Development, characterization, and multiplex reaction of microsatellite loci in Lithraea molleoides (Anacardiaceae), a dominant tree from the mountains of Gran Chaco

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Short Report

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Abstract

We develop and characterize 14 microsatellite primers in *Lithraea molleoides,* a dominant dioecious native tree to Gran Chaco mountains. Five of them were optimized for multiplex PCR and evaluated in natural populations of the species. In order to analyze the level of polymorphism, 63 individuals of *L. molleoides* were assessed from two localities of central Argentina. Two to six alleles per locus were found with an average effective number of 1.82 alleles. The mean expected heterozygosity in each studied locality was 0.405 and 0.436. In view of generating tools for the conservation of species in a context of ongoing habitat loss, this set of newly developed microsatellite markers will be fundamental for assessing the genetic diversity and differentiation, as well as gene flow patterns of *L. molleoides* populations throughout its distribution range.

Key Message

Addressing Gran Chaco's pressing conservation needs, we established microsatellite markers for *L. molleoides*, a pivotal mountain tree. By developing 14 markers from enriched genomic libraries and optimizing multiplex PCR, we offer a potent tool to counter habitat loss and declining populations in this critical ecosystem.

Introduction

Tropical and subtropical dry forests worldwide are experiencing large-scale, rapid land use changes and associated biodiversity loss. The Gran Chaco ecoregion, the most extensive and threatened seasonally dry forest of South America, exemplifies this concerning trend, as it is a global deforestation hotspot (Kuemmerle *et al.* 2017). The lowland areas are significantly impacted by soybean cultivation and cattle ranching, and mountains are affected by fire, grazing, and the non-native species encroachment (Cabido *et al.* 2018; Piquer-Rodríguez *et al.* 2018). The Gran Chaco harbors high biodiversity, many endemic species, and has urgent conservation needs.

Genetic diversity, alongside ecosystem and species diversity, constitutes a fundamental aspect of biodiversity (DeWoody *et al.* 2021). Unfortunately, the intraspecific genetic diversity of plant species from Gran Chaco has been poorly explored and particularly, a small number of species-specific molecular markers has been developed for their emblematic native tree species (e.g., *Prosopis* sp., Pomponio *et al.* 2015, Bessega *et al.*, 2013, Mottura *et al.* 2005, *Bulnesia sarmientoi*, Camps *et al.* 2018). Hypervariable genetic markers such as microsatellites (SSR) and single nucleotide polymorphism (SNP) are necessary for assessing genetic erosion within and among populations, fine-scale population genetics and developing effective conservation management and recovery plans for at-risk species (Hussain and Nisar 2020).

Lithraea molleoides (Vell.) Engl. (Anacardiaceae) is a dioecious evergreen tree native to South America, and a dominant species of the mountains from Gran Chaco (Chaco Serrano region, following Cabido *et al.* 2018). In this study, we developed and characterized species-specific microsatellite markers for *L.*

molleoides based on enriched genomic libraries. In addition, we optimized a multiplex polymerase chain reaction to combine the polymorphic microsatellite loci chosen. In order to determine the genetic variability of polymorphic microsatellite loci, we genotyped and characterized two localities of *L. molleoides* in the Chaco Serrano, in Córdoba province, Argentina. Estimates of population genetic metrics generated from these microsatellite markers will be of great value to assess the effect of rapid land-use changes in the conservation status of the species.

Materials and Methods

Microsatellite development

For the isolation of polymorphic loci, we first extract genomic DNA from L. molleoides individuals using the DNeasy Plant Mini Kit (Qiagen). Then, we send the extracted DNA to Genetic Marker Services (Brighton, UK); a company specializing in microsatellite development. For the microsatellite isolation process, an enriched library was constructed and then, a hybridization capture protocol was used. Enrichment was achieved by incubating adaptor-ligated, restricted DNA with synthetic repeat motifs such as [AC]17, [AG]17, [AAC]10, [AAT]10, [CCG]10, and [CTG]10, that were bonded to filters. Optimization of the enrichment was attained by washing the filters at various temperatures (50-60°C) and SSC stringency levels (0.5X-2X). Transformation of the library into Escherichia coli JM109 was performed by plating onto LB-agar/ampicillin plates. Then, screening of motif-positive clones was conducted, followed by their isolation, culturation, and sequencing. Finally, sequence editing was performed to remove plasmid sequence, and the software Tandem Repeats Finder (Benson, 1999) was utilized to locate repeated regions of different sizes.

