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MICROSATELLITE LETTERS

Development and characterization of twenty-two polymorphic microsatellite markers for the leafcutter ant, *Acromyrmex lundii*, utilizing Illumina sequencing

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Abstract We isolated and characterized a total of 22 microsatellite loci for the leafcutter ant, *Acromyrmex lundii*. The loci were screened for 24 individuals from southern Brazil and Uruguay. The number of alleles per locus ranged from 5 to 20, the observed heterozygosity ranged from 0.417 to 0.917, and the probability of identity values ranged from 0.011 to 0.38. These genetic markers will be useful for understanding the population and conservation biology of the leafcutter ant *A. lundii* and closely related species, and will provide novel insights into the evolutionary biology of social parasitism and leafcutter ant mating systems.

Keywords Leafcutter ants · Fungus-growing ants · Attini · *Acromyrmex* · Polygyny · Polyandry · Social parasitism · Microsatellites · Illumina · PAL_FINDER · PCR primers · SSR

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Primer Note

The leafcutter ants of the genera *Acromyrmex* and *Atta* represent the most derived clade within the fungus-growing ants and their colonies are genetically complex societies governed by either one or multiple queens, which can be inseminated by multiple males (Boomsma et al. 1999). Their complex colony structure makes leafcutter ants well-suited study organisms for investigating the evolution of cooperation and conflict in social insect societies and their mating systems. Furthermore, *Acromyrmex* colonies are exploited by at least four species of obligate social parasites (see Rabeling and Bacci 2010 for a brief review) and polygyny, the presence of more than a single reproductively active queen per colony, is thought to be a precondition for the evolution of social parasitism. Lastly, most *Acromyrmex* species include multiple subspecies that have restricted and in some cases disjunct geographic distributions (Gonçalves 1961). Therefore, these geographically isolated subspecies could potentially comprise cryptic species that were not recognized previously, which makes *Acromyrmex* ants an interesting system to investigate the population genetic conditions of cryptic species and the conservation genetics of social insects in general.

To investigate the evolution of mating systems and social parasitism in leafcutter ants and to test whether six geographically separate subspecies represent reproductively isolated cryptic species that are new to science, we developed 22 polymorphic microsatellite markers for the leafcutter ant, *Acromyrmex lundii*. Genomic DNA used to isolate the microsatellite loci was extracted from single individuals of *A. lundii*, utilizing a QIAamp DNA Micro Kit (QIAGEN). An Illumina paired-end shotgun library was prepared by shearing 1 μg of DNA using a Covaris S220 and following the standard protocol of the Illumina

Table 1 Details for 22 polymorphic microsatellite loci developed for the leafcutter ant *Acromyrmex lundii*

Locus	Primer sequence 5′ → 3′	Repeat motif	Size (bp)	N	*K*	H_o	H_e	PI	TD
Aclu 3	F[a]: AAGTTGTCACAGGTGCTCGC	TGCG	238–274	24	10	0.750	0.788	0.069	65
	R: AATCTTTGAAGTTCGCGATGG								
Aclu 6	F[a]: CTATGAGCGTACAGCAGCGG	TGCG	312–364	24	14	0.833	0.812	0.053	65
	R: ACGCACGACCACACATACG								
Aclu 8	F[a]: TTATTGTTTCAATTAACGCGATTCC	AACG	315–356	24	10	0.750	0.724	0.11	65
	R: AGAAACGAGAGAGGGTGGATAGC								
Aclu 9[b]	F[a]: TGAATTCCTTGCCGAACTCC	TGC	266–326	24	20	0.750	0.924	0.011	65
	R:AGGACGGATCGACAGTGAGC								
Aclu 11	F[a]: GATCCCGACTGACCTGTGG	TGCG	218–230	24	5	0.792	0.739	0.11	65
	R: CATCAGTGCAGCGTTACTCG								
Aclu 12	F[a]:TGAGAGTCCACTCTAGTAATAAATGCTCC	ATCT	275–315	24	10	0.792	0.872	0.03	65
	R:CATTAACCCATATTTATGAACTCAATAGC								
Aclu 13	F[a]: ACGAATTAGTCCACGATGCC	ATACC	289–344	23	10	0.870	0.831	0.05	65
	R: CCACGTGACATGTATTCGC								
Aclu 14	F[a]: TACACGTTCCGCGTGTCC	AACG	238–279	24	8	0.625	0.735	0.12	65
	R: CCTAATCCACGTACTCACGTCC								
Aclu 15	F[a]: GTCGCCGCGATTATGATACC	ACCG	351–395	24	8	0.542	0.692	0.14	65
	R: CGCAGCGATTGTAACGTCG								
Aclu 20[b]	F[a]: TAGCCCATCGAGAGCGACG	TCGCG	238–266	24	7	0.750	0.837	0.048	65
	R: GACCGATACCGCATCCTC								
Aclu 21	F[a]: CCTCCTCGCACATAATTCGC	ATAC	283–348	24	15	0.917	0.910	0.015	65
	R: GATACTTCGAACGACCTTGATCC								
Aclu 25	F[a]: TGAAGGCTGTCTGTTCTAAATTCG	TCCG	267–302	24	5	0.625	0.694	0.15	65
	R: GCGATACGATTCCCAAGTCC								
Aclu 29	F[a]: CAAGTCCCGATGATTTGCG	TTCC	114–134	24	6	0.625	0.707	0.14	65
	R: GGAAGGGAGAGAACGCGG								
Aclu 31	F[a]: TAGCGTGTTCGTGTTCCCG	TGCG	316–342	24	7	0.917	0.778	0.082	65
	R: GGGAAGAGGCAAGAAGATCG								
Aclu 32	F[a]: GCTTACTCATTCGCATTCGC	TGCG	166–214	24	9	0.708	0.798	0.064	65
	R: GGTAATACCATCGGACTTTGCG								
Aclu 34	F[a]: AGGCCAGACGGGTAGACG	TGCC	276–296	24	6	0.583	0.636	0.2	65
	R: GTTCGATTAATGGTCCCGC								
Aclu 36[b]	F[a]: AGCAGAAATCTCATATTAGGCACC	TGCG	248–310	24	10	0.542	0.851	0.038	65
	R: TCAGTGTATCTCTGGTTGAACGC								
Aclu 39	F[a]: CCTCCATGAAATGAGCATCG	TGCG	286–354	24	9	0.417	0.627	0.16	65
	R: TGAATCAATTCGTGTTAGTGCG								

