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Polymorphic microsatellite markers for the symbiotic fungi cultivated by leaf cutter ants (Attini, Formicidae)

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Abstract

We developed 23 polymorphic microsatellite markers for the symbiotic fungi cultivated by leaf cutter ants, then assessed allelic variation in North American leafcutter-fungus populations (Mexico, Cuba, USA). Polyploidy was indicated by 21 of the 23 loci, consistent with the multinucleate nature of leafcutter fungi. Microsatellite fingerprinting can now assess fungal genetic variation within leafcutter nests to test for monoculture of the cultivated fungi.

Keywords: Attini, fungus-growing ant, *Leucocoprinus gongylophorus*, mutualism, symbiosis

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Leafcutter ants (genera *Atta* and *Acromyrmex*) grow symbiotic fungi for food (fungal anamorph *Attamyces bromatificus*, teleomorph *Leucocoprinus gongylophorus*; Mueller 2002). *Attamyces* fungi appear to be obligate symbionts, as they have not been found growing independently of the ants (Mueller *et al.* 1998; Vo *et al.* 2009). The diverse leafcutter ant species are thought to associate with a single *Attamyces* lineage in a many-to-one co-evolutionary relationship (Mikheyev *et al.* 2006; Mueller & Rabeling 2008). Attine fungi are clonally propagated by the ants within and between nests, but recombination occurs occasionally (Mikheyev *et al.* 2006, 2007).

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Fungal genomic libraries were constructed by Genetic Identification Services (Chatsworth, CA) from pooled DNA extracted from pure cultures isolated from gardens of three leafcutter species (*Atta cephalotes*, *Atta colombica*, *Acromyrmex octospinosus*) and two *Trachymyrmex* species (*cornetzi*, *zeteki*) collected near Gamboa, Republic of Panamá. Fungal mycelium was isolated from gardens following previously published protocols (Mueller *et al.* 1996, 1998).

Pooled genomic DNA was partially restricted with an enzyme cocktail (*Rsa*I, *Hae*III, BsrB1, *Pvu*II, *Stu*I, *Sca*I, EcoRV). Size-selected fragments (300–750 bp) were linked to adapters containing a *Hin*dIII site, and then captured with magnetic beads (CPG, Inc.). Four enriched libraries were prepared using Biotin-AAC$_{12}$,

Table 1 Locus information, primer sequences, recommended annealing temperature (T_m), size range of PCR products (base pairs) and diversity statistics for 23 microsatellite markers for the symbiotic fungi (*Attamyces*) cultivated by leaf cutter ants (*Atta* and *Acromyrmex*, Attini, Formicidae)

