

## MULTIPLE PATERNITY IN THE ORIENTAL-AUSTRALIAN REAR-FANGED WATERSNAKES (HOMALOPSIDAE)

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**Abstract.**—We used species-specific microsatellite loci to detect multiple paternity in two species of homalopsid snakes, *Enhydris enhydris* and *Enhydris subtaeniata*. We collected data from nine loci for *E. subtaeniata*, and four for *E. enhydris*. Four *E. subtaeniata* litters and two *E. enhydris* litters were genotyped. All litters showed multiple paternity with three to five fathers typically detected. This is the first report of multiple paternity from a tropical Asian snake taxon. We discuss the significance of the results with respect to squamate behavioral ecology and compare our results to other studies on multiple paternity in reptiles.

**Key Words.**—*Enhydris enhydris*; *Enhydris subtaeniata*; Homalopsidae; microsatellites; mud snakes; multiple paternity

### INTRODUCTION

Multiple paternity has now been demonstrated in many animal taxa, including insects, fish, reptiles, birds, and mammals (Birkhead and Moller 1998). The investigation of proximate and ultimate factors explaining why females of so many species mate with multiple males has contributed to the study of sexual selection, mating systems, sperm competition, and related topics (e.g., Jennions and Petrie 2000).

Evidence for multiple paternity in snakes has accumulated through the successive refinement of techniques for detecting multiple fathers in a given litter or clutch. Initial evidence came from classical genetic analysis of offspring phenotypes (e.g., Blanchard and Blanchard 1941; Gibson and Falls 1975), followed by the application of increasingly powerful molecular genetic approaches, including analysis of allozymes (e.g., Zweifel and Dessauer 1983; Schartz et al. 1989), DNA fingerprinting (Höggren and Teglström 1995; Höggren and Tegelström 2002), and microsatellite DNA analysis (e.g., McCracken et al. 1999; Blouin-Demers et al. 2005).

We have been able to locate 18 papers published between 1941 and 2005 (15 of these since 1985) that investigate multiple paternity in snakes (Table 1): *Thamnophis sirtalis* (6 papers); *T. butleri* (1), *T. elegans* (1); *Nerodia sipedon* (3); *Elaphe obsoleta* (1); *Lampropeltis getula* (1); *Vipera berus* (3); *Agkistrodon contortrix* (1); and *Liasis fuscus* (1) (Garner and Larsen 2005; Rivas and Burghardt 2005). All of these studies documented multiple paternity in the taxa studied.

These studies include three taxonomic families (Pythonidae, Viperidae, and Colubridae; Lawson et al. 2005) and nine species, and suggest that multiple paternity is phylogenetically widespread among snakes (Olsson and Madsen 1998; Garner and Larsen 2005; Kissner et al. 2005).

In order to expand the phylogenetic and geographic context of our understanding of the mating system of squamates, we examined mating patterns in two freshwater homalopsid species. The Oriental-Australian rear-fanged watersnakes (Homalopsidae) includes ten genera and 34 species of snakes distributed from Pakistan across Southeast Asia to northern Australia (Gyi 1970; Murphy and Voris 1994; Greene 1997). All homalopsids are amphibious, primarily nocturnal, and usually associated with mud substrates. Eight of the 34 (24%) species are coastal marine species living in mangrove forests, tidal mudflats, near-shore coastal waters, and estuarial habitats (Heatwole 1999). The freshwater species are found in ponds, streams, wetlands, agricultural wetlands (e.g., rice paddies), and lakes (Gyi 1970). Most homalopsids eat fish, frogs, or tadpoles, but feeding on crustaceans is well documented in three of the coastal marine species (Voris and Murphy 2002). The Homalopsidae are especially interesting from a phylogenetic perspective because current evidence suggests that they are a basal colubroid family (Voris et al. 2002; Lawson et al. 2005; Vidal et al. 2007). Here, we report on the development of novel microsatellite markers to examine multiple paternity in two homalopsids, *Enhydris enhydris* (Schneider) and *Enhydris subtaeniata* (Bourret). Further, we document

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**TABLE 1.** Summary of 18 papers published between 1941 and 2005 that investigated multiple paternity in snakes. Multiple paternity was documented in all 18 studies (9 species); the frequency of multiple paternity varied from 37.5-100% of the litters or clutches tested in any given study. The method of determination of paternity is indicated in column two. The third column shows the percentage of litters in the study that exhibited multiple paternity, the number of fathers determined per litter, the range of litter or clutch sizes recorded, and the conditions under which snakes were obtained (wild caught or captive breeding). All of this information was not available for some of the studies cited.

