Isolation and characterization of 15 new microsatellite markers for the globally endangered Lear's macaw *Anodorhynchus leari*

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Abstract

25 **Background:** In 2016, a set of 16 microsatellite markers was characterized for Lear's macaw (*Anodorhynchus leari*) using DNA samples from captive individuals. Extending this molecular toolkit, including the use of samples from wild individuals, is expected to provide the required power of resolution for pedigree inference of both wild and captive individuals, and could support research on the genetic structure of wild populations.

Methods and Results: We characterize a set of 15 microsatellite markers optimized for the Lear's macaw, developed from a microsatellite-enriched library in a three-step procedure. Primer pairs were initially designed for 30 62 microsatellites with >7 tandem repetitions. After amplification of DNA of five wild individuals from different localities, 22 loci seemed to be polymorphic and were further tested on 12 wild nestling samples. Fifteen unlinked loci showed unambiguous peaks and low to moderate polymorphism levels. The combination of the four most polymorphic markers allowed individual identification even of putative sibs.

Conclusions: These markers complement previously described microsatellites developed for *A. leari* and constitute 35 a fundamental genetic toolkit for the investigation of the genetics of both wild and captive populations, thus assisting integrated management plans for the conservation of this globally endangered species.

Keywords: microsatellite loci; parrots; conservation genetics; Lear's macaw; endangered species

Introduction

Wild populations of parrots are threatened by habitat loss due to multiple anthropogenic impacts like overgrazing, deforestation, introduction of alien species, or disturbance of nesting and roosting sites [1]. These impacts, along with the persistence of poaching for the pet trade [2], affect their ecological relationships and population dynamics, potentially causing dramatic population declines [3]. Such declines can in turn cause depletions of genetic diversity, which can expose relict populations to the detrimental consequences of inbreeding and genetic drift [4]. In the case of endemic species with small distribution ranges, genetically impoverished populations may ultimately rely on rescue management to persist [5,6].

Assessing demographic parameters such as effective population sizes, genetic diversity and inbreeding levels in natural populations of parrots is paramount for their conservation. However, estimating these parameters in wild populations is challenging, especially in dense tropical forests and other habitats where parrot tissue sampling is difficult [7,8]. Another challenge to obtain such genetic information is the availability of specific molecular markers such as microsatellites [9]. This is the case of the globally endangered Lear's macaw *Anodorhynchus leari*, which presents a relict population with an extremely reduced distribution range [10]. An additional population, isolated from the main nucleus and considered as functionally extinct, is requiring urgent management actions for its persistence [11]. In this scenario, information on genetic parameters from both wild and captive populations is necessary to plan population management actions and to support the captive breeding program [6,12].

Jan & Fumagalli [13] published primer sets of microsatellites for seven endangered parrot species, including 16 markers for *A. leari* optimized from DNA samples of captive individuals. The present study complements this molecular toolkit with an additional set of 15 microsatellite markers specific for *A. leari*, which we tested on DNA samples from blood and feathers of wild macaws in a 3-step optimization procedure. Our new set, together with previous markers, supports the development of a genetic monitoring program to be applied for the integrated conservation management of this emblematic species.

Materials & Methods

We obtained tissue samples of wild *A. leari* individuals from five localities representing the species' entire range of distribution (Fig. 1A, 1B). We extracted blood samples (0.1 ml) from the wing brachial vein of 15 nestlings > 30 days old (Fig. 1C), captured according to Pacífico et al. [14] in two breeding localities: 1) Toca Velha, in the *Estação Biológica de Canudos*, and 2) Barreiras, in a private land (Fig. 1B, Table 1). Additionally, we collected molted wing and tail feathers (Fig. 1D), following Gebhardt et al. [15], in three locations: 1) roosting sites of Serra Branca in the *Estação Ecológica do Raso da Catarina*, 2) roosting site of Baixa do Chico in the *Terra Indígena do Brejo do Brugo*, and 3) foraging site of the farmland Cercadinho, in the surroundings of the *Parque Nacional do Boqueirão da Onça* (Fig. 1B, Table 1). We stored blood samples in 100% ethanol and feathers in craft paper envelopes, frozen at -18°C until laboratory analyses. For DNA extraction, we digested blood and feather samples in 100 mM NaCl, 50 mM Tris pH 7.5, 50 mM EDTA pH 8.0, 1% SDS and 25µl of proteinase K (20 mg/ml) at 55 °C