We selected loci for further development according to their clean repeat structure and consistency, adequate flanking sequence, and absence of regions of larger cryptic repetitive DNA. We designed primers for the chosen loci using PRIMER 3.0 software (Rozen and Skaletsky, 2000). Our aim was to seek primer pairs that could amplify products ranging from 100 to 250 bp, thus minimizing overlap ambiguities during sequencer genotyping. To evaluate the performance of the primers, we conducted touchdown PCR experiments using a total of seven individuals previously sampled from five different sites in the Chaco Serrano, Córdoba, Argentina, with a minimum distance of 5 kilometers between each site.

PCR amplifications included 7 pmol of each primer, 1.5 mM of MgCl2, 0.2 mM of each dNTP, 1X PCR buffer, 0.8 µg/µL BSA, 0.5 U Taq Polymerase, and 1.5 µL of DNA diluted 20-fold, in a final volume of 25 µL. The touchdown PCR protocol consisted of 32 cycles, starting with denaturation at 95°C for 60 s, followed by annealing for 60 s (two cycles at 64–59°C, 10 cycles at 58°C, and another 10 cycles at 57°C), elongation at 72°C for 60 s, and a final extension step at 72°C for 5 min.

We assessed the specificity, active polymorphism, and presence of null alleles in the PCR products using cooled high-resolution agarose gels containing 4% MetaPhor (Lonza) agarose in TAE. Gel

electrophoresis was performed in a cold room at 10°C. Among the seven individuals used for testing polymorphism, a total of fourteen loci exhibited clear and specific bands, as detailed in Table 1.

Table 1. Characteristics of 14 microsatellite primers developed for *Lithraea molleoides*. The five pairs selected for the multiplex reaction and the genotyping are shown with asterisks. The fluorochromes used for labeling the forward primers for genotyping are indicated in parentheses. **Bp**: base pairs.

N°	Primer	Forward 5'-3'	Reverse 5'-3'	Bp
1*	*LMO1* (FAM)	TAT CTT CAC CGT CGA ACT GC	GAG CCC AAG TTG TGT TGG TT	198
2*	*LMO5* (FAM)	CCC TTA CTC GCA TAG CAG GT	TCA CAC CTA TGT TGT CTT AAA TCT TG	137
3*	*LMO90* (HEX)	TGG ATT GGA GAT GGA AAT GG	CCG TTC AGA TTC TCA AAG CA	176
4*	*LMO93* (FAM)	GTG TGC CTG TGT GTG CTT GT	GGG TCC ACA CCT AAG CAT TG	104
5*	*LMO95* (HEX)	AAA GGC CTC TCA GCA ACA TC	TCC AAT CAA AAG CAT TCT TCC T	127
6	*LMO7*	TGA CAA GTT TGC TGA GGG AAG	CAC GCA CAC ACA CAA ACA CG	168
7	*LMO9*	CCA TTT GGG TTG CTT TGT CT	TTG GAC AAG TTT GTA TGT GGT TG	185
8	*LMO10*	AGT GAA AGA CAG TTT GAA GT	GTA CTC ACC TAC TAC ATA ATC	102
9	*LMO11*	ACA TTT GGT GGT TTG AAA TCT T	GTA CCC ATC CTC CTC TAG ACA A	134
10	*LMO12*	CCC AAT CAA GTT TTG TGA TGC	CAA CTG TTT GCG TTA CGT TCA	171
11	*LMO16*	GAT TCT TGG GCT TGG AAT TG	CAA CAC GCA CAC ACT CAC AC	193
12	*LMO19*	CGC TGA GCA TTA AAG GGT GT	CAC ATG CAC ACA CAC ACG TA	123
13	*LMO92*	CTC CAG GTC GCT TCA ATG AT	GGT TCT TGT AAG CGC AGA GA	127
14	*LMO94*	TCT CAG TAT CTC CCG TGA ATA GA	GTA CAA GTT AAA ACA TGG CAG C	128

Multiplex amplification and microsatellite marker analysis

We optimized a multiplex PCR in order to make the genetic analysis of different populations more efficient. Due to limited funding and currency depreciation (Petherick 2017; Catanzaro 2018), we were able to use five of the 14 primer pairs developed for this purpose. For multiplex PCR we used an

equimolar mixture of five fluorescent-labeled polymorphic microsatellite primers. In order to determine the genetic variability of these loci, we genotyped 31 and 32 individuals of *L. molleoides*, from two localities, totaling 63 individuals. These samples were collected from the mountains of Chaco Forest of central Argentina (locality 1: 31° 7' 52.55"S and 64° 19' 52.82"W, locality 2; 31° 10' 37.26" and 64° 16' 12.49"W). We performed DNA extractions following a modified CTAB method (Wilkie *et al.* 1997) with the addition of PVP (polyvinylpyrrolidone) optimized for plants with a high concentration of phenolic compounds.