TAXON	METHOD	PATERNITY	REFERENCE
<b><u>Colubridae; Natracinae</u></b>			
<i>Thamnophis sirtalis sirtalis</i>	Offspring phenotype	2 litters discussed Wild Caught	Blanchard and Blanchard 1941
	3 loci	2 and 3 father litters Litter = 6-19 Wild caught	
<i>Thamnophis sirtalis</i>	Offspring phenotype	13/22 litters (59.1%) Litter = 10-34 Wild caught	Gibson and Falls 1975
<i>Thamnophis sirtalis sirtalis</i>	Microsatellite DNA 4 loci	6/8 litters (75.0%) 1 litter with 3 fathers 5 litters with 2 fathers Litter = 4-13 Wild caught	McCracken et al. 1999
<i>Thamnophis sirtalis sirtalis</i>	Allozyme data 4 loci	16/32 litters (50.0%) Est. up to 72% Litters with 2 fathers Litter = 6-40 Wild caught	Schwartz et al. 1989
<i>Thamnophis sirtalis</i>	Microsatellite DNA 4-6 loci	4/4 litters (100.0%) Min of 2 fathers for 3 litters Min of 3 fathers for 1 litter Litter = 14-21 Wild caught	King et al. 2001
<i>Thamnophis butleri</i>	Referenced in Rivas and Burghardt (2005)		Albright 2001
<i>Thamnophis elegans</i>	Microsatellite DNA 3 loci	3/6 litters (50.0%) 1 litter with 3 fathers Litter = 8-24 Wild caught	Garner and Larson 2005
<i>Nerodia sipedon</i>	Allozyme data 7 loci	12/14 (85.7%) ≥ 2 fathers Litter = 8-37 Wild caught	Barry et al. 1992
<i>Nerodia sipedon</i>	Microsatellite DNA 8 loci	26/45 litters (57.8%) Up to 3 fathers/litter Litter = 5-28 Wild caught	Prosser et al. 2002
<i>Nerodia sipedon</i>	Microsatellite DNA 7 loci	25/46 litters (54.3%) 2 or 3 fathers/litter Captive breeding	Kissner et al. 2005
<b><u>Colubridae; Colubrinae</u></b>			
<i>Elaphe obsoleta</i>	Microsatellite DNA 10 loci	30/34 clutches (88.2%) 9 litters with 3 fathers 21 litters with 2 fathers 4 litters with 1 father Clutches from the wild	Blouin-Demers et al. 2005
<i>Lampropeltis getulus</i>	Allozyme data	1/1 clutch (100.0%) 1 litter with 2 fathers Clutch = 6 viable/8 Captive Breeding	Zweifel and Dessauer 1983

**TABLE 1.** *Continued.* Summary of 18 papers published between 1941 and 2005 that investigated multiple paternity in snakes. Multiple paternity was documented in all 18 studies (9 species); the frequency of multiple paternity varied from 37.5-100% of the litters or clutches tested in any given study. The method of determination of paternity is indicated in column two. The third column shows the percentage of litters in the study that exhibited multiple paternity, the number of fathers determined per litter, the range of litter or clutch sizes recorded, and the conditions under which snakes were obtained (wild caught or captive breeding). All of this information was not available for some of the studies cited.

