Genetic Variation at Microsatellite Loci in the Tropical Herb Aphelandra aurantiaca (Acanthaceae)

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Primer Note

Genetic variation at microsatellite loci in the tropical herb *Aphelandra aurantiaca* (Acanthaceae)[1]

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- *Premise of the study:* To assess the effect of forest fragmentation on genetic variation and population structure of *Aphelandra aurantiaca* (Acanthaceae), a tropical and ornamental herbaceous perennial plant, we developed the first microsatellite primers for the species.
- *Methods and Results:* Fourteen microsatellite markers were isolated and characterized from *A. aurantiaca* genomic libraries enriched for di-, tri-, and tetranucleotide repeat motifs. Polymorphism was evaluated in 107 individuals from four natural populations. Twelve out of 14 genetic markers were polymorphic. The number of alleles per locus ranged from two to 12, and the observed and expected heterozygosities ranged from 0.22 to 0.96 and from 0.20 to 0.87, respectively. Fixation indices ranged from −0.41 to 0.44.
- *Conclusions:* These newly developed microsatellite markers for *A. aurantiaca* will be useful for future population genetic studies, specifically to detect the possible loss of genetic diversity due to habitat fragmentation.

Key words: Acanthaceae; *Aphelandra aurantiaca*; gene flow; genetic structure; Los Tuxtlas tropical rainforest; outcrossing rate.

Aphelandra R. Br. is one of the largest genera of Acanthaceae, comprising ca. 175 species of perennial herbs, shrubs, and small trees restricted to the Neotropics (Wasshausen, 1975; Daniel, 1991). Species in this genus have colored flowering spikes (Wasshausen, 1975), and the genus is well known to horticulturists because some species are cultivated for ornamental purposes (Daniel, 1991). However, until now, no studies of molecular genetic diversity in this genus have been carried out. We focus on the understory herb *A. aurantiaca* (Scheidw.) Lindl., distributed from southern Mexico through Central and South America (Daniel, 1991). In Mexico, its distribution is restricted to regions with abundant rainfall such as Los Tuxtlas rainforest (Daniel, 1991), where it is one of the dominant understory species (Calvo-Irabién, 1997). The region of Los Tuxtlas, considered the northernmost limit of rainforests in the Americas, has been heavily impacted by deforestation and fragmentation (Dirzo and Miranda, 1991; Dirzo and García, 1992). Because fragmentation produces isolation between populations, it could impact their genetic structure (Chávez-Pesqueira et al., 2014), reducing genetic variation and gene flow, and increasing genetic divergence and inbreeding (Young et al., 1996). *Aphelandra aurantiaca* is a suitable model to study the genetic consequences of rainforest fragmentation due to the life history characteristics of the species. For example, it has a relatively short life span, which means that some generations have passed since the onset of fragmentation, and it depends on canopy cover, which is usually reduced in forest fragments. Furthermore, because *A. aurantiaca*'s attractive, nectar-producing flowers are pollinated by birds (Calvo-Irabién, 1997), its mating system can be affected by habitat fragmentation if this reduces species richness and abundance of pollinators (Aguilar et al., 2006). To date, little is known about its genetic structure, particularly in the context of rainforest fragmentation. Therefore, we aimed to develop variable genetic markers to elucidate the genetic diversity and structure of *A. aurantiaca*.

METHODS AND RESULTS

Using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA), we extracted genomic DNA from a single individual of *A. aurantiaca* for use in the isolation of microsatellite loci. A paired-end library was prepared by shearing 1 µg of genomic DNA following the standard protocol of the Illumina TruSeq DNA Library Kit (Illumina, San Diego, California, USA). Illumina sequencing was conducted on the HiSeq (Illumina) with 100-bp paired-end reads. Ten million of the resulting sequences were analyzed with the program PAL_FINDER_v0.02.03 (Castoe et al., 2012), extracting positive reads that contained di-, tri-, tetra-, penta-, and hexanucleotide microsatellites and sending to the program Primer3 (version 2.0.0; Rozen and Skaletsky, 1999) for primer design. To avoid duplicated loci, data were filtered and only primers that occurred one or two times were included; 24 loci out of 1722 that met this criterion were chosen. Primer pairs were tested for amplification and polymorphism using DNA obtained from five different individuals from the four Los Tuxtlas

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populations sampled (Appendix 1), and amplified PCR products were then separated on 4% Metaphor agarose gels (Lonza, Rockland, Maine, USA). After excluding loci that did not amplify, we selected 14 potential polymorphic loci and marked these with fluorescent labels (Table 1). The PCR amplification was carried out in a 20-μL reaction containing 2 μL of 10× PCR buffer (KCl 500 mM, Tris-HCL pH 8.3, gelatin 100 μg/mL, 1% triton, bovine serum albumin [BSA] 1.5 mg/mL), 1 μL of $MgCl_2$ (30 mM), 2 μL of dNTPs (0.2 mM), 2 μL of DNA, 0.5 μL of each of the two primers (10 mM), 0.5 μL of *Taq* DNA polymerase (5 U/μL), and 12 μL of water (BIOTECMOL, Mexico City, Mexico), performed on a Thermo Scientific Hybaid Px2 thermal cycler (Thermo Scientific, Waltham, Massachusetts, USA) using the following conditions: 94°C for 10 min; followed by 35 cycles of 94°C for 1 min, at temperatures between 55–61°C for 1 min, and 72°C for 1 min; and a final extension step of 72°C for 7 min.

