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PHYLOGENETICALLY WIDESPREAD MULTIPLE PATERNITY IN NEW WORLD NATRICINE SNAKES

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***Abstract.*—We used microsatellite DNA markers to identify the extent to which multiple paternity within litters occurs among species of New World natricine snakes. We selected seven species to represent the three major clades of Natricinae and all three subclades of the gartersnake clade. Microsatellite DNA genotyping of dams and litters confirmed multiple paternity within litters of six species, including *Thamnophis radix, T. sauritus, Storeria dekayi, S. occipitomaculata, Nerodia rhombifer*, and *Regina septemvittata.* Multiple paternity was not evident in one litter of nine *Thamnophis melanogaster*. Together with published data documenting multiple paternity in *T. bulteri, T. elegans, T. sirtalis*, and *N. sipedon*, these results confirm the phylogenetically widespread occurrence of multiple paternity among New World natricines, emphasizing the need to consider phylogenetic (historical) explanations when analyzing snake mating systems.**

Key Words.—microsatellite DNA; multiple paternity; *Nerodia*; *Regina*; sperm storage; *Storeria*; *Thamnophis*

INTRODUCTION

Molecular genetic techniques have revolutionized the analysis of mating systems, frequently revealing the occurrence of promiscuity or polygynandry (multiple mating by both males and females) in species previously thought to be monogamous or polygynous (Avise et al. 2002; Griffith et al. 2002; Westneat and Stewart 2003; Simmons 2005; Eccard and Wolf 2009). Among reptiles, such analyses have mostly focused on the occurrence of multiple paternity within clutches of eggs or litters of young produced by individual females and have demonstrated its prevalence among turtles, lizards, and snakes (Uller and Olsson 2008; Voris et al. 2008; Refsnider 2009). As a result, reptilian examples have been used to evaluate adaptive explanations for polygynandry in general and multiple paternity in particular, including turtles (Pearse et al. 2002), lizards (Olsson and Madsen 2001; Calsbeek et al. 2007; LaDage et al. 2008), and snakes (Prosser et al. 2002; Blouin-Demers et al. 2005; Kissner et al. 2005; Madsen et al. 2005; Dubey et al. 2009), although with somewhat mixed results (see contrasting viewpoints in Uller and Olsson 2008 vs. Madsen 2008). Its widespread occurrence has led some authors to suggest the need to consider multiple paternity in a phylogenetic context (e.g., Voris et al. 2008), but aside from the suggestion by Rivas and Burghardt (2005) that polyandry, not polygyny, may be ancestral in snakes, phylogeny has largely been ignored in interpreting reptilian patterns of multiple paternity.

In this paper, we examine multiple paternity within New World natricine snakes in an effort to understand whether multiple paternity is phylogenetically widespread and conserved or evolutionarily labile within this group. New World natricines consist of approximately 55 species of live-bearing North American snakes within nine genera (*Thamnophis*, *Nerodia*, *Regina*, *Virginia*, *Storeria*, *Clonophis*, *Seminatrix*, *Tropidoclonion*, *Adelophis*). New World natricines are a useful focal group for a number of reasons. Their phylogenetic relationships are well resolved (Alfaro and Arnold 2001; de Queiroz et al. 2002). Also, their reproductive behavior has been well characterized at least in some species and includes male placement of copulatory plugs within the female's cloaca (Devine 1975; Devine 1977; Crews 1980; Rossman et al. 1996; Shine et al. 2000a, b), pheromonal signaling of prior mating (Ross and Crews 1977), and multiple paternity (first demonstrated using molecular markers by Schwartz, et al. 1989, reviewed by Voris et al. 2008). In addition females store sperm within the vagina and infundibulum of the oviducts for weeks to months before fertilization (Blanchard and Blanchard 1941; Halpert et al. 1982; Gist and Jones 1987; Andren et al. 1997; Sever and Ryan 1999) creating a situation where postcopulatory male intrasexual selection (sperm competition) and cryptic female choice may be important determinants of male reproductive success (Birkhead and Parker 1997; Birkhead and Møller 1998; Birkhead 2000; Olsson and Madsen 1998; Arnqvist and Rowe 2005). Finally they have been frequent subjects

for quantitative genetic analysis (reviewed by Brodie and Garland 1993), analyses which are often predicated on the assumption that litters are singly-sired and thus represent sets of full-sibs (King et al. 2001).

