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Microsatellite markers and multiple paternity in the garter snake *Thamnophis sirtalis*

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Abstract

Data from four microsatellite loci developed for the common garter snake, *Thamnophis sirtalis*, show that multiple paternity is common in a natural population on Beaver Island, Michigan. Six of eight litters tested, and all litters with five or more neonates, were multiply sired. At least triple paternity was documented in the largest litter examined (n = 13 neonates). Inheritance patterns and genotype frequencies in the wild population indicate the presence of null allele(s) at one of the microsatellite loci. Garter snakes are widely used in quantitative genetics research, and paternity testing is essential in studies that rely on sibling analysis.

Keywords: behavioural genetics, mating systems, microsatellites, multiple paternity, null alleles, *Thamnophis sirtalis*

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Introduction

Garter snakes (*Thamnophis*) are model species for studies of behaviour, genetics and development (Burghardt 1993). These viviparous snakes can have large litters and, starting at birth, neonates can be tested for differences in a variety of behavioural traits. Significant genetic differences have been demonstrated among litters of neonatal garter snakes in several antipredator, foraging, social and locomotor responses (reviewed in Brodie & Garland 1993; Burghardt & Schwartz 1999). Single paternity and thus full-sibship among littermates has been widely assumed in estimating heritabilities (e.g. Arnold 1981). However, the demonstration that multiple paternity is common in at least some populations of *T. sirtalis* (Schwartz *et al*. 1989) points to the importance of testing assumptions, regarding paternity, in all species and populations used in behavioural genetic studies.

Using four polymorphic allozyme loci, Schwartz *et al*. (1989) documented multiple paternity in 16 of 32 litters examined, and estimated that multiple paternity actually occurred in 72% of these litters. Regression analysis of the allozyme genotypes estimated average sibling relatedness within multiply sired litters at $r = 0.390$, consistent with litters being mixtures of full- and half-sibs (Schwartz 1989). Given a real within-litter average relatedness of $r = 0.390$, calculations based on the false assumption of full-sibship would underestimate true heritabilities by ≈ 35%, and increase the standard errors of these estimates.

Here we report the development of microsatellite genetic markers that enhance the ability to detect multiple paternity within litters of neonatal *T. sirtalis*. We document the utility of these markers in an analysis of litters from Beaver Island, MI, and we report the first documentation of at least triple paternity within a single litter.

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Materials and methods

Samples

Pregnant female garter snakes (*Thamnophis sirtalis*) were captured in July 1996 in a 2 ha old-field habitat on Beaver Island, Charlevoix County, MI. Snakes were transported to the University of Tennessee where they were maintained in individual cages until they gave birth. After giving birth, ≈ 1 ml of blood was collected by venipuncture and 1 cm of the tip of the tail was dissected from each adult female to provide tissue for DNA extraction. Neonates were housed individually and tested for a suite of behavioural traits (Burghardt & Schwartz 1999; Burghardt & Krause 1999). After behavioural testing, ≈ 0.05 ml of blood was collected from each neonate for paternity analysis. Muscle and liver tissues were removed from snakes that died during or after behavioural testing.

Table 1 Attributes of four microsatellite loci developed for *Thamnophis sirtalis* (GenBank accession nos AF135963–AF135966)

Locus	Primer sequences (5′ to 3′)	Product size range (bp)	Repeat sequence in original clone	No. of alleles	H_O*
Ts1	CGGCATAAATCTTATCTAGC ACTTTTTCAGGCTGATGTTC	112–163	$(AAT)_{25}$	17	0.82
Ts2	GGCTAGCCCCTGTGTCCTT CACAACTCCAAATATTGAAGATTA	114–204	$(AAT)_{10} + (AAT)_3$	18	0.49
Ts3	CAACTGGCSGCTGTGATACAA GTGTTAATGTGTTGGACAGGGC	97–112	$(AAT)_7$	4	0.27
Ts4	ACTGAACAAGTTGGGTGTAG GCAAGAAGATGGCTATCTTG	168–186	$(GT)_{21}$	6	0.54

*Calculated for the subset of wild-caught adults. For all adults and neonates examined, H_O values were as follows: Ts1, 0.74; Ts2, 0.50; Ts3, 0.36; and Ts4, 0.36.

