	U	U	U	U	HS	U	U	U	U	U	U	U	U	-	
Abo 15	U	U	U	U	U	U	U	HS	U	U	HS	U	U	U	-

(Abo 1 to Abo 7) collected between 2003 and 2005, (Abo8 to Abo15) between 2014 and 2016. U = unrelated; HS = half siblings; FS = full siblings; PO = Father or Mother/offspring.

The values of heterozygosity (Ho; He) measured are very similar to each other, not statistically diverging by Tukey's test. For the HWE index, only Locus 06 showed a significance level of $p<0.01\%$, where the others did not differ in significance. As for the Fis de Wrigh (Fis) statistic, the average of the 12 loci corresponds to a value very close to zero (0.041). According to the graph in figure 1, it is noticeable that these indexes (Fis) are negative when analyzed with a smaller number of samples (20 and 40 samples) and positive over 80 samples.

In table 2 it was described the PI Identification Probability indexes, the Identification Probability among PIsibs siblings, these same indexes with the locus combination, the Polymorphic Informative Content and the information classification based on the information probabilities. In its characterization the PIC ranged from 0.607 to 0.831. The locus that presented the highest PIC value was Locus 2, being the locus that occupies the first place in the most informative rank according to the probability of identification.

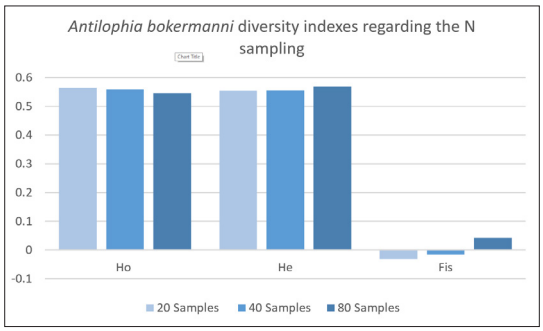

Figure 1: Indexes of Genetic Diversity of Populations of *Antilophia bobermanni* (12 microsatellite loci) with sampling number variation and application of Tukey statistics to describe sampling consensus that did not differ statistically.

PI values ranged from 0.01 to 0.48 and PIsibs from 0.40 to 0.71. For the combination of all 12 loci the PI and PIsibs are 2.1x10-8 and 4.1x10-4 respectively after the product rule. In the exclusion probability (P2Exc) the combined loci values ranged from 0.17 to 0.93. We observed that PI and P2Exc values improve with the increase in Loci number, as shown in (Figure 2).

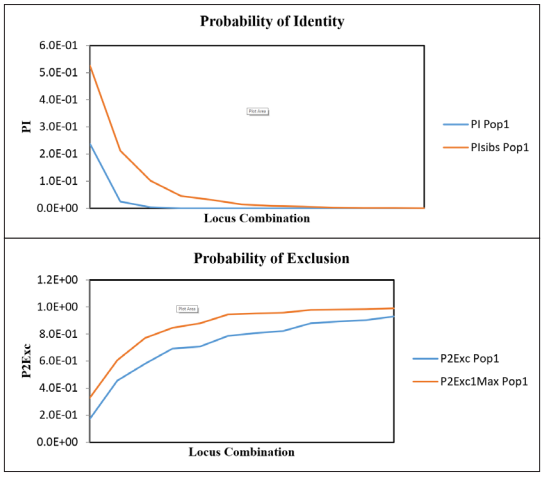

Figure 2: The log values of identification probabilities (log PI and PIsibs) and exclusion probability (P2Exc)

The multiple focus correspondence of the loci for increasing combinations shows that the 12 microsatellites are capable of individually identifying all 82 specimens analyzed through exclusive genotypes and that, in addition to identifying all samples of A. bokermanni, this technological kit has the potential to find up to 3321 unique genotypes (Figure 3). In the principal component analysis (PCA) it was possible to observe that when plotting the multivariate data of the microsatellite markers of 15 individuals (Abo 01 to Abo 15) there was no overlapping of samples (Figure 4).