<b>Viperidae: Viperinae</b>			
<i>Vipera berus</i>	DNA Fingerprinting	6/6 litters (100.0%) 3 litters with 3 fathers 3 litters with 2 fathers Litter = 2-7 Captive breeding	Höggren and Tegelström 1985
<i>Vipera berus</i>	DNA Fingerprinting	6/8 litters (75.0%) 2 or 3 fathers	Höggren and Tegelström 2002
<i>Vipera berus</i>	Allozyme analysis	2/3 litters (66.7%) 2 fathers Captive breeding	Stille et al. 1986
<i>Agkistrodon contortrix</i>	Offspring phenotypes	7/12 clutches (58.3%) Clutch = 3-9 Captive breeding	Schuett and Gillingham 1986
<b>Pythonidae</b>			
<i>Liasas fuscus</i>	Microsatellite DNA 3 loci	12/14 (85.7%) ≥ 2 fathers Clutch = 6-20 Wild Caught	Madsen et al. 2005

that multiple paternity does occur in these taxa and discuss our results in the context of the behavioral ecology of squamates and other studies on multiple paternity in reptiles.

#### MATERIALS AND METHODS

**Study species.**—*Enhydryis enhydryis* is a widely distributed freshwater homalopsid found from eastern India, around the Bay of Bengal, across Indochina, to the Greater Sunda islands of Borneo and Java. *Enhydryis enhydryis* is a medium-sized snake with an adult snout-vent length (SVL) typically between 0.5 and 0.75 m, which exhibits sexual size dimorphism (females are larger than males). It is found in wetlands, streams, ponds, and rice paddies and eats primarily fish (Voris and Murphy 2002). Litter size varies from 6 to 39 offspring (Murphy et al. 2002). This species is extremely abundant at the sites we studied in Thailand, typically comprising over 80% of the snakes collected (Karns et al. 1999-2000; Karns et al. 2005). Preliminary analysis of DNA sequence data indicates that this widespread taxon consists of more than one species (Harold Voris and Daryl Karns, unpubl. data).

*Enhydryis subtaeniata* is a freshwater homalopsid associated with the drainage basin of the lower Mekong River (northeastern Thailand, Cambodia, Laos and Vietnam). Relatively little is known about this species, complicated by the fact that, historically, it has been confused with *E. enhydryis* and *Enhydryis jagorii* (Murphy and Voris 2005). It is found in the same type of aquatic habitats as *E. enhydryis* and eats fish and frogs (Karns et al. 2005). Litter size reported in this study ranges from 14 to 25.

**Specimen collection.**—Gravid females of both species were collected from wetland habitats by local fishermen as gill net by-catch in April of 2006. The specimens we used in this study came from two areas in Thailand. *Enhydryis enhydryis* (HKV field numbers and Chulalongkorn University Museum of Zoology (CUMZ) numbers as follows: HKV 33394, CUMZ (R,H) 2006.1; HKV 33397, CUMZ (R,H) 2006.4; HKV 333402, CUMZ (R,H) 2006.9) and *E. subtaeniata* (HKV 33395, CUMZ (R,H) 2006.2; HKV 33396, CUMZ (R,H) 2006.3; HKV 33404, (R,H) 2006.11; HKV 33405, CUMZ (R,H) 2006.12) came from a reservoir area about 50 km northeast of the city of Khon Kaen, Khon Kaen province, in northeast Thailand. Two other *E. enhydryis* (HKV 33403, CUMZ (R,H) 2006.10 and HKV 33406, CUMZ (R,H) 2006.13) came from the area around Thale Noi, a fresh water lake about 20 km north of the city of Phthalung, Phthalung province, in peninsular Thailand.

Snakes were transported alive to Chulalongkorn University in Bangkok, where they were euthanized by cardiac injection of Euthasol (pentobarbital sodium and phenytoin sodium solution) and processed. We measured SVL and tail length to the nearest mm and weighed snakes to the nearest 0.1 gm. We took tissue samples (liver and heart) from euthanized snakes and preserved them in 95% ethanol. We removed the oviducts from the female snakes and the embryos were then removed from the oviducts and preserved in 95% ethanol. The female snakes were then preserved in 10% buffered formalin and deposited in the herpetological collection of the Natural History Museum of Chulalongkorn University.