Locus	GenBank accession number of clone	Number of times sequence was cloned	Repeat motif	Primer sequence (5′–3′)	T_m (°C)	Size range	No. of samples screened	No. of alleles	No. of alleles per individual
A11	EF471764	1	$(CT)_{11}$	F: CGTGCGTTTCTTCTTCTTC R: CCCATTGTTCAGGAATCC	61	161–177	28	6	2–4
A41	EF471747	1	$(CA)_{13}$	F: TGACCCATGCCATACTCTCTC R: CATGGAAATAAAACAAGTGATAGGG	60	261–273	28	6	1–3
A119	EF471754	17	$(TG)_{12}$	F: AGGACAGAGGCAAGGTGATG R: CGTCTCATATCCGCCTTCTC	60	206–226	28	4	2–4
A128	EF471759	2	$(AC)_{11}$	F: CTCGATGGCTGGACTCAACT R: ACGTCATCCATCAATCAACG	63	200–216	220	9	2–5
A435	EF471748	4	$(GT)_9$	F: AAACTCGGCGTCTTCCACTA R: GGCAGAGACGAGAGAAGGTG	60	212–232	28	7	1–3
A447	EF471762	1	$(CA)_{11}$	F: CTCGGGCATGCCTAAAAATA R: AGGTATCTGGGACCCAAAGG	59	174–182	28	5	1–3
A1030	EF471763	8	$(AC)_{11}$–C–$(CT)_8$	F: CTCCATCCCTCCCCTCTTAG R: GTGCTTGAACTCTGGGCTTT	60	204–222	220	7	1–3
A1132	EF471742	4	$(TCA)_{11}$	F: ATTGTTGGACCAGCTCTTGG R: CGAAAGGGAACGGAAATACA	64	196–229	220	11	2–4
A1151	EF471741	3	$(CA)_{11}$	F: CACCCACCACTAAACCCATC R: TGGATTGACGATGGAGAACA	58	152–175	220	11	2–5
B12	EF471753	1	$(GA)_{13}$	F: GAGGGCGACGATGAATAGA R: GCATTTGAAAAACCACAACC	60	230–240	220	4	2–3
B312	EF471746	2	$(GA)_{18}$	F: CAGTGACTTCTTCCCTGTGG R: GAGCCAGCGATTCGATCTAC	64	167–177	28	9	1–4
B319	EF471756	3	$(AG)_{13}$	F: GTGCTGATTGAAGGGGAGAG R: GCTGTCGGCTCTTGTTTCTC	60	195–203	220	5	1–2
B150	EF471744	5	$(CT)_{15}$	F: CATCCGCAGCCTGAATGTAG R: GGCGAAGATCGAAGGAGTG	58	162–186	220	10	1–5
B430	EF471757	1	$(TC)_{17}$	F: GGAAGCGATCTCAACAGTGATAC R: GGACCTACGGAGACCAATCTAAG	56	145–167	220	12	1–5
B447	EF471761	1	$(AG)_9$	F: GCTCCAGTACCCGTCCATCC R: CTCCCTTCATCTCCGTCTTC	65	216–224	28	5	1–3
C101	EF471752	1	$(CAG)_6$ $(CAA)_5$	F: TCGGATGCTTCCTGTCTC R: CGTTGTAGGTCCTCCATTTG	59	100–118	220	7	2–5
C117	EF471758	1	$(CAG)_8$ $(CAC)_7$	F: GGACAGCCTCGATCTCAGTT R: GGGAAAGCCATAGGGTTCAT	58	239–254	220	6	1–4
C126	EF471750	3	$(CTG)_7$	F: CAGACCTCGACCTCTGAACC R: TTCGCTCAACGGAGAGAACT	60	244–250	220	3	1–2
C606	EF471760	2	$(CAT)_{12}$	F: ACCTGCGCCATGATTTATTT R: CAGGAGCCTACGGTGAGAAG	60	148–163	28	6	1–5

Table 1 (Continued)

Locus	GenBank accession number of clone	Number of times sequence was cloned	Repeat motif	Primer sequence (5′–3′)	T_m (°C)	Size range	No. of samples screened	No. of alleles	No. of alleles per individual
C625	EF471749	1	$(TCA)_{14}$	F: GTCCACCTCGTCTGTGGATT R: TGATGAGGACGATGATGAGG	58	159–186	220	10	1–5
C647	EF474466	15	$(TC)_8$-GT-$(CTG)_6$	F: GTCCTGCCAGGTGTCAAAAT R: AGGTACGCGACGAGAGTGTT	60	210–216	28	4	2–4
C1133	EF471755	3	$(TGC)_8$–A–$(GGA)_7$	F: GCCGATGATGATGTGATTGAC R: CCAATGGATCAGCAATGTATG	63	226–238	28	5	2–3
D115	EF471743	2	$(TCA)_6$–TCG–$(TCA)_4$	F: CGACGTGACTTCATTTGACC R: AGCAAAGGTTGACTCGGAAA	62	216–234	28	4	2–3

Twenty-eight cultivar samples were profiled for all 23 loci in an initial screen. Based on this screen, the 12 most reliable loci were chosen to characterize an additional 220 cultivars.

Biotin-CAG_{10}, Biotin-$CATC_8$ and Biotin-$TAGA_8$ to capture fragments. After removal of the adapters with *Hin*dIII, fragments were ligated into the *Hind*III site of the plasmid pUC19. Plasmids were propagated into *Escherichia coli* DH5α and stored in 20% glycerol at −80 °C.

Cells from the glycerol stock were spread on X-gal/IPTG/ampicillin plates, picked after incubation and heated to 100 °C for 10 min in 10-μL PCR master mix (1× PCR buffer, 30 nmol $MgCl_2$, 3 nmol of each dNTP, 15 pmol M-13 cloning-site primers). Five microlitres of polymerase solution (0.075 μL 5 U *Taq* DNA polymerase, 0.5 μL 10× PCR buffer, 4.425 μL ddsH_2O) was added to amplify the insert using a PTC-200 Cycler (MJ Research) (94 °C for 3 min; 35 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 30 s; 72 °C for 4 min). Overall, 1112 PCR products (291, 680, 106 and 35 for the CA, GA, CAG and TAGA libraries respectively) were sequenced on an ABI 3100 Genetic Analyzer using BigDye™ Terminator chemistry.