To assess the extent of multiple paternity within New World natricines, we generated microsatellite DNA genotypes for dams and offspring of seven previously untested species. *Storeria occipitomaculata* and *S. dekayi* were chosen to represent the semifossorial clade, *Thamnophis melanogaster*, *T. radix*, and *T. sauritus* the gartersnake clade (Mexican, widespread, and ribbon and common gartersnake subclades, respectively), and *Nerodia rhombifer* and *Regina septemvittata* the watersnake clade. When combined with the four species in which multiple paternity is already known *(T. sirtalis* - ribbon and common gartersnake subclade, *T. butleri* and *T. elegans* - widespread subclade, *N. sipedon* - watersnake clade), this study brings the total number of species tested to 11 with all three clades and two of three subclades represented by two or more species (only the Mexican subclade is represented by a single species).

MATERIALS AND METHODS

Tissue sampling.—We collected gravid female *Thamnophis radix* and *S. dekayi* from DeKalb County, Illinois, and *N. rhombifer* from Jackson County, Illinois; *T. sauritus* and *S. occipitomaculata* were collected from Charlevoix County, Michigan. We maintained each snake individually in a terrarium with food and water *ad libitum* on a 12:12 light/dark cycle until parturition. We collected tissue samples (blood or tail tips) immediately after parturition to ensure dam identification. In total, we sampled seven dams and 128 offspring. We released snakes at their respective capture sites following tissue collection. Colleagues provided dam and offspring tissues following parturition from *T. melanogaster* from San Pedro Tlatizapan, Mexico and *R. septemvittata* from Ottawa County, Ohio.

Molecular techniques.—We extracted total genomic DNA from blood, frozen tail tips, or ethanol-preserved tail tips using the DNeasy® Tissue Kit (QIAGEN), ethanol precipitation, or chloroform extraction (Wusterbarth 2009). The concentration of DNA in the extracts was determined spectrophotometrically and subsequently diluted to 10ng/µl for amplification. We amplified six microsatellite DNA loci using primers for loci cloned from *T. sirtalis* (*2Ts* and *3Ts*; Garner et al. 2002) and *N. sipedon* (*Nesi2*, *Nesi3*, *Nesi9*, and *Nesi10*; Prosser et al. 1999). Each 10.0 µl amplificationreaction included 1.0 µl 25 mM Promega $MgCl_2$, 1.0 µl Promega 10X buffer, 0.4 µl 10 mM Promega dNTPs, 1.0 µl 10 ng/µl template, 1.0 µl 10ng/µl forward primer and 1.0 µl 10 ng/µl reverse primer with fluorescent tags (Invitrogen), and 0.1 µl 5 units/µl Promega *Taq* polymerase. Reactions were incubated at 94 C for 4 min, cycled 35 times through 94 C for 30 s, 54 C for 30 s, 72 C for 60 s, with a final elongation step at 72 C for 5 min. We genotyped amplification products using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, California, USA). After identifying maternal alleles in offspring genotypes, we counted the number of paternal alleles per litter. The presence of more than two paternal alleles (the maximum when a sire is heterozygous) within any single litter indicated multiple paternity.

RESULTS

Primers for microsatellite DNA loci cross-amplified well despite their having been cloned from other species. Alleles amplified with *Nesi2*, *Nesi3*, *Nesi9* and *Nesi10* primers had lengths within ten base pairs (bp) of the allele length ranges observed by Prosser et al. (1999). Allele lengths for the *2Ts* locus, however, were at least 60 bp shorter than those reported by Garner (1998). Similarly, most alleles detected at the *3Ts* locus were shorter by ca. 80-140 bp than those reported by Garner (1998). To verify proper gene amplification, the *3Ts* PCR product for *T. radix* was sequenced and the published microsatellite repeat (Garner et al. 2002) was present. Although we did not conduct formal tests for genotyping error rates, retyping a subset of individuals during PCR optimization gave identical results.

Microsatellite DNA genotyping of dams and litters revealed three or more paternal alleles among offspring, confirming multiple paternity within litters of six species, including *T. radix*, *T. sauritus*, *S. dekayi*, *S. occipitomaculata*, *N. rhombifer*, and *R. septemvittata* (Table 1). Given the presence of three to five paternal alleles within these litters, each was sired by a minimum of two to three males (Table 1). In cases where more than one microsatellite DNA locus was analyzed (*T. sauritus*, *S. dekayi*), multiple paternity was confirmed by both loci (see Table 1). Multiple paternity was not evident in a single litter of nine *T. melanogaster*. At both the *3Ts* locus and the *Nesi10* locus, we detected just two paternal alleles (Table 1). When plotted on the phylogeny of New World natricines, these results confirm the widespread occurrence of multiple paternity across divergent clades (Fig. 1).