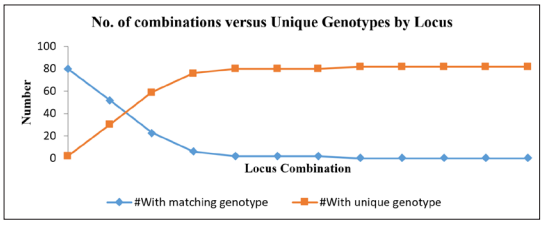

Figure 3: Number of combinations versus single genotype by locus.

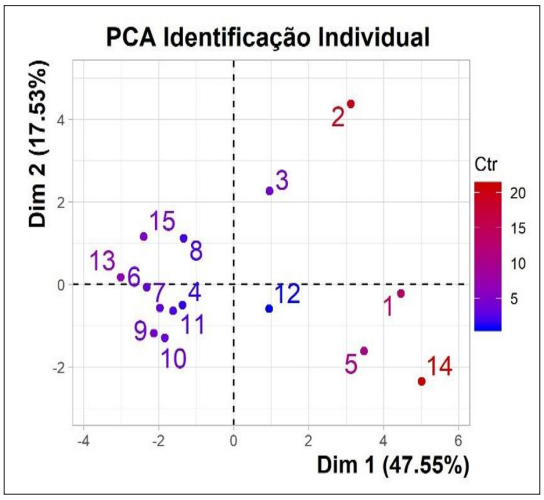

Figure 4: Principal Component Analysis (PCA) with a representation of the population of A. *bokermanni* in 15 samples.

The same samples used in the PCA were also used in the kinship matrix. Most relationships showed unrelated individuals, but it was also possible to observe a case of parents/offsprings, siblings and half-siblings, due to the resolution power of the set of 12 markers (Table 5). Among the fifteen individuals selected for a possible determination of kinship, we have a possible couple (female Abo08 and male Abo09) from which have been identified two of their possible offsprings (Abo 06 and Abo 13). This couple, according to the matrix, is unrelated (U) with logarithmic probability of (LnL= -32.12), which rules out a consanguineous mating in this example (Table 5). The calculated relationship matrix describes a logarithmic probability of (LnL= -25.55) and a Delta Ln (L) value equal to 0.92, between full siblings (FS) Abo 06 and Abo 13. Parents were captured over a decade after the capture of their possible offspring (Abo 06) and the offspring (Abo 13) was captured in the same period as the parents. It is still possible to observe an occurrence of 5 half siblings (HS) according to the significant values of Delta Ln (L) and they are: Abo 09 with Abo 07; Abo 11 with Abo 10; Abo 13 with Abo 10; Abo 15 with Abo 8 and Abo 15 with Abo 11.

Discussion

Resolution Ability of Microsatellites

The potential applicability of a set of microsatellite markers in a conservation study is directly linked to its ability to measure relevant indexes that characterize the population of the species in question, such as its diversity and genetic structure, flow of individuals within its area of occupation , level of inbreeding and others. In the case of Antilophia bokermanni (Soldadinho-do-Araripe), the reasons that lead to its “critically endangered” status will probably require that interventions be carried out in the near future, thus requiring the characterization of relationships in family groups with the definition of pedigrees and even an individual identification process. The panel of microsatellites evaluated here for A. bokermanni proved to be sufficiently capable of generating such information, being a strategic tool in its conservation process.

The success rate of studies with microsatellite markers is usually related to their frequency in the genome [18]. The abundance of microsatellite sequences varies considerably from taxon to taxon with some groups, such as birds, having much lower frequencies

in their genomes compared to other organisms, such as humans having a three-fold higher incidence [19]. A study with bears in Europe also used this number of 12 loci and it was also the best option used by Coetzer when performing a multiplex test with microsatellites for individual identification in Poicephalus robustus (Cape Parrots), seeking to resolve issues related to the definition of paternity and trade monitoring. The resolution ability, in these cases, is also directly related to the genetic diversity present in the population, in wich a high number of loci, but with low diversity within the population, can compromise the understanding of the process of flow of individuals, inbreeding events and definition of kinship relationships. Similarly with the results presented here, Nielsen and Chakraborty advocate that when obtaining loci with high heterozygosity rates (approaching 0.50) it provides the greatest power for paternity analysis. Such aspect corroborates with this research since our results indicated that both observed and expected heterozygosities, on average of the 12 markers, are greater than 0.5.