Table 1 continued

Locus	Primer sequence 5′ → 3′	Repeat motif	Size (bp)	N	*K*	H_o	H_e	PI	TD
Aclu 41	F[a]: AAGACCGAAGGTATCCCAACC R: TGCCCTAGAGCCATAATAAATGC	AATC	280–296	24	5	0.625	0.689	0.15	65
Aclu 43	F[a]: GTTGTGTCGTGCAGAACTGG R: TGGTCCGTCTTCTTGCCC	TGTC	276–308	24	7	0.625	0.694	0.12	65
Aclu 44	F[a]: TATACACACCAATCGCGTCG R: TGTATGTGCATTGATAGTACACGC	TGCG	184–216	24	9	0.792	0.796	0.068	65
Aclu 45	F[a]: CTTACGTGTCGCTCTCCCG R: TTACACGTATCTCTCGCGTCG	TGCG	220–267	24	12	0.875	0.851	0.38	65

The size indicates the range of observed alleles in base pairs and includes the length of the CAG tag; number of individuals genotyped is *N*; *k* is number of alleles observed; H_o and H_e are observed and expected heterozygosity, respectively; PI is the probability of identity for each locus, and TD refers to the touchdown protocol used for PCR (see text)

[a] indicates CAG tag (5′-CAGTCGGGCGTCATCA-3′) label

[b] indicates significant deviations from Hardy–Weinberg expectations after Bonferroni corrections

TruSeq DNA Library Kit and using a multiplex identifier adaptor index. Illumina sequencing was conducted on the HiSeq with 100 bp paired-end reads. Five million of the resulting reads were analyzed with the program *PAL_FINDER_v0.02.03* (Castoe et al. 2012) to extract those reads that contained di-, tri-, tetra-, penta-, and hexanucleotide microsatellites. Once positive reads were identified in *PAL_FINDER_v0.02.03*, they were batched to a local installation of the program Primer3 (version 2.0.0) for primer design. To avoid issues with copy number of the primer sequence in the genome, loci for which the primer sequences only occurred one or two times in the 5 million reads were selected. Forty-eight loci of the 5,487 that met this criterion were chosen. One primer from each pair was modified on the 5′ end with an engineered sequence (CAG tag 5′-CAGTCGGGCGTCATCA-3′) to enable use of a third primer in the PCR (identical to the CAG tag) that was fluorescently labeled. The sequence GTTT was added to primers without the universal CAG tag addition.

Forty-eight primer pairs were tested for amplification and polymorphism using DNA obtained from eight individuals. PCR amplifications were performed in a 12.5 μL volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 μg/ml BSA, 0.4 μM unlabeled primer, 0.04 μM tag labeled primer, 0.36 μM universal dye-labeled primer, 3.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 units AmpliTaq Gold® Polymerase (Applied Biosystems), and 20 ng DNA template) using an Applied Biosystems GeneAmp 9700. Touchdown thermal cycling programs encompassed a 10 °C span of annealing temperatures ranging between 65 and 55 °C. Touchdown cycling parameters consisted of an initial denaturation step of 5 min at 95 °C followed by 20 cycles of 95 °C for 30 s, highest annealing temperature (decreased 0.5 °C per cycle) for 30 s, and 72 °C for 30 s; and 20 cycles of 95 °C for 30 s, lowest annealing temperature for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were run on an ABI-3130xl sequencer and sized with Naurox size standard prepared as described in DeWoody et al. (2004). Results were analyzed using GeneMapper version 3.7 (Applied Biosystems). Twenty-two of the tested primer pairs amplified high quality PCR product that exhibited polymorphism.

We assessed the variability of the 22 polymorphic loci in 24 specimens from southern Brazil and Uruguay (Table 1). We estimated the number of alleles per locus (*k*), observed and expected heterozygosity (H_o and H_e), and probability of identity (PI) using GenAlEx v6.4. Tests for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium were conducted using GENEPOP v4.0. After Bonferroni correction for multiple comparisons, three loci showed significant deviations from expectations under HWE, and no linkage disequilibrium was detected for any of the 231 paired loci.

These newly developed microsatellite loci will assist in studying the evolution of mating systems and social parasitism in the leafcutter ant *A. lundii*, and preliminary analyses indicate that these markers cross amplify in other closely related species, such as *Acromyrmex heyeri*. In general, the developed genetic markers will aid in understanding the evolution of cooperation and conflict in socially complex societies as well as in testing whether the species currently recognized as *A. lundii* consists of multiple cryptic species.

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