**DNA extraction and marker development.**—We extracted genomic DNA from the liver or heart tissue of adults and from approximately 3 mm of the midsection from each embryo using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minnesota) following the manufacturer's protocol. DNA from one individual of each species was subsequently used to screen for microsatellite markers. For all individuals, we made 1/10 dilutions of extractions for subsequent polymerase chain reactions (PCR).

Microsatellite development followed an enrichment protocol of Glenn and Schable (2005). This protocol employs biotinylated probe repeats captured by streptavidin-coated magnetic beads (Dynabeads® M-280 Invitrogen, Carlsbad, California, USA). Briefly, genomic DNA is cut with the restriction enzymes *RsaI* and *XmnI*. Single-stranded SuperSNX24 linkers (FOR: 5'-GTTTAAGGCCTAGCTAGCAGAATC-3', REV: 5'-GATTCTGCTAGCTAGGCCTTAAA CAAA -3') are double stranded and then ligated to the ends of the cut gDNA fragments. These linker sites serve as PCR priming sites throughout the protocol. Five biotinylated tetranucleotide probes (AAAT, AACT, AAGT, ACAT, AGAT) were hybridized to gDNA. Magnetic beads were added to this mixture and the resultant bead-probe-DNA complex was captured by a magnetic particle collecting unit. After a series of increasingly stringent washes, enriched fragments were removed from the biotinylated probe by denaturing at 95°C and precipitated with 95% ethanol and 3M sodium acetate. To increase the amount of enriched fragments, we performed a "recovery" PCR in a 25 µl reaction containing 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM MgCl<sub>2</sub>, 10X BSA, 0.16 mM of each dNTP, 0.52 µM of the SuperSNX24 forward primer, 1 U *Taq* DNA polymerase, and approximately 25 ng enriched gDNA fragments. Thermal cycling, performed in a MJ Research DYAD, was done as follows: 95°C for 2 min followed by 25 cycles of 95°C for 20s, 60°C for 20 s, and 72°C for 90 s, and a final elongation step of 72°C for 30 min. We cloned subsequent PCR fragments using the TOPO-TA Cloning® kit (Invitrogen, Carlsbad, California, USA) following the manufacturer's protocol (Invitrogen). We used bacterial colonies as a template for subsequent PCR in a 25 µl reaction containing 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM MgCl<sub>2</sub>, 4X BSA, 0.12 mM of each dNTP, 0.25 µM of the M13 primers (M13FOR: 5'-TGTAACACGACGGCCA GT-3', M13REV: 5'-CAGGAAACAGCTATGACC-3'), and 1 U *Taq* DNA polymerase. We performed thermal cycling as follows: an initial denaturing step of 95°C for 7 min was followed by 35 cycles of 95°C for 20s, 50°C for 20 s, and 72°C for 90 s. PCR products were cleaned using MultiScreen-PCR Filter Plates following the manufacturer's protocol (Millipore, Billerica, Massachusetts, USA). DNA sequencing was performed using the BigDye® Terminator v3.1 Cycle

Sequencing Kit (Applied Biosystems, Foster City, California, USA). We precipitated sequencing reactions with ethanol and 125mM EDTA, which were run on an ABI 3730 DNA Analyzer. Primers flanking core microsatellite repeats were developed using Primer3 (Rozen, S, and H. Skaletsky. 2007. Primer3. Available from [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [Accessed 17 February 2008]). Potential hairpin formation and self-annealing sites were checked in the oligonucleotide properties calculator (Accessed from <http://www.basic.northwestern.edu/biotools/oligocalc.html>).