Table 5: List of A. bokermanni population probabilities in 15 samples from two different breeding seasons for the same locality.

Specimen 1	Specimen 2	R	LnL(R)	U	HS	FS	PO	Delta Ln(L)
Abo 02	Abo 01	U	-37.87		-	1.73	5.36	9999
Abo 03	Abo 01	U	-40.10		-	0.44	3.41	9999
Abo 03	Abo 02	HS	-36.81		1.26	-	0.56	9999
Abo 04	Abo 01	U	-34.67		-	2.10	4.22	9999
Abo 04	Abo 02	U	-31.82		-	1.63	4.51	9999
Abo 04	Abo 03	U	-34.05		-	1.58	4.74	9999
Abo 05	Abo 01	U	-38.67		-	0.26	2.37	9999
Abo 05	Abo 02	U	-35.25		-	1.84	4.41	9999
Abo 05	Abo 03	U	-38.05		-	2.33	5.00	9999
Abo 05	Abo 04	U	-31.23		-	0.62	2.44	9999
Abo 06	Abo 01	U	-35.61		-	0.76	2.72	9999
Abo 06	Abo 02	U	-32.19		-	0.89	4.57	9999
Abo 06	Abo 03	U	-34.99		-	0.90	2.47	9999
Abo 06	Abo 04	U	-28.17		-	0.42	1.47	9999
Abo 06	Abo 05	U	-32.17		-	0.88	2.74	9999
Abo 07	Abo 01	U	-38.84		-	2.71	5.93	9999
Abo 07	Abo 02	U	-35.99		-	1.56	5.19	9999
Abo 07	Abo 03	U	-38.22		-	0.08	3.73	0.89
Abo 07	Abo 04	U	-31.40		-	0.58	2.29	9999
Abo 07	Abo 05	U	-35.40		-	2.62	7.39	9999
Abo 07	Abo 06	U	-32.34		-	0.69	1.66	1.91
Abo 08	Abo 01	U	-36.40		-	1.64	4.73	9999
Abo 08	Abo 02	U	-32.99		-	0.19	1.33	9999
Abo 08	Abo 03	U	-35.78		-	2.01	6.18	9999
Abo 08	Abo 04	U	-28.96		-	1.02	3.01	9999
Abo 08	Abo 05	U	-32.96		-	2.42	6.66	9999
Abo 08	Abo 06	PO	-28.24		1.66	0.72	1.07	-
Abo 08	Abo 07	U	-33.13		-	0.71	1.42	9999
Abo 09	Abo 01	U	-37.83		-	2.41	5.69	9999
Abo 09	Abo 02	U	-34.98		-	3.72	8.87	9999
Abo 09	Abo 03	U	-37.21		-	3.28	8.08	9999
Abo 09	Abo 04	U	-30.39		-	0.08	1.14	9999
Abo 09	Abo 05	U	-34.39		-	2.98	7.50	9999
Abo 09	Abo 06	PO	-30.26		1.07	0.29	0.68	-
Abo 09	Abo 07	HS	-34.18		0.38	-	0.80	0.10
Abo 09	Abo 08	U	-32.12		-	0.74	1.53	9999
Abo 10	Abo 01	U	-37.43		-	3.40	7.04	9999

