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PRIMER NOTE

Using the incomplete genome of the ectomycorrhizal fungus *Amanita bisporigera* to identify molecular polymorphisms in the related *Amanita phalloides*

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Abstract

We describe the cross-genomic isolation of 13 single nucleotide polymorphisms (SNPs) and one variable microsatellite from five loci for the death cap mushroom *Amanita phalloides*. Microsatellite repeats were identified by searching the partial *Amanita bisporigera* genome. Flanking primers were designed for 25 of these microsatellite loci and tested for cross-amplification in *A. phalloides*. One locus contained an interrupted, compound microsatellite, and four loci contained one to six SNPs. These results demonstrate the usefulness of even an incomplete genome to identify molecular markers for population studies in nonmodel organisms.

Keywords: *Amanita phalloides*, ectomycorrhizal fungus, fungal genome, single nucleotide polymorphism

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Intraspecifically variable genetic markers have provided empirical data for the study of phylogeography, population genetics and conservation. Single nucleotide polymorphisms (SNPs) have broad utility because of their abundance in genomes and because their evolution is easily modelled (Moran *et al*. 2004). While SNP isolation strategies have been developed for nonmodel organisms — for example, sequencing of amplified fragment length polymorphisms (AFLP) (Nicod & Largiadèr 2003) — whole genomes have been shown to be valuable resources for SNP isolation only when the same gene regions can be aligned across several different mammal species (so-called 'CATS' loci, Lyons *et al*. 1997). Here we report the usefulness of even a single incomplete genome for developing population markers in a related taxon. Microsatellite loci isolated from the genome of *Amanita bisporigera* were tested for cross-amplification in *Amanita phalloides*, resulting in the isolation of one microsatellite locus and four loci containing a total of 13 SNPs. *Amanita phalloides* is a putatively invasive ectomycorrhizal fungus and, like several other *Amanitas*, deadly poisonous. The SNP markers described here will be used as population genetic markers to investigate its biology in North America.

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The Amanita Genome Project is an ongoing collaboration to sequence *A. bisporigera* in order to isolate the genes responsible for its toxins. As of August 2004, approximately 10% of the genome (over 5.5 million bp) was sequenced (Hallen & Walton, unpublished, http://www.prl.msu.edu/walton/amanita.htm). The *Amanita* genome sequence completed at the time of undertaking this project was publicly available as text in FASTA format. Initially, microsatellite loci with a minimum of seven contiguous repeats were isolated using the 'Find' function in Microsoft Word and primers designed in the flanking region using OLIGO 4.0 (National Biosciences). Later, we used MSATMINER (Thurston & Field 2005) to scan the *Amanita* genome, identify microsatellite loci with contiguous repeats of at least eight dinucleotides or six trinucleotides, and design primers for a polymerase chain reaction (PCR) product length of 200 bp.

PCR was carried out in a 50-μL volume containing 2 μL genomic DNA (concentration not determined), 10× PCR buffer (final concentrations: 50 mM KCl, 10 mM Tris, 2.5 mM $MgCl_2$), 200 μM of each dNTP, 200 nM of each primer and 0.01 U/μL *Taq* polymerase (Roche). Cycling was performed on a MJ PTC-100 Thermal Cycler (MJ Research). The thermocycling program consisted of an initial denaturing step at 94 °C for 1 min followed by 35 cycles at 94 °C for 1 min, annealing temperature (Table 1) for 1 min and 72 °C for 1

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Table 1 Characterization of 13 SNPs from four loci and one microsatellite locus in the death cap mushroom *Amanita phalloides*

Locus	GenBank Accession no.	OLIGO sequence (5′–3′)	T_a (°C)	Amplicon length (bp)	Sequence (position no.)	Heterozygosity* sample size H_E	H_O
CA11	DQ1333971†	F: CGCTCTCCAATCAAAACTT	55	277	A/G(40)	0.19 (60)	0.12 (60)
	DQ1333972	R: GATGGGAGAGTTGGACG			C/T (85)	0.36 (60)	0.13 (60)
					C/T (88)	0.18 (60)	0.10 (60)
					G/T (190)	0.22 (60)	0.02 (60)
					G/T (221)	0.15 (60)	0.03 (60)
GGT8	DQ133974	F: TCCGACGAGGGTGAACAGCA	58	288	G/C (76)	0.10 (60)	0.10 (60)
	DQ133975	R: CCGCCGCCGTATAGCAAAAC			C/T (78)	0.29 (60)	0.22 (60)
					A/G (101)	0.32 (60)	0.20 (60)
					A/G (134)	0.03 (60)	0.03 (60)
					A/C (135)	0.32 (60)	0.20 (60)
					A/G (196)	0.22 (60)	0.15 (60)
GT9	DQ133976	F: GGACAAACGAAATGGGTGTC	55	213	A/G (129)	0.50 (61)	0.51 (61)
	DQ133977	R: TTGACCAGACGCCATATGAA					
TC9	DQ133978	F: CTACGCTTGAAATGGGCAAT	55	190	C/T (64)	0.45 (54)	0.43 (54)
		R: AATAATGCAGGGAGGCTTTC					
CCA7c	DQ133973	F: GGCGGGTCGGAGTCTAACG	58	211–220	$(CCA)_4CCC(CAA)_5$	0.53‡	0.00‡
		R: TTGGAAGGTGAGGCAGGCG			(99)§	(5)	(5)