for 8 hours. We then purified genomic DNA using a customized silica-based method in an automatic robotic system (Freedom EVO 100, from TECAN), using a homemade magnetic bead-based DNA purification system.

We implemented a 3-step procedure to isolate and characterize microsatellites (Fig. 2). First, we developed a microsatellite-enriched library (motifs: AGG, AAG, ACAT and ATCT) using DNA of one individual (Table 1) and Nextera XT DNA kit (Illumina) at the AllGenetics & Biology SL. Lab. Sequencing was performed on an Illumina MiSeq platform (PE300), rendering 5,606,286 paired end reads which we processed in Geneious 8.1.5. using customized scripts [16]. We designed 633 primer pairs using Primer3 in Geneious 8.1.5 (Table S2).

In a second step, we selected a subset of 62 microsatellites presenting more than seven motif repeats to test amplification on DNA samples from five individuals, one from each locality (Fig. 1; Table 1). PCRs were composed by forward and reverse primers and either an M13-tail (5'GGA AAC AGC TAT GAC CAT) labeled with HEX dye, or an oligonucleotide CAG-tail (5'CAG TCG GGC GTC ATC) labeled with the FAM dye, to allow multiplexing primer pairs (up to four) and increase cost-efficiency of runs [17].

The total volume of each PCR was 12.5 μ L, containing 1 μ L of DNA (10 ng/ μ L), 6.25 μ L of the Qiagen Type-it® Master Mix for Microsatellite PCR, 1.25 μ L of primer mix and 4 μ L of PCR-grade water. The optimal PCR protocol consisted of an initial denaturation step at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30s, 56 °C for 90s and 72 °C for 30s; 8 cycles of 95 °C for 30s, 52 °C for 90s and 72 °C for 30s; and a final extension step at 68 °C for 30 min. PCR products were genotyped on an ABI 3130XL sequencer with the GeneScan 500 LIZ size standard (Applied Biosystems). We scored alleles using Geneious 8.1.7 and GENEMAPPER® 5.0 (Applied Biosystems™). From the 62 tested microsatellites, 31 were monomorphic and nine presented null alleles, thus rendering 22 polymorphic microsatellites, ranging from two to four alleles (Table S3).

In the third step, we tested the 22 markers on 12 blood samples from wild nestlings (Table 1) using the same protocol and PCR conditions described above, except for the annealing temperature (between 56 °C and 63 °C). After DNA fragment size genotyping, 15 loci produced unambiguous allele peaks (one to five alleles per locus in this final step, Table 2) and seven (*Ale292*, *Ale421*, *Ale514*, *Ale624*, *Ale324*, *Ale500* and *Ale624*) did not show clear assignable peaks or had a weak signal, rendering them inefficient for future studies with degraded DNA samples (such as molted feathers). Only two of the 15 assignable markers failed to amplify more than two samples (*Ale504*: 3 samples and *Ale361*: 4 samples), and one of them (*Ale361*) was monomorphic (Table 2).

We used GIMLET® 1.3.2 [18] to calculate the number of alleles (A), observed (H_o) and expected (H_e) heterozygosity, and polymorphism information content (PIC) in the final set of 15 loci and to estimate the probability of individual identification (PI) both accounting or not for the possibility of presence of siblings in the sample (PI sibs) for each marker. We tested for linkage disequilibrium and deviance from Hardy-Weinberg proportions using GENEPOP 4.7.0 [19] in R (R Core team program), applying the sequential Bonferroni correction [20].