To encompass the most genetic diversity of *A. aurantiaca* in the Los Tuxtlas rainforest, we collected leaf tissue of 107 individuals from four populations (Appendix 1). Genomic DNA was extracted following the cetyltrimethylammonium bromide (CTAB) MiniPrep protocol (Doyle and Doyle, 1987). We selected a subset of loci to function well together in four multiplex reactions (QIAGEN Multiplex PCR Kit) with labeled primers (Applied Biosystems, Foster City, California, USA) (Table 1). Each multiplex PCR mixture (10 μL) contained 2 μL of DNA template (20 ng), 0.2 μL of each fluorescent-labeled forward primer (0.2 μM), 0.2 μL of each reverse primer (0.2 μM), 5 μL of QIAGEN Reaction Mix (1×), and 2.6 μL of RNase/DNase-free water (the volume of water varied depending on the number of primers in each multiplex reaction) (QIAGEN). Multiplexed reactions were carried out on a Hybaid Px2 thermal cycler (Thermo Scientific) and a Veriti 96-Well Thermal Cycler (Applied Biosystems). PCRs were performed through touchdown reactions, starting with initial heat activation at 95°C for 10 min, followed by 31 cycles with denaturation of 94°C for 60 s, annealing for 60 s, and 60 s of extension at 72°C. Annealing cycling temperature began at 57°C and decreased 1°C every cycle for six cycles (to 51°C), followed by two stages of 12 cycles each (with annealing temperatures of 55°C and 54°C). To check amplification, 5 μL of the PCR products were subjected to electrophoresis in a 1.5% agarose gel with 1× TBE buffer and stained with ethidium bromide. The remaining PCR products (5 μL) were diluted in 10 μL of water. One or two microliters of these PCR products (20–50 ng) were run on ABI Prism 310 and ABI 3730xl (Applied Biosystems) automated capillary sequencers; allele sizes were scored manually using GeneScan 500 LIZ Size Standard (Applied Biosystems) in GeneMarker version 2.4.0 (SoftGenetics LLC, State College, Pennsylvania, USA).

Of the 14 primers tested, 12 were polymorphic and two were monomorphic with high-quality amplification (Table 2). For each polymorphic locus, we calculated the number of alleles (A), observed heterozygosity (H_o), and expected heterozygosity (H_e); tests of deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed using the software Arlequin version 3.5.1.3 (Excoffier and Lischer, 2010). Fixation indices (F_{IS}) were estimated by GenAlEx version 6.5 (Peakall and Smouse, 2006). The probability of null alleles was estimated using MICRO-CHECKER software (van Oosterhout et al., 2004). We detected higher probabilities of null alleles between two loci (5250 and 1071) as suggested by the general excess of homozygotes (Table 2). There was LD at 10 of 90 paired loci comparisons, and significant departure from HWE was inferred at six loci, although this figure varied depending on the studied population (Table 2). A ranged from two to 12 across the studied populations. H_o and H_e ranged from 0.22 to 0.96 and from 0.20 to 0.87, respectively, and F_{IS} ranged from –0.41 to 0.44.

CONCLUSIONS

We developed and characterized 12 polymorphic and two monomorphic novel microsatellite markers for the herb *A. aurantiaca*. The primers will be useful for assessing population genetic structure and mating system of *A. aurantiaca* in both

TABLE 1. Characteristics of 14 microsatellite loci developed in *Aphelandra aurantiaca.*