T_a, annealing temperature; H_E, expected heterozygosity; H_O, observed heterozygosity.
*Determined including all individuals in all populations.
†Sequences for one European individual, Qs6, were deposited to GenBank. Two Accession nos indicate that this individual is heterozygotic for that locus, and each Accession no. represents a haplotype.
‡Observed and expected heterozygosities were calculated despite limited sample size.
§Microsatellite repeat starts at this base.

min, followed by an additional incubation step of 7 min at 72 °C. PCR products were purified with either Montáge PCR Centrifugation Filter Devices (Millipore) or QIAGEN 96 PCR Purification Kit and were sequenced in a total volume of 5 μL containing either 2 μL DNA template or 1:1 DNA template/water dilution, 1 μL 5 μM primer, 1 μL BigDye version 3.1 terminator reaction ready mix and 1 μL BigDye 5× sequencing buffer (Applied Biosystems). Sequencing thermal profile was 1 min at 96 °C followed by 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min on the same thermal cycler. Sequencing reactions were purified by ethanol precipitation and subjected to capillary electrophoresis on an ABI 3100 Genetic Analyser (Applied Biosystems). The forward and reverse strands were aligned and edited using SEQUENCHER 4.2 (GeneCodes). For those individuals with more than one heterozygous site per locus, haplotypes were determined using the TOPO TA Cloning Kit for Sequencing (Invitrogen).

Twenty-five *A. bisporigera* microsatellite loci were tested for cross-amplification in *A. phalloides*. Twelve loci did not amplify. Of the 13 primer pairs that produced a PCR-amplified product, nine generated a single band. Four of these were invariant, one contained an interrupted, compound microsatellite and four contained at least one SNP. The 13 SNPs and the microsatellite locus are characterized in Table 1. The SNP loci were amplified and sequenced in a total of 61 *A. phalloides* individuals. The natural history of this ectomycorrhizal fungus is only partially understood, but for this analysis we have grouped the individuals into five populations based on geography: western North America (California, Washington, British Columbia), eastern North America (New Jersey and Pennsylvania), southern Europe (France), the island of Corsica (France) and northern Europe (Denmark and Norway). Loci were tested for Hardy–Weinberg equilibrium and linkage disequilibrium (LD) using the software GDA (Lewis & Zaykin 2001). Two of the polymorphic loci (CA11 & GGT8) contained more than one SNP, and for these two loci haplotypes were used to test for disequilibrium within each population. A total of five haplotypes were found for locus CA11 and six for GGT8. Locus CA11 showed a significant departure from Hardy–Weinberg expectation in the northern European population, and significant LD was observed in the western North America population between loci GT9 and TC9. We treated the microsatellite distinctly because we know mutational processes at microsatellite loci are different

Table 2 Cross-species amplification data for one to two individuals of five additional species of *Amanitas* using the primers developed from the *Amanita bisporigera* genome. Successful amplification is indicated with a plus (+)

Species	Locus				
	CA11	GGT8	GT9	TC9	CCA7c
A. bisporigera	+	+	+	+	+
A. muscaria	–	–	+	–	–
A. ocreata variant 1	+	–	+	–	–
A. ocreata variant 2	+	–	+	–	–
A. pantherina	–	–	–	–	–
A. velosa	–	–	–	–	–
A. virosa	+	–	+	+	+

than those at SNP loci (Brumfield *et al*. 2003). We chose not to include the microsatellite for our study on *A. phalloides* invasion biology and therefore characterized it for only a subset of individuals. However, the microsatellite locus may prove useful in other studies. The five primer pairs were tested for cross-amplification without further optimization in five other *Amanita* species (Table 2).

While multiple genome alignments are becoming more available for a variety of organisms, for example, five genomes of the fungus *Cryptococcus*, the discussion of cross-species marker isolation has been limited to mammals (Lyons *et al*. 1997). Here, we found that isolating microsatellite regions in *A. bisporigera* led us not toward regions of variable number tandem repeats but to SNPs in *A. phalloides*. We have demonstrated that targeting potentially variable regions in the genome of one taxon may be helpful in isolating variable regions in another closely related taxon.

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