DISCUSSION

Multiple paternity has now been documented in 10 of 11 New World natricine snake species tested. Furthermore, multiple paternity is phylogenetically widespread within New World natricines, occurring in multiple species within the watersnake, gartersnake, and

TABLE 1. Microsatellite DNA genotypes of dams and offspring from seven New World natricine snakes. Genotypes are identified based on the size (in base pairs) of amplified fragments. Maternal alleles are underlined. Numbers of offspring exhibiting a given genotype are shown in parentheses.

Species	Locus	Maternal Genotype	Offspring Genotypes				Inferred Paternal Alleles
Thamnophis radix	*3Ts*	382/394	241/382 (6)	249/382 (4)	260/382 (3)	241/394 (6)	241, 249, 260
			249/394 (6)				
Thamnophis sauritus	*Nesi3*	168/199	168/187 (2)	176/199 (1)	187/199 (3)	199/199 (1)	176, 187, 199
	Nesi9	167/185	158/167 (1)	160/167 (2)	160/185 (2)	167/167 (2)	158, 160, 167, 185
			167/185 (1)				
Storeria dekayi	*Nesi2*	157/165	152/157 (1)	154/157 (1)	154/165 (1)	157/159 (2)	152, 154, 157, 159, 165, 180
			157/165 (2)	159/165 (5)	165/180 (1)		
	Nesi3	161/165	159/161 (1)	161/163 (3)	161/168 (2)	161/216 (2)	159, 163, 168, 216
			163/165 (2)	165/216 (1)			
Storeria occipitomaculata	*3Ts*	240/244	207/240 (2)	207/244 (2)	236/240 (1)	240/240 (1)	207, 236, 240
Nerodia rhombifer	*Nesi3*	170/176	160/170 (4)	160/176 (2)	164/170 (7)	164/176 (10)	160, 164, 172
			170/172 (3)	172/176 (4)			
Regina septemvittata	*2Ts*	196/207	188/196 (1)	188/207 (4)	196/196 (3)	196/209 (4)	188, 196, 207, 209
			207/207 (1)	207/209 (2)			
	3*Ts*	281/289	281/281 (5)	281/289 (5)	281/293 (2)	289/289 (2)	281, 289, 293
			289/293 (1)				
Thamnophis melanogaster	*3Ts*	264/264	252/264 (5)	264/264 (4)			252, 264
	Nesi10	118/120	118/120 (5)	120/120 (3)			118, 120