Locus	Primer sequences (5′–3′)	Allele size range (bp)	Fluorescent label	Repeat motif	GenBank accession no.
0432[a]	F: AGGCTGAAGAGATTTGCAGG R: AAGACAGGCTGATGCAGTCG	113–124	NED	$(AGCC)_{24}$	SRR1816885
1233[a]	F: GTTGCATTTGAGGCATGAGG R: TGTAATTGAACTAGGTCTTGTACTCGC	116–126	PET	$(AT)_{22}$	SRR1514097
4343[a]	F: TGTAAAGGAAAGTTGAAGAAATAAGGG R: TGATTCGTTGGAGACACATGC	150–172	6-FAM	$(ATT)_{27}$	SRR1817142
4914[a]	F: AGGAATTGTCCGGTCTTCCC R: CCGGCTGATTCTGCTTCC	130–152	VIC	$(AT)_{22}$	SRR1817143
5490[a]	F: GGTGTACGTAGCCCACAACG R: TGAAGAAGTTGTTCCAAGGTACG	174–184	NED	$(ATGC)_{24}$	SRR1816884
1810[b]	F: TGGCACTTATAGCCACATCCC R: GAACCAGTGTTGCGTGTCC	194–207	PET	$(AC)_{26}$	SRR1817168
4378[b]*	F: GAGAATATAGAGGCCACCGGG R: TCCGGTACATGCTCCAAAGG	216	VIC	$(TTC)_{18}$	SRR1817171
1721[c]*	F: TCCTCCTCTCTCATTACAAGTGG R: TGTTCTTTAGTTTGCACACGC	180	PET	$(TC)_{20}$	SRR1817170
4483[c]	F: GATGGAGGCAGTGGAGATAGC R: GCAGAATCTTCTGGAACCACC	206–229	NED	$(TC)_{30}$	SRR1817184
5250[c]	F: TTCCTTCTTGTTGTTATTCTTGGC R: GGAACAAAGAGTCATGATTGAAGC	208–293	6-FAM	$(TC)_{28}$	SRR1817169
1071[d]	F: TTGTATTTGAATTGAACCCTTTCG R: CGAATTGAAGTCCAATGTGGC	272–304	PET	$(AT)_{26}$	SRR1817193
1808[d]	F: TGCGTGTCTTTGTTGTACTATCTGG R: AATGCTCAAGGCATGCACC	294–318	NED	$(AGT)_{30}$	SRR1817198
4536[d]	F: AAGAATTGTAATCCTTGAAAGCCC R: GGAAATTTATATGGAATGCCGC	187–193	6-FAM	$(TGC)_{21}$	SRR1817191
5441[d]	F: CAAAGACCTGTAATAGATATAAGGAAGCC R: AACTTAATGGACCATGTCGGC	200–300	VIC	$(TC)_{30}$	SRR1817260

Note: Annealing temperature was the same for all primers (T_a = 57°C). For genotyping, we used: (a) one quintuplex reaction (loci 0432, 1233, 4343, 4914, and 5490), (b) one duplex reaction (loci 1810 and 4378), (c) one triplex reaction (loci 1721, 4483, and 5250), and (d) one quadruplex reaction (loci 1071, 1808, 4536, and 5441).

*Monomorphic locus.

TABLE 2. Genetic properties of the newly developed polymorphic microsatellite loci of *Aphelandra aurantiaca*.[a]

	F1 (n = 27)			Selva 1 (n = 21)			Selva 2 (n = 31)			Bambú (n = 28)			
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	F_{IS}[b]
0432	4	0.74	0.72	4	0.33	0.51	4	0.61	0.64	3	0.46	0.59	0.14
1233	7	0.66	0.63	5	0.66	0.68	5	0.61	0.65	9	0.60	0.73	0.04
4343	5	0.62	0.73	7	0.61	0.64	7	0.74	0.72	7	0.46	0.75	0.12
4914	4	0.59	0.51	4	0.52	0.55	4	0.48	0.60	7	0.57	0.65	0.05
5409	3	0.96	0.54*	4	0.42	0.47	3	0.51	0.54	3	0.46	0.59	–0.12
1810	7	0.92	0.62*	4	0.85	0.60	5	0.96	0.67	4	0.85	0.68	–0.41
4483	10	0.70	0.86*	7	0.76	0.74	9	0.58	0.73	7	0.42	0.59	0.14
5250	7	0.51	0.76*	6	0.38	0.48	7	0.63	0.74	7	0.39	0.76‡	0.28
1071	7	0.48	0.73	8	0.45	0.84‡	10	0.46	0.85*‡	6	0.31	0.73‡	0.44
1808	6	0.40	0.59	7	0.57	0.66	9	0.45	0.61	7	0.46	0.50	0.18
4536	4	0.22	0.27	5	0.35	0.54*	2	0.22	0.20	3	0.44	0.41	0.11
5441	7	0.81	0.80	11	0.73	0.87	11	0.87	0.82	12	0.67	0.83	0.05

Note: A = number of alleles; F_{IS} = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity.
[a] All values are based on 107 samples representing Los Tuxtlas rainforest located in southern Mexico. See Appendix 1 for locality and voucher information.
[b] Fixation index of each locus across populations.
*Loci that were not in Hardy–Weinberg equilibrium ($P < 0.001$).
‡Null alleles.

preserved and fragmented rainforest. Likewise, we expect these microsatellite loci could be useful for other *Aphelandra* species.

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APPENDIX 1. Geographic location and voucher information of populations of *Aphelandra aurantiaca* from Los Tuxtlas tropical rainforest. A voucher was collected only at the largest forest fragment (ca. 700 ha).

Species	No. of individuals	Voucher specimen[a]	Collection locality	Geographic coordinates
A. aurantiaca	27	—	Site F1	18°34.648′N, 95°4.105′W
A. aurantiaca	21	TUXsno3239	Site Selva 1	18°35.153′N, 95°4.609′W
A. aurantiaca	31	—	Site Selva 2	18°35.269′N, 95°6.023′W
A. aurantiaca	28	—	Site Bambú	18°36.607′N, 95°8.363′W

[a] Voucher specimen is deposited at the herbarium of the Instituto de Biología, Universidad Nacional Autónoma de México (MEXU); subcollection of Los Tuxtlas herbarium.