Library construction and screening

DNA for library construction was isolated by proteinase K digestion followed by phenol–chloroform–ethanol extraction (Sambrook *et al*. 1989) from 5 g of muscle tissue dissected from an adult *T. sirtalis* that had died in captivity. Ten micrograms of DNA was digested with *Mbo*I, and fragments smaller than ≈ 125 bp were removed using microcon 100 concentrators. The digested DNA was ligated to a pBluescriptII-KS phagemid vector that had been dephosphorylated and digested with *Bam*HI. Plasmid insert DNA was transformed into *Epicurian coli* XL2-blue ultracompetent cells and plated on nitrocellulose membranes (Scheichter & Snell Ltd) on Luria Broth (LB) + ampicillin medium agar plates. Two libraries were probed with cocktails of simple sequence repeat motifs $(AAT)_8$ $(GT)_{12}$, which had been previously end-labelled with [γ^{32}P]-ATP using T4 polynucleotide kinase (New England Biolabs). Positive clones were identified with autoradiography and subjected to two rounds of replicate plating and selection. Seventeen clones of less than 600 bp were manually sequenced (Biorad Systems Ltd) using the Sanger dideoxy chain-termination method and M13 and/or T3 primers. Primers (Integrated DNA Technologies) were identified using DNAStar and Oligo software. Microsatellites were amplified for four loci with at least seven uninterrupted repeats in the original clone (Table 1).

Polymerase chain reaction (PCR) amplification, electrophoresis and autoradiography

DNA for paternity analysis was isolated from blood using phenol–chloroform extraction, purified in microcon 30 concentrators and resuspended in 0.1× Tris-EDTA buffer. PCR amplifications were carried out in 12-μL reaction volumes containing 1.2 μL of 10× PCR buffer (Promega), 0.06 μL (5 mM) each of dATP, dGTP, dTTP and dCTP (Promega), 0.07 μL (1 pmol) of each primer, 0.2 μL (5 units/μL) of *Taq* DNA polymerase (Promega) and 1 μL (≈ 10 ng/μL) of template DNA. The optimal $MgCl_2$ volume (concentration) was 0.29 μL (0.6 mM) for locus 1 and 0.38 μL (0.8 mM) for the other loci. In each reaction, the upstream primer was end labelled (with γ^{32}P) using T4 polynucleotide kinase. Sterile, ultrapure water was added to obtain the final reaction volume. Each reaction was overlaid with 1 drop of mineral oil to prevent evaporation.

PCR reactions were carried out in an Ericomp (Twin Block Easy Cycler) thermocycler. Amplification conditions consisted of 2 min at 95 °C, followed by 27 cycles of 1 min at 95 °C and 1 min at 56 °C (locus 1), 54 °C (locus 2), 57 °C (locus 3), or 55 °C (locus 4), and 1 min at 72 °C. Reaction products were electrophoresed on 4% polyacrylamide (19:1 *bis*-acrylamide), 8 M urea gels at 60–80 W constant power (gel temperature 45–50 °C) for ≈ 2.5 h. Gels were adhered to Whatman 3 mm chromatography paper, covered with mylar wrap, vacuum dried at 80 °C for 1 h and autoradiographed with X-ray film for 24–96 h. Sequencing reactions (λ fmole DNA cycle sequencing system; Promega) were run on each gel to facilitate the sizing of PCR products.

Results

Genetic diversity

High levels of polymorphism were observed at the four microsatellite loci. The total number of alleles observed per locus ranged from four at locus Ts3 to 18 at locus Ts2 (Table 1). Wild-caught adults, exclusive of their progeny, were examined to assess heterozygosity and allele frequencies in the Beaver Island population. In the subset of adults, 15 alleles were observed at loci Ts1 and Ts2, and four and six alleles were observed at loci Ts3 and Ts4, respectively. Observed per-locus heterozygosities (H_O) in the adult sample ranged from 0.82 (Ts1) to 0.27 (Ts3; Table 1).