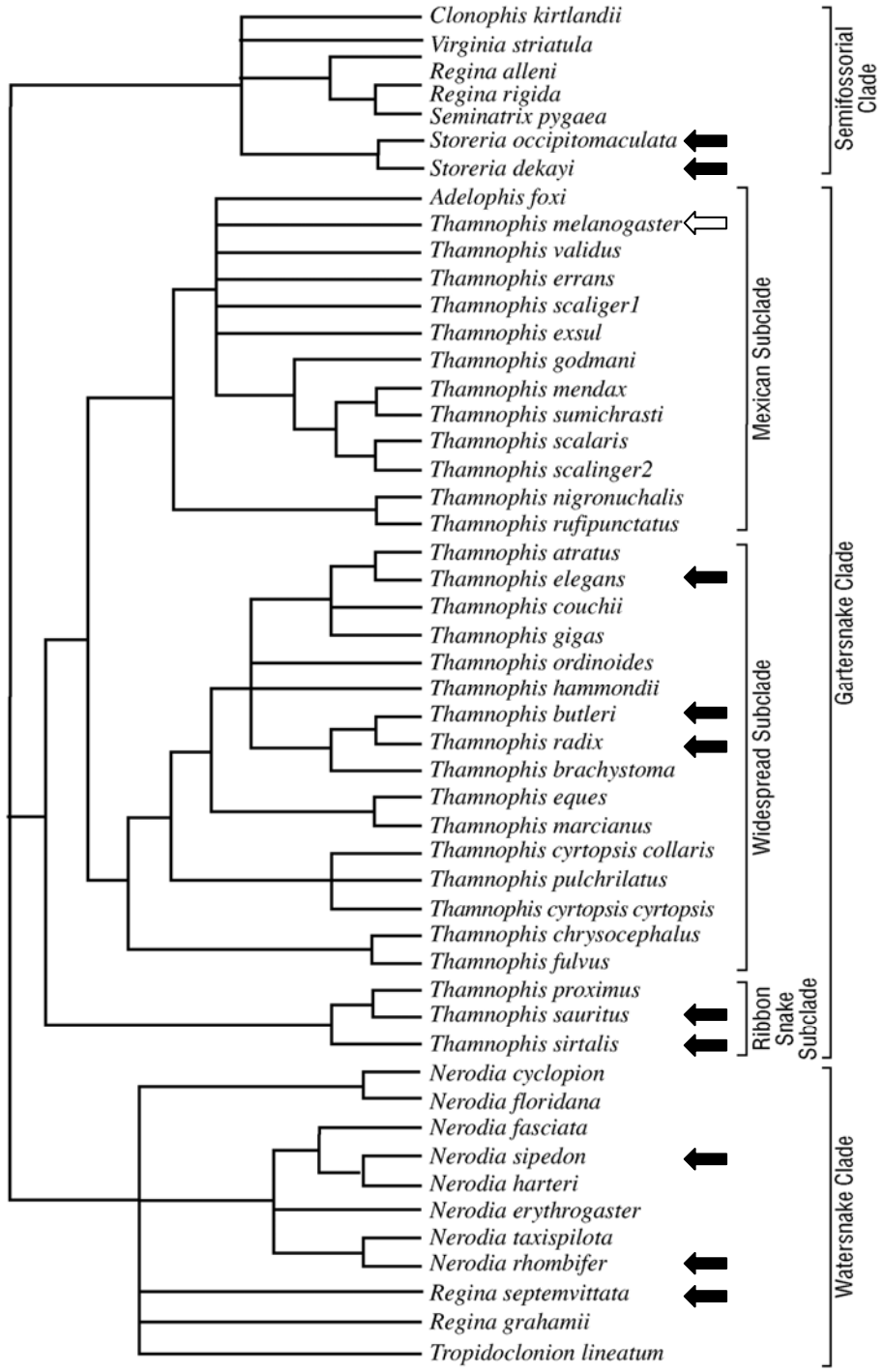

FIGURE 1. Phylogenetic relationships of New World natricine snakes adapted from analyses of de Queiroz et al. (2002, *Thamnophis* and *Adelophis*) and Alfaro and Arnold (2001, other genera). Tree shown is based on combined information from strict consensus (Fig. 1 in de Queiroz et al. 2002) and maximum likelihood (Fig. 5 in Alfaro and Arnold 2001) with poorly supported nodes (bootstrap proportions <65%) collapsed. Species in which multiple paternity has been confirmed are indicated by black arrows. *Thamnophis melanogaster*, in which multiple paternity has been tested but not confirmed, is indicated by a white arrow. Published sources of information on multiple paternity include the following: *Nerodia sipedon* (Barry et al. 1992; Prosser et al. 2002), *Thamnophis butleri* (Albright 2001), *T. elegans* (Garner and Larsen 2005), *T. sirtalis* (Gibson and Falls 1975; Schwartz et al. 1989; McCracken et al. 1999; King et al. 2001).

semifossorial clades, and among gartersnakes, within both the ribbon and the widespread subclades (Fig. 1). The Mexican subclade of gartersnakes is the only group of New World natricines within which multiple paternity has not been documented. The single Mexican subclade member tested here showed no evidence of multiple paternity but sample size was low (one litter of just nine offspring) and further investigation seems warranted. Indeed, given the widespread occurrence of multiple paternity within New World natricines demonstrated here, documentation of a species with exclusively single paternity of litters would seem novel.

The widespread occurrence of multiple paternity among New World natricines suggests that a promiscuous or polygynandrous mating system is ancestral in this group. Future investigations encompassing natricines from Europe, Africa, Asia and Australia would aid in providing a broader phylogenetic scope. Given that multiple paternity also occurs in members of the Colubrinae, Homalopsidae, Pythonidae, and Viperidae (reviewed by Voris et al. 2008), studies of other snake families, especially basal groups, would be of interest.