Results and Discussion

The genetic diversity values obtained for each of the final 15 microsatellites are shown in Table 2. The resulting set showed adequate amplification in both invasive (blood) and non-invasive (molted feather) samples from wild Lear's

macaws. Most of the selected markers showed moderate levels of polymorphism, except for one monomorphic locus and two biallelic loci showing low heterozygosity and PIC values. With the observed levels of genetic variation (Table 2), the combination of the two most polymorphic loci (*i.e.*, the product of their PI values) was sufficient for individual identification below a 0.05 alpha error threshold. The combination of the four most polymorphic loci was necessary to reach the same level of individual identification when accounting for the possible presence of siblings in the sample (product of PI sibs indices, see Table 2). None of the markers showed evidence of linkage disequilibrium or deviance from Hardy-Weinberg equilibrium after applying the sequential Bonferroni correction.

Although informative, our markers showed lower levels of polymorphism than the set developed by Jan and Fumagalli [13]. Both studies used a similar number of markers (15 *vs.* 16, respectively), although Jan and Fumagalli [13] tested more DNA samples (26). Our results represent a sample from the whole wild population, while Jan and Fumagalli [13] used samples from captive individuals. Given the species' demographic history and its association with the pet trade [10,21], there is the possibility that the captive population may include lineages that are now extinct in the wild, and in this case the captive population could retain a higher genetic diversity than the current remaining wild population. In such a case, the wild population of *A. leari* might be facing the serious threat of genetic erosion caused by inbreeding and genetic drift. This hypothesis should be further tested by analyzing larger sample sizes from both the captive and remaining wild populations.

The main purpose for developing these new molecular tools is to assist the conservation of the Lear's macaw. The use of a high proportion or the complete set of markers now available is expected to provide the required power of resolution for pedigree inference of wild and captive individuals. These markers should help to optimize the genetic composition of captive breeding pairs within the *ex situ* conservation program, aimed to release captive-bred individuals to reinforce the isolated, functionally extinct population [CITA]. Moreover, once the genetic structure of the wild population will be evaluated, the microsatellite markers could also help to identify the geographic origin of individuals recently seized [22-24] and allow the identification of breeding sites vulnerable to nest poaching that need protection [25].

As a conclusion, the microsatellites developed in this study can be used for assessing the genetic diversity of captive and wild populations, estimating effective population sizes of breeding colonies and detecting demographic bottlenecks, which are paramount goals in the Lear's macaw conservation action plan [21]. This information is urgently required to support adequate conservation management programs for this endangered species [21]. Moreover, the application of our microsatellites to identify individuals may assist research on the Lear's macaw ecology, including the verification of nest-site fidelity among years, addressing sex ratio sampling bias (*i.e.* exclusion of replicated samples from molted feathers), and dispersal (through genetic tagging) [7, 26]. Altogether, conservation programs for the Lear's macaw will benefit from pilot studies combining both sets of markers to test their performance for the specific goals to be addressed, and so design cost-effective monitoring protocols [27]. Our results set the framework to incorporate genetic information into integrative management for the conservation of this globally endangered species.

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Declarations

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Conflict of interest

The authors declare they have no conflict of interest.

Ethical approval

Legal permits to conduct the field research, access protected areas in Brazil, handle birds and collect biological material were extended by SISBIO, permit n. 12763. CITES permit n. 14BR016156/DF allowed the transport of blood samples and feathers from Brazil to Spain for genetic analyses. Birds were handled following the guidelines of the Sistema Nacional de Anilhamento (SNA, CEMAVE, ICMBIO, MMA) under the authorization 2991 and bird banding license 458295, according to IN-IBAMA no 27/2002 of 23/12/2002. Blood sample extraction was approved by the ethical committee CEUA (Comissão de Ética no Uso de Animais, Brazil), Protocol 188/2013.

Informed consent

All authors consent to publication.