Heterozygote and homozygote frequencies in the adults were in agreement with Hardy–Weinberg expectations

Table 2 Genotypes of mothers and neonates for litter nos 1, 3 and 5

	Genotypes			
	Ts1	Ts2	Ts3	Ts4
Litter 3				
Mother	151/148	123/114	112/97	186/168
N1	151/121	123/123	112/97	186/186
N2	148/130	114/114	112/112	186/186
N3	148/121	114/114	109/97	—
N4	151/130	123/123	112/97	172/168
N5	148/121	114/114	112/97	186/172
N6	—	123/123	112/112	172/168
N7	151/130	—	112/112	—
N8	148/121	114/114	112/97	186/186
N9	151/121	123/123	97/97	172/168
N10	151/121	168/123	112/112	168/168
Paternal alleles	130, 121	168, null (or 168, 123, 114)	112, 109, 97	186, 172, 168
Litter 5				
Mother	154/118	126/114	112/97	186/168
N1	154/139	114/114	112/97	168/168
N2	154/118	162/114	112/97	—
N3	148/118	126/126	100/97	—
N4	—	—	112/97	168/168
N5	154/148	126/114	100/97	168/168
N6	139/118	114/114	—	—
Paternal alleles	148, 139 (154 or 118)	162, 126, 114 or (162, null, and either 126 or 114)	100, (112 or 97)	168
Litter 1				
Mother	148/133		97/97	170/168
N1	163/133		—	174/168
N2	148/142		97/97	
N3	133/133		100/97	168/168
N4	148/142		97/97	168/168
N5	148/148		97/97	174/170
N6	148/148		97/97	
N7			100/97	170/170
N8	148/133		100/97	
N9	148/133		97/97	170/168
N10	148/148		100/97	
N11	163/148		97/97	168/168
N12	148/148		100/97	170/168
N13	151/133		100/97	168/168
Paternal alleles	163, 151, 148 142, 133		100, 97	174, 170, 168

The lack of 123/114 heterozygotes at locus Ts2 in litter no. 3 suggests the possible presence of a null allele. The presence of five paternal alleles at locus Ts1 in litter no. 1 demonstrates at least three fathers. The paternal allele is underlined.

at loci Ts1, Ts3 and Ts4 (*G*-test, $P > 0.05$). A significant deficiency of heterozygotes was observed at locus Ts2 (*G*-test, $P = < 0.001$), suggesting the possible presence of one or more nonamplifying null alleles at this locus. The inheritance of maternal alleles did not differ from Mendelian expectations at any locus in any litter (Binomial test, $P > 0.39$ in all cases). The presence of a null allele (or alleles) at locus Ts2 was also supported by the genotypes of neonates and mothers. This is evidenced in litter no. 3 by the apparent absence of a unique paternal allele in eight of nine neonate genotypes (Table 2).

Paternity analysis

The microsatellite genotypes of mothers and neonates were examined for eight litters in which four to 13

Table 3 Multiple paternity, the numbers of paternal alleles detected at each microsatellite locus, and the estimated proportional contribution of the most likely male genotypes

Litter	No. of paternal alleles at each locus				No. of neonates	Multiple paternity	Contribution of likely males $\bar{x}$ (range)
	Ts1	Ts2	Ts3	Ts4			
1	5	—	2	3	13	Yes	0.46 (0.39–0.54)
2	4	3	1	1	11	Yes	0.83 (0.81–0.91)
3	2	2 or 3*	3	3	10	Yes	0.83 (0.77–0.88)
4	3	2	—	—	7	Yes	0.77 (0.57–0.91)
5	3	3	2	1	6	Yes	0.75 (0.71–0.78)
6	3	1	1	1	5	Yes	0.67 (0.50–0.84)
7	2	1	1	1	4	No	
8	2	—	2	1	4	No	

*See Table 2.

neonates per litter could be scored. Three or more paternal alleles at a locus were detected in six of these litters, confirming multiple paternity (Table 3). At least two different loci demonstrated multiple paternity in four litters, and multiple paternity was detected in all of the litters for which the genotypes of six or more neonates were determined. The presence of five paternal alleles at locus Ts1 in litter no. 1 confirms that paternity in this litter involved at least three males (Table 2).