Abo 10	Abo 02	U	-36.66		-	3.75	8.67	9999
Abo 10	Abo 03	U	-38.89		-	2.13	5.97	9999
Abo 10	Abo 04	U	-32.07		-	1.10	3.26	9999
Abo 10	Abo 05	U	-36.07		-	2.57	5.99	9999
Abo 10	Abo 06	U	-33.01		-	1.33	3.24	9999
Abo 10	Abo 07	FS	-33.15		3.09	1.28	-	0.80
Abo 10	Abo 08	U	-33.80		-	0.88	2.22	9999
Abo 10	Abo 09	PO	-34.51		0.73	0.03	0.29	-
Abo 11	Abo 01	U	-36.00		-	3.32	7.44	9999
Abo 11	Abo 02	U	-33.97		-	2.30	5.21	9999
Abo 11	Abo 03	U	-36.76		-	2.55	5.26	9999
Abo 11	Abo 04	PO	-28.82		1.13	0.30	1.18	-
Abo 11	Abo 05	U	-33.94		-	0.33	2.45	9999
Abo 11	Abo 06	U	-30.88		-	0.95	3.69	2.22
Abo 11	Abo 07	U	-34.12		-	1.73	5.85	3.95
Abo 11	Abo 08	U	-31.67		-	1.27	3.44	9999
Abo 11	Abo 09	U	-33.11		-	0.11	2.12	0.95
Abo 11	Abo 10	HS	-34.14		0.64	-	1.42	0.21
Abo 12	Abo 01	U	-39.18		-	1.33	3.35	9999
Abo 12	Abo 02	U	-35.77		-	1.47	4.90	9999
Abo 12	Abo 03	U	-38.56		-	0.23	1.45	9999
Abo 12	Abo 04	U	-31.74		-	1.32	4.19	9999
Abo 12	Abo 05	PO	-31.22		4.52	1.96	2.09	-
Abo 12	Abo 06	U	-32.68		-	0.42	1.40	9999
Abo 12	Abo 07	U	-35.91		-	2.73	6.68	9999
Abo 12	Abo 08	U	-33.47		-	1.48	4.76	9999
Abo 12	Abo 09	U	-34.90		-	1.66	4.89	9999
Abo 12	Abo 10	U	-36.58		-	2.29	6.18	9999
Abo 12	Abo 11	U	-34.46		-	0.61	3.14	9999
Abo 13	Abo 01	U	-34.03		-	1.61	4.18	9999
Abo 13	Abo 02	U	-31.17		-	0.45	3.14	9999
Abo 13	Abo 03	U	-33.41		-	0.87	2.15	9999
Abo 13	Abo 04	U	-26.59		-	0.09	0.72	9999
Abo 13	Abo 05	U	-30.59		-	2.73	6.77	9999
Abo 13	Abo 06	FS	-25.55		1.98	1.25	-	0.92
Abo 13	Abo 07	U	-30.76		-	0.37	1.26	1.11
Abo 13	Abo 08	PO	-27.36		0.96	0.32	1.33	-
Abo 13	Abo 09	PO	-27.88		1.87	0.65	0.48	-
Abo 13	Abo 10	HS	-31.19		0.24	-	0.46	0.14
Abo 13	Abo 11	U	-29.30		-	0.54	2.39	1.65
Abo 13	Abo 12	U	-31.10		-	1.57	4.84	9999
Abo 14	Abo 01	U	-46.25		-	1.25	3.88	9999
Abo 14	Abo 02	U	-44.78		-	2.88	7.03	9999
Abo 14	Abo 03	U	-47.01		-	3.97	8.94	9999
Abo 14	Abo 04	U	-40.19		-	2.84	5.93	9999
Abo 14	Abo 05	HS	-43.14		1.04	-	0.20	9999
Abo 14	Abo 06	U	-41.13		-	2.79	6.87	9999
Abo 14	Abo 07	U	-44.37		-	1.55	5.18	9999

Abo 14	Abo 08	U	-41.92		-	2.98	7.30	9999
Abo 14	Abo 09	U	-43.35		-	2.93	6.80	9999
Abo 14	Abo 10	U	-45.03		-	2.59	5.97	9999
Abo 14	Abo 11	U	-42.91		-	2.61	6.48	9999
Abo 14	Abo 12	U	-44.70		-	1.24	5.36	9999
Abo 14	Abo 13	U	-39.55		-	2.97	6.49	9999
Abo 15	Abo 01	U	-37.95		-	2.84	6.61	9999
Abo 15	Abo 02	U	-32.74		-	0.08	2.16	9999
Abo 15	Abo 03	U	-37.33		-	1.26	3.23	9999
Abo 15	Abo 04	U	-30.51		-	0.27	1.60	9999
Abo 15	Abo 05	U	-34.51		-	2.35	6.08	9999
Abo 15	Abo 06	U	-31.45		-	0.87	2.14	2.50
Abo 15	Abo 07	U	-34.68		-	0.77	2.38	2.09
Abo 15	Abo 08	HS	-31.89		0.35	-	1.32	0.37
Abo 15	Abo 09	U	-33.67		-	0.06	1.99	0.70
Abo 15	Abo 10	U	-35.35		-	0.64	3.18	1.77
Abo 15	Abo 11	HS	-32.84		0.38	-	1.27	0.80
Abo 15	Abo 12	U	-35.02		-	1.68	5.04	9999
Abo 15	Abo 13	U	-29.87		-	0.06	0.01	0.77
Abo 15	Abo 14	U	-43.47		-	4.12	9.89	9999