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FIGURE CAPTIONS

255 **Figure 1.** (A) Geographical extension of the Caatinga Dry Forest Biome (grey) in Northeast Brazil. (B) Location of
the five sampling localities (n. 1 to 5, see Table 1) where the remaining population of the Lear's macaw aggregates
in roosting sites, in the central-north of Bahia State, Brazil. Map obtained using QGIS v.3.8. (QGIS Development
Team, 2019). (C) Lear's macaw nestling (Photo by Maura Fernanda Lacerda). (D) Lear's macaw molted feathers
collected at roosting and foraging sites (Photo by Thiago Filadelfo).

Figure 2. Flow-chart of the three-step isolation and characterization of specific microsatellites for the Lear's macaw.

First step: the microsatellite enriched library was obtained using the DNA sample of a single wild individual.

Second step: 62 microsatellite primer pairs were tested on five individuals from five localities; nine primer pairs

presented null alleles, 31 resulted in monomorphic, and 22 in polymorphic microsatellites. Third step: the

polymorphic primer pairs were tested on 12 nestlings from different nests from the same locality; five primers

produced low quality profiles with many stutter bands, two were discarded due low signal, and 15 were informative.

TABLES

Table 1. Samples of Lear's macaw used in the three phases of the study: sample identification code, locality (numbers in concordance with Fig. 1), geographical coordinates (latitude and longitude), sample tissue type, month and year of collection. *Sample field number; blood samples are deposited at Museu de Zoologia da Universidade de São Paulo (MZUSP), DNA samples extracted from feathers are stored at Laboratorio de Ecología Molecular (LEM-EBD) and DNA samples from blood are also stored at Laboratório de Genética e Evolução Molecular de Aves (LGEMA-IBUSP).

Genetic sampling	Sample	Locality (n.)	latitude	longitude	type	month	year
Library enrichment	MZUSP 85891	Toca Velha (2)	S 9° 57.233	W 38° 59 588	blood	April	2008
Primers selection and optimization 1	LEM-EBD-adn254*	Cercadinho (1)	S 10° 08.266'	W 41° 08.080'	feather	May	2014
	183, F1-N02*	Toca Velha (2)	S 9° 57.233	W 38° 59 588	blood	April	2015
	181, F1-N55*	Barreiras (3)	S 9° 25.732'	W 38° 35.478'	blood	April	2015
	LEM-EBD-adn255*	Serra Branca (4)	S 9° 52.296'	W 38° 37.976'	feather	May	2014
	LEM-EBD-adn256*	Baixa do Chico (5)	S 9° 27.804'	W 38° 36.768'	feather	Jul	2014
Polymorphic primers selection and optimization 2; Individual identification	003, F1-N12*	Toca Velha (2)	S 9° 56.867'	W 38° 58.701'	blood	April	2008
	026, F1-N23*	Toca Velha (2)	S 9° 57.407'	W 38° 59.949'	blood	April	2009
	030, F2-N27*	Toca Velha (2)	S 9° 56.770'	W 38° 58.696'	blood	April	2009
	073, F1-N14*	Toca Velha (2)	S 9° 56.867'	W 38° 58.701'	blood	March	2011
	086, F1-N07*	Toca Velha (2)	S 9° 57.299'	W 38° 59.540'	blood	April	2011
	093, F2-N37*	Toca Velha (2)	S 9° 57.407'	W 38° 59.949'	blood	April	2011
	099, F1-N03*	Toca Velha (2)	S 9° 57.407'	W 38° 59.949'	blood	February	2012
	112, F2-N02*	Toca Velha (2)	S 9° 57.407'	W 38° 59.949'	blood	April	2012
	153, F2-N24*	Toca Velha (2)	S 9° 57.407'	W 38° 59.949'	blood	February	2014
	155, F2-N13*	Toca Velha (2)	S 9° 56.867'	W 38° 58.701'	blood	April	2014
	165, F1-N38*	Toca Velha (2)	S 9° 57.407'	W 38° 59.949'	blood	April	2014
	166, F1-N26*	Toca Velha (2)	S 9° 57.407'	W 38° 59.949'	blood	April	2014

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Table 2. Characterization of 15 microsatellite loci for the Lear's macaw: *N*: Number of successfully amplified samples, *A*: observed number of alleles, H_O : observed heterozygosity, H_E : expected heterozygosity, PIC: polymorphism information content, PI: probability of individual identification. PI sibs: probability of individual identification when accounting for the possibility of siblings in the sample.