Maximum likelihood analysis was used to estimate the proportional contributions of all possible multilocus paternal genotypes in each multiply sired litter (G. Russell, G. F. McCracken and G. M. Burghardt, manuscript in preparation). Among all possible paternal genotypes, the estimated contribution of the 10 most probable males averaged between 0.67 and 0.83 in the litters for which there was at least double paternity (litter nos 2–6), and was 0.46 in litter no. 1, which had at least triple paternity (Table 3). This analysis indicates that paternity is not shared equally among males in multiply sired litters.

Discussion

Our results demonstrate a high incidence of multiple paternity in *Thamnophis sirtalis*, closely approximating Schwartz *et al*.'s (1989) earlier estimate of multiple paternity in 72% of the litters that they examined. Also, as reported by Schwartz *et al*. (1989), our ability to detect multiply sired litters increased with the number of alleles present at a locus (Tables 1 and 3). Unlike the mainland populations of *T. sirtalis* that were studied by Schwartz *et al*. (1989), the population from Beaver Island has substantially smaller litters. In Schwartz *et al*.'s (1989) study, the litters in which multiple paternity was detected ranged from 11 to 40 neonates, whereas litters in which multiple paternity was not detected ranged from five to 12 (Schwartz 1989). The correlation of litter size with the number of loci at which multiple paternity was detected was highly significant ($r = 0.81$, $P < 0.0001$). In the present study, multiple paternity was detected in substantially smaller litters (range five to 13, $x = 8.6$ neonates/litter, Table 3). The reasons for the high frequency of multiple mating in this species are unknown; however, multiple mating in adders (*Vipera berus*) is known to increase offspring viability, and offspring quality may be enhanced by intrauterine competition among sperm of different males (Madsen *et al*. 1992). Beaver Island is near the northernmost limit of the range of *T. sirtalis*, and the smaller size of litters on Beaver Island may result from ecological factors. Litter sizes in *T. sirtalis* are known to show geographical variation (Gregory & Larsen 1993), but the reasons for this have not been established. However, larger females are known to have larger litters (Rossman *et al*. 1996). In the present study, there was a positive but nonsignificant relationship between total litter size and female snout-vent length ($r = 0.408$, $P = 0.248$). If larger females attract more males, a higher incidence of multiple paternity might be expected in larger litters. Actually determining whether larger litters have a higher incidence of multiple paternity, however, is complicated because multiple sires are simply more difficult to detect with smaller numbers of neonates, a difficulty that is alleviated with the greater allelic diversity provided by microsatellites. Future studies will explore multiple paternity in relation to female size, offspring number and offspring viability in *T. sirtalis*.

Although Schwartz *et al*. (1989) examined a larger number of larger litters, none of these litters required more than two sires to account for the paternal allelic contributions. Here, as another consequence of high allelic diversity, we document seven alleles (two maternal, five paternal) in a single litter (Table 2), confirming at least triple paternity.

Other studies have documented the presence of null

alleles at microsatellite loci, either by the apparent non-inheritance of paternal alleles in pedigrees (Callen *et al*. 1993) or by pedigree analysis and observed heterozygote deficiencies in population samples (Paetkau & Strobeck 1995; Pemberton *et al*. 1995). Although the presence of null alleles can inhibit the utility of microsatellites for parentage studies, the presence of null alleles can be recognized and accommodated so as not to preclude this utility (Pemberton *et al*. 1995). For example, the neonate genotypes observed at locus Ts2 in litter no. 5 require the presence of at least three paternal alleles and hence multiple paternity, whether or not the null allele was contributed (Table 2).

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This study is the first of a series in which microsatellite markers will be used to better assess relatedness and the inheritance of behavioural traits in garter snakes. This work is a collaboration between Gordon M. Burghardt and his laboratory of comparative ethology, which focuses on reptilian behaviour, and Gary F. McCracken and Sylvia E. Houts, who use genetic markers to assess kinship and the genetic structure of animal social groups.