R: Kinship Relationship; LnL(R): Probability of the log of R; U: unrelated; HS: half siblings; FS: full siblings; PO: Father or Mother/offsprings; Delta Ln(L) : Neperian delta of possible probabilities. (A Delta Ln(L) of '9999' indicates that the relationship is not possible).

Individual Identifications and Kinship Relationships

Waits point out that sibling identification probabilities (PIsibs) have values between the limits of 0.001 and 0.0001, and when reaching these values for microsatellite markers, they would then be suitable for individual differentiation. Therefore, the profile of the microsatellites studied here fits perfectly for this forensic purpose, since for this index of probability of identification between siblings (PIsibs) we found a value of 4.1x10-4 when the 12 loci are combined, remaining within the indicated range by researchers. In addition, it was possible to identify individuals and determine some kinship relationships. Also, selection of high heterozygosity loci (approaching 0.50) provided greater capacity for kinship analysis. Among the loci, there are those that assume a superior position in the identification ranking based on the high value of the polymorphic information content (PIC) which, in turn, are the ones with the highest heterozygosity index. The related probabilities and analyzes assume that the loci are independent (unrelated) and that the allele frequencies in the population are accurately estimated [20,21].

Waits described that the indexes (PI and PIsibs), necessary to obtain a probability <0.0001, with codominant loci and when heterozygosity, is approximately equal to 0.4, it would be approximately from 11 to 22 markers to reach significant values. These results are similar to that of this study, with just 5 loci the probability of identifying individual PI reaches the level <0.0001 and with 11 loci PIsibs reaches the same mark in the analysis of A. bokermanni.

A tool that helps in forensic interpretations is the kinship matrix that determines the possible genealogies between individuals. According to Woltmann it was possible to identify a dyad of possible parents/offspring (PO) in their samples of Myrmeciza exsul (Sclater, PL 1859). However, they were not consistent in predicting full-sibling (FS) and half-sibling (HS) relationships due to inconclusive evidence of migration between population sites. As for the kinship matrix found in the present work, full sibling (FS) and half sibling (HS) relationships appeared more frequently when compared to parent/offspring (PO) relationships in the 15 analyzed samples, which by the way this PO relationship only was determined in a couple (Abo 08 and Abo 09). The fact that some individuals were known for their gender made it easier to understand the correlations of family, as well as the interpretation of these parental relationships. The samples mentioned above were collected in different periods with an interval of 10 years, in the same source area (Grangeiro), which leads to interpret that they are more related specimens. Characterizing as a philopatric species. Because they maintain a family group that remains inhabiting the same locality (source) for more than a decade.

Biological Diversity

Genetic diversity is a key aspect that allows natural populations to adapt and survive in changing environments [22]. A low diversity can make it difficult for populations to adapt, decreasing their competence in several aspects, such as effects of inbreeding and inbreeding depression [23]. In the case of Soldadinho-do-Araripe, having access to and knowing such indexes, it is made possible to assemble strategies more effectively for its monitoring, creating adequate conditions for its maintenance and also possible future processes of translocation of specimens. he technological kit offered can provide the necessary information

for this purpose.. First, because of the fact that, according to Turchetto-Zolet microsatellites have become the class of genetic markers widely used in a wide range of applications, among them, measuring diversity indices, distinguishing family groups, as well as quantifying their differences and similarities based on the level of species and individuals. A relevant result, that it was possible to visualize the resolving capacity of the markers, in addition to making individual identifications of entire sample bank of this study with exclusive genotype, refers to the multiple focus matching capacity of the loci for increasing combinations (Figure 3). With these 12 markers it was possible to create a prediction that approximately 3,321 unique genotypes will be potentially determined. This number contemplates a reality with an estimated population of less than 800 specimens, which will facilitate studies and understanding of their biological diversity and genetic variability for the next generations.