Of the 1112 sequences, 307 contained at least eight microsatellite repeats (111, 118, 75 and 3 for the CA, GA, CAG and TAGA libraries respectively). Sequences are deposited at Genbank under accessions EF451159–EF451542. However, 247 of these sequences were duplicate. Of the 60 unique sequences, 15 were judged unsuitable (e.g. small flanking regions). Primer sets were designed for the remaining 45 loci (15, 11, 18, and 1 for the CA, GA, CAG and TAGA libraries respectively) using Primer3 (Rozen & Skaletsky 2000).

Of the 45 loci, 31 primer pairs produced products in the expected size range (100–350 bp) under the following PCR conditions: 94 °C for 3 min; 30 cycles of 94 °C for 10 s, 50 °C for 20 s, 72 °C for 25 s; 72 °C for 10 min; 10 μL reaction mix (1× PCR buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 10 μg BSA, 2 nmol of each primer, 0.25 U of Taq polymerase and 10–30 ng template DNA). Annealing temperatures were optimized for these 31 loci using a temperature gradient programme (50–65 °C).

We eliminated eight of the 31 loci because they were either monomorphic ($n = 1$) or exhibited significant stutter that precluded reliable scoring ($n = 7$). The remaining 23 loci showed allelic polymorphisms that could be reliably scored (Table 1). Allelic variation was examined initially for 28 samples (21 *Attamyces* from *Atta texana*; seven *Attamyces* from *Acromyrmex versicolor*). Based on this initial screen, the 12 most reliable loci with strongly amplifying markers were chosen to characterize 220 *Attamyces* from five leafcutter species (35 *Acro. versicolor* from Arizona; 165 *Atta texana* from Texas and Louisiana; seven *A. mexicana* and eight *A. cephalotes* from Mexico; five *A. insularis* from Cuba). Products were analysed on an ABI 3100 Genetic Analyzer and scored using GENESCAN version 3.5 and GENOTYPER version 3.6 NT.

Multiple-allele patterns at 21 of the 23 loci indicate that *Attamyces* is polyploid, consistent with the multinucleate cells of *Attamyces* found in ultramorphological studies (Hervey *et al*. 1977; Mohali 1998). That is, rather than containing two haploid nuclei like a typical dikaryotic, basidiomycete cell, each *Attamyces* cell harbours a population of more than two haploid nuclei. The exact ploidy level remains unknown. Up to five alleles/locus, verifiable in blind repeat genotyping, were recovered from a single fungal isolate (Table 1). Because of the unknown ploidy level, expected heterozygosities cannot be calculated.

Commingling of several fungal genotypes in the same mycelium can be ruled out as an explanation for the multi-allele patterns, because multiple-allele patterns breed true under repeated subculturing of single-cell lines cut from the mycelial growth front. Likewise, PCR artefacts can be ruled out because genotyping is highly repeatable. Specifically, 88 samples with unusual alleles were blindly re-genotyped for 12 loci (starting with extraction); only 0.9% of 5130 alleles showed discrepancies between the blind re-genotyping. Moreover, fungi from different ant nests can share the identical multi-allele marker profiles across all loci (i.e. different nests can share clonally identical fungi), consistent with clonal fungal propagation between mother and offspring nests. Specifically, among the 35 fungi from *Acro. versicolor* (Arizona), the markers from the 12 most reliable loci could distinguish 25 genotypes (i.e. distinct fungal clones differing by at least one marker, verifiable in repeat genotyping), and among the 165 fungi from *A. texana* (Texas and Louisiana), the markers could distinguish 49 clones (no *Attamyces* genotype was identical between Arizona and Texas/Louisiana populations). In all cases of identical marker profiles, the respective fungi were collected from nests in close spatial proximity (e.g. neighbouring nests; sometimes nests from the same locality within 50 km of each other), consistent with the expected clonal propagation of fungi within ant lineages and the limited dispersal of queens carrying *Attamyces*. A detailed analysis of these biogeographical patterns will be published elsewhere (U. G. Mueller, S. M. Brushi, A. S. Mikheyev, S. E. Solomon, H. D. Ishak *et al*., unpublished data).

Two hypotheses can explain the multi-allele patterns: (1) The same primer pair amplifies multiple loci in each haploid nucleus; this is possible if *Attamyces* underwent recent genome duplications. (2) Variation exists within the population of haploid nuclei in the multinucleate mycelium. If monokaryon mycelium can be created experimentally, microsatellite genotyping will be able to distinguish between these two hypotheses.

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