PCR reaction mix contained 1 µL (~10 ng) of DNA, 1X of Kapa load-stained buffer (Biosystems), .5 mM of MgCl2, 0.2 mM of each dNTP, 0.8ug/µl of bovine serum albumine (BSA), 0.2 mM of each primer, 0.5U of Kapa Taq DNA polymerase (Biosystems), for a total of 25 µL. The PCR amplification conditions were as follows: initial denaturation for 1 min at 95°C; followed by 32 cycles (denaturation for 1 min at 95°C, alignment 1 min (two cycles at 64 - 59°C, ten cycles at 58°C, ten cycles at 57°C and elongation for 1 min at 72°C), and a termination phase of 5 min at 72°C. The process was carried out in an Eppendorf Mastercycler thermal cycler. To test for DNA amplicons after Multiplex PCR, 5 µl of PCR products were loaded onto agarose gel stained with 1% Sybr Safe (Invitrogen). PCR products were genotyped by Macrogen Inc., South Korea (www.macrogen.com). Lastly, genotypes of each individual were determined using PeakScanner 1.0 software (Applied Biosystems).

Genetic diversity analyses

GenAlEx v. 6.5 (Peakall and Smouse 2012) was used to estimate total number of alleles, average number of alleles per locus, effective number of alleles, observed and expected heterozygosity.

Results and Discussion

In this study, we developed the first microsatellite primers for the native and dominant tree species *Lithraea molleoides* from Gran Chaco, five of which were grouped in a multiplex PCR, and further tested in natural populations of the species. These primer pairs revealed clear and reproducible variable loci in *L. molleoides.* The number of alleles per locus (A) ranged from 2 to 6, with the value of effective alleles (Ae) ranging between 2.818 and 1.111 (Table 2). The mean of Ae values was 1.868 and 1.78 alleles per locality. The mean observed heterozygosity (Ho) value was 0.328 and 0.729 per locality, and expected heterozygosity (He) value was 0.436 and 0.405 per locality.

Table 2. Descriptive statistics for polymorphic microsatellites in two localities of *Lithraea molleoides*. **N**: number of individuals, **A**: number of different alleles, **Ae**: number of effective alleles, **Ho**: observed heterozygosity, **He**: expected heterozygosity, **SE**: standard deviation.

	Locality 1					Locality 2				
Locus	**N**	**A**	**Ae**	**Ho**	**He**	**N**	**A**	**Ae**	**Ho**	**He**
LMO1	31	4	1.615	0.387	0.381	32	3	1.661	0.638	0.398
LMO5	29	3	1.591	0.241	0.372	29	4	1.111	0.260	0.100
LMO90	30	3	1.504	0.300	0.335	31	5	2.034	1.024	0.508
LMO93	31	6	2.818	0.355	0.645	32	5	1.938	0.895	0.484
LMO95	31	2	1.811	0.355	0.448	26	3	2.156	0.830	0.536
Mean	**30.4**	**4**	**1.868**	**0.328**	**0.436**	**30.0**	**4**	**1.780**	**0.729**	**0.405**
SE	**0.40**	**1**	**0.243**	**0.026**	**0.055**	**1.14**	**0**	**0.186**	**0.133**	**0.08**

Conclusion

The development of these molecular markers is of vital importance for studying this species, given the current scenario of accelerated habitat loss and fragmentation in the Gran Chaco (Kuemmerle *et al.* 2017). These markers will enable the study of intraspecific genetic diversity at different geographical scales, as well as the study of species' patterns of gene flow. This valuable resource will greatly contribute to conservation strategies for this dominant species.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Lithraea molleoides microsatellite data will be submitted to NCBI Genebank, and accession numbers will be provided prior to final acceptance of the manuscript. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Author contributions

ALC, Investigation, Project administration, Writing - original draft, Formal analysis.
MCA: Conceptualization, Formal analysis, Writing - review & editing, **NH** Investigation**, RA** and **LA:** Conceptualization, Methodology, Resources, Supervision, Project administration, Writing - review & editing, Funding acquisition. All authors contributed to critical revision of the manuscript, read and approved the final version.

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Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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