Possibly the species A. bokermanni has undergone a recent speciation process, and even with this evolutionary historicity, its average heterozygosity, in the 12 loci of 0.550, was not supported, which makes the general use of microsatellites in population studies of biological diversity [24,3,4]. In view of this recent speciation event with a satisfactory maintenance of population heterozygosity, it is possible that the population did not go through a historical event that compromised its population genetic variability (gene pool), such as gene drift (founder effect or bottleneck). What draws attention, regarding the diversity indexes, mainly the expected and observed heterozygosity of the population, is that the measured values are very similar to each other, to the point of not statistically differing, which this consensus demonstrates the operational potential of the technological kit of the microsatellites, and consequently also reflects in the Wrigh's F statistic (Fis) in wich the average of the 12 loci corresponds to a value very close to zero (0.041). That leads to the understanding that inbreeding effects are not common in this population.

Conservationist Considerations

Recent studies prove that measuring to reduce the rate of extinction, or creating a metric for this rate, is a necessary element of any post-2020 biodiversity policy goal, and that it provides clarity on the main concerns that most people have about the loss of biodiversity. And they also suggest that the understanding of the general post-2020 impact on the structure of biodiversity should have as a vision the concept proposed by the Convention on Biological Diversity (CBD) for 2050 of: "Living in harmony with nature" [25]. However, measuring this progress towards this goal is challenging because of a largely political and science-based construction and a single biodiversity metric that can measure the rate of extinction is justified when setting a global objective only on the rate of species extinction based on some scientific principles [25].

It has fundamental importance the understanding that the fact that the Soldadinho-do-Araripe is classified as Critically Endangered by the IUCN puts at risc not only its extinction, but an entire ecosystem that may be a new biodiversity conservation hotspot embedded in the sedimentary basin of Araripe, devoid of conservation planning that projects the future of this species and others that were recently discovered and that are already threatened with extinction, such as the crab Kingsleya attenboroughi, Pinheiro & Santana (Guajá do Araripe). The concept of extinction, in the context of this research, is interesting because it fully incorporates the most fundamental aspect of biodiversity loss. The extinction of a species represents an irreversible loss, a measurable reduction in the diversity of life on Earth, and is a major concern for conservation [23]. Thus, the tool presented here is capable of carrying out the necessary measurements and estimation for adequate population monitoring, trying to avoid yet another loss in global biodiversity.

In this perspective, the conservationist impact of this research stems from the fact that we are facing a particular biological case, in which the entire population is restricted to an area of approximately 30 km², unevenly distributed in the small strip of humid forest [1]. Recent studies have shown that, as a result of local fires and other anthropogenic actions, there is the possibility of formation of islands of habitat as a consequence of the fragmentation of its natural habitat [1]. Soon this technological kit of microsatellite markers will be the ideal technology for monitoring actions that seek to avoid the process of habitat fragmentation.

In the current biodiversity crisis, with many species increasingly threatened, it is important to develop methods to determine how best to allocate conservation resources. Genetic studies, such as the one in this research, with endangered species, are important not only in monitoring their diversity, but also because they enable intervention measures (translocations, pair assembly, flow measurements) with greater effectiveness. This study will provide support in obtaining predictions of one or more possible sites (Springs) that may serve as a source of rare alleles and, from this, make possible for translocation programs to be implemented and, consequently, ecological stations function as in situ germplasm banks [26-38].

Conclusion

The twelve microsatellite loci proposed by this technological panel were efficient in the genotyping of the 82 samples of Antilophia bokermanni, in addition to the possibility of expanding its sampling number. It was possible to determine and characterize each individual particularly as well as perform a kinship analysis within a family group, even determining possible parents/offsprings and siblings. It was shown that with microsatellites their forensic applications are relevant and effective, although it was also aimed to emphasize that the same set of markers can be applied for the purpose of population, phylogenetic and taxonomic studies due to their satisfactory diversity indexes. This should help the current and future monitoring of the Soldadinho-do-Araripe whose maintenance and conservation will help preserve the biodiversity of Chapada do Araripe.

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