Promiscuous and polygynandrous mating systems present a situation in which sperm competition is likely to play an important role in determining male reproductive success (Birkhead and Parker 1997; Birkhead and Møller 1998; Olsson and Madsen 1998; Arnqvist and Rowe 2005). In such systems, the benefits of multiple mating to males are clear: males that mate more frequently and with more females achieve higher fecundity (Darwin 1871; Dubey et al. 2009; Ursenbacher et al. 2009). In contrast, the benefits of multiple mating to females are less clear (Uller and Olsson 2008). In the Water Python (*Liasis fuscus*), brood hatching success is positively correlated with the number of paternal microsatellite alleles observed, suggesting that multiple paternity, and the genetic variability it engenders, increases female reproductive success (Madsen et al. 2005). Information on post-hatching survival and reproduction in singly- vs. multiply-sired broods would provide a direct test of this possibility. For example, New World natricines often show genetic polymorphisms in prey preference at birth (Burghardt 1975; Arnold 1981; Burghardt et al. 2000) coupled with a high frequency of multiple paternity (McCracken et al 1999; King et al. 2001; Garner et al. 2002; Garner and Larsen 2005). Because neonatal snakes should search for alternative prey in different habitats and using different tactics, multiple paternity could be a bet-hedging strategy for females in habitats that undergo climatic and prey availability shifts across years. Alternatively, some females may mate with multiple males in the absence of such benefits, simply as a response to physiological stress when courted by males (Shine et al. 2003, 2005; Shine and Mason 2005). Female *T. sirtalis* in Manitoba sometimes gape their cloacas, allowing intromission, in response to lung compression stress caused by the weight of courting males (Shine and Mason 2005). Whether this may occur in populations or species with less intense mating aggregations is unknown. Finally, given the widespread occurrence of multiple paternity in snakes generally and New World natricines in particular, phylogenetic (historical) explanations for multiple mating should also be considered (Duvall et al. 1993). The extent of multiple paternity in the closest lizard relatives to snakes, and the relationship of multiple mating to snake social systems, which generally differ from the territorial and hierarchical forms of social organization common in lizards, also merit attention (Rivas and Burghardt 2005).

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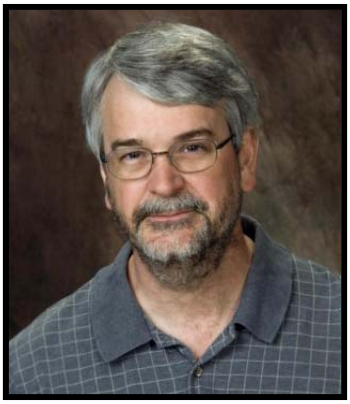
THERESA WUSTERBARTH is a human anatomy and physiology instructor at Northeast Wisconsin Technical College, Green Bay, Wisconsin. This article is based on a chapter from her Ph.D. dissertation, completed at Northern Illinois University in 2009, which also included an analysis of multiple paternity within an Illinois *Thamnophis radix* population and sperm characteristics of *T. radix* and *T. butleri*. (Photographed by Debra Olbrich)

MEL DUVALL is an Associate Professor at Northern Illinois University. He studies molecular evolution to investigate ancient divergences as well as recent events shaping the systematics and biogeographies of species clusters. One aspect of this work is the study of small genomes, such as the chloroplast genomes of cereals and other plants, to explore adaptation and infer phylogenies. (Photographed by Don Butler)

RICHARD KING is an Associate Professor at Northern Illinois University, DeKalb, Illinois. His interests center on ecological and evolutionary processes at local and regional scales and the conservation biology of Midwestern amphibians and reptiles. He is pictured measuring a Lake Erie watersnake on Kelleys Island, Ohio as part of a long-term (30 year) study. (Photographed by Deb Jacobs)



SCOTT GRAYBURN is Director of the Molecular Core Laboratory at Northern Illinois University. He studies gene expression in diverse organisms using quantitative real-time PCR and collaborates extensively with faculty in the Department of Biological Sciences and elsewhere. (Photographed by Don Butler)

GORDON BURGHARDT is an Alumni Distinguished Service Professor in the Departments of Psychology and Ecology & Evolutionary Biology at the University of Tennessee. His research focuses on the relationship between genetics and early environments in the development of behavior patterns and sensory processes, concentrating on natricine snakes with feeding, antipredator, and social behavior as the target systems. (Photographed by Gisela Kaufmann)