Locus	Forward primer sequence	Reverse primer sequence	Repeated motif	Anneal. temp. (°C)	<i>N</i>	Allele size range (bp)	<i>A</i>	H_O	H_E	PIC	PI	PI sibs
<i>Ale066</i>	GAAAGCCTGCCAT GCCAAAG	TCTTGCACTTCCCT GAACCC	(AC)16	56	12	174-178	3	0.917	0.692	0.589	0.153	0.465
<i>Ale103</i>	TGGAACGCAATGG AGGACAG	GGCTGTCTGCTCTT TGATGC	(AC)12	59	12	189-193	2	0.083	0.083	0.077	0.811	0.923
<i>Ale176</i>	AACAGCTTCCTCA GTGTGGG	TCCTTCCTTCCCTCT TCCACT	(AAGGC)9	59	12	153-174	4	0.750	0.743	0.663	0.084	0.427
<i>Ale281</i>	GCATAGGAATCCA ACATCGGC	TTGCTCTGGGATGC TGCTTC	(AG)24	57	12	115-145	4	0.583	0.736	0.649	0.101	0.433
<i>Ale309</i>	TGAGGTTCCAAGA GACTCTCCT	TTCAGATCCTTGGC CCAGTG	(AC)9	57	11	144-146	2	0.091	0.091	0.083	0.796	0.916
<i>Ale327</i>	GTGATCTGGTGTG AGGACCA	GACAGTCTGCTTCA CTCCAGA	(AC)10	59	12	170-182	4	0.750	0.746	0.663	0.090	0.426
<i>Ale361</i>	ACAGGGACTTGAA TGTAGCAGT	ATGATGGTGTGATC CCAGCC	(AC)7	56	8	175	1	0.000	0.000	0.000	1.000	1.000
<i>Ale363</i>	TGGGAGGGTTTAT TGGTTGGG	TGTGGGATTGGCCA CTATAGG	(AC)12	59	12	106-130	3	0.417	0.540	0.420	0.294	0.574
<i>Ale409</i>	GGTTTCAGTCCTC CCTTGACT	TCCCTTCTTCTCCCA TTCCC	(AG)10	57	12	124-126	2	0.583	0.518	0.373	0.364	0.596
<i>Ale418</i>	CGCTTGCATGTAC TCTTACCC	TCAAGGGCAGTTGT GGGAG	(AC)16	56	12	203-224	3	0.417	0.453	0.369	0.315	0.629
<i>Ale504</i>	GCTTCTTAGCCAT GCAACCC	ACAAAGCCAACTTC CTACAACAA	(AT)7	59	9	148-150	2	0.556	0.425	0.321	0.386	0.659

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<i>Ale517</i>	TTGTAGTAAGTGT GGGTAAAGCC	CATGGAGCTCTGGG CAGAAA	(ACT)16	56	11	155-167	4	0.545	0.515	0.451	0.206	0.579
<i>Ale529</i>	TGATAAGCAGAGG AGGAATCCT	AGATGGAGGTGCTG TGAGAC	(AC)16	59	12	128-150	5	0.667	0.710	0.628	0.101	0.448
<i>Ale606</i>	AATCCCAACTCAA CAGGGTTGT	CTTTCTACCTTCTCA AACACCCT	(AC)11	59	11	104-106	2	0.364	0.312	0.253	0.464	0.736
<i>Ale628</i>	AGAGGGAATGTCT CTTCCCATT	TGCTAGATAACATA CACAGCCAG	(AC)9	56	11	135-147	3	0.545	0.450	0.385	0.280	0.628