**Primer testing and genotyping.**—We developed primer pairs for 12 and 11 microsatellite loci for *E. enhydris* and *E. subtaeniata* respectively. To fluorescently-label PCR products, we followed the protocol of Schuelke (2000) where an M13 sequence (5'-TGTAACACGACGGCCAGT-3') is added to the 5' end of the forward primer in each species-specific primer pair. An M13-labeled primer is then included in each PCR to add a fluorescent tag. Amplification was carried out by polymerase chain reaction (PCR) in 10 µl reactions containing 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>), 10X BSA, 2 mM of each dNTP, 0.16 µM of each of the fluorescently-labeled M13 primer and species-specific reverse primer, 0.04 µM of the species specific M13-tailed forward primer, 0.6 U *Taq* DNA polymerase, and 1 µl genomic DNA. We performed the reactions on an MJ Research Dyad thermocycler under the following cycling conditions: an initial denaturing step of 4 min at 94 °C; followed by 30 cycles of 94°C for 30 sec, primer-specific annealing temperature (Table 2) for 30 sec, and 72°C for 45 sec, then 8 cycles of 94°C for 30 sec, 53°C for 30 sec, and 72°C for 45 sec; a final extension step of 10 min at 72°C concluded each profile. Fluorescently-labeled PCR products were run on an Applied Biosystems 3730 DNA Analyzer along with an internal size ladder (LIZ-500, Applied Biosystems, Foster City, California, USA). We scored fragments with the aid of Genmapper v.4.0 (Applied Biosystems, Foster City, California, USA).

We tested primer variability using 18 specimens for *E. subtaeniata* and 19 specimens for *E. enhydris*, including the gravid females used in this study and other specimens previously collected for another study (Voris et al. 2002). Of the primer pairs tested, we used nine (Esu17, Esu24, Esu31, Esu51, Esu53, Esu54, Esu57, Esu70, and Esu74) for *E. subtaeniata* litters and four (Een162, Een167, Een198, and Een166) for *E. enhydris* litters (Table 2). Other primers that we tested either were not variable or exhibited multiple peaks and were not used for genotyping mothers and offspring.

If one male sired each litter, we would see a maximum of four alleles at each locus in the genotypes of the

**TABLE 2.** Characteristics of the microsatellite primers used in this study for *Enhydris subtaeniata* and *E. enhydris*. The annealing temperature ( $T_A$ ), the number of alleles from wild caught snakes, and the observed heterozygosity ratios ( $H_o$ ) are given.

Locus	Primer Sequence (5' to 3')	Core Repeat	$T_A$ (°C)	Number of Individuals	Number Alleles	$H_o$
<i>E. subtaeniata</i>						
Esu17	GGGAGATGGGGTGGTATAGAA GCTCCACCATGTTTCTCCAT	(TAGA)21	63	18	8	0.71
Esu24	TTGTCAAAGAACCCGGGTAG GGAGACCCATAAATTCCAA	(TAGA)18	60	18	8	0.65
Esu31	AGCAAAGGGGGAAAAGTCAT GCCCTACCAACAGCAAGCTA	(TAGA)14	58	17	7	0.75
Esu51	TCAAAGGCTCTCTCCACCAC TGGTTTGGTGAAATGGGATT	(TAGA)15	56	18	13	0.76
Esu53	GGGTTCGGTTTCTTTCCTTC CACCTTTCCCAAGAGTTCA	(TAGA)17(CAGA)6	58	18	14	0.82
Esu54	TGCTATTTAAACTGATCCCTCAGA TGGTTAAGAACAGCTTTGAAAGAA	(TATC)13TAT(TATC)12	58	18	14	0.88
Esu57	TGCGTATTTACCATGCACCA AGACTGTTTTGTGGCCATACTT	(TATC)16	58	18	8	0.82
Esu70	CATACTGGTGAAAAGACTGTG CCCTAACGCCAGGAAATACC	(TAGA)17	60	18	7	0.81
Esu74	CTCCATCCCCTCTGGGTTTC CTTTCGGCTGTTCCCATAG	(TGA)18	58	17	5	0.50
<i>E. enhydris</i>						
Een162	TCTAAATGCCATATGTATACCTTCA CCTGTTTTAATCAACACCCTCTTT	(TATC)22	58	16	14	0.94
Een166	CAGCTAAGGTTGTGCTCATCA ACTCTATATTGTGGATTTTTGTTATCC	(AAG)8...(AAG)26	63	17	20	1.00
Een167	GCTGAAAAGGTTAGCCACCA TCCTATGGGAAAAATAGGCAGA	(TATC)21	60	18	10	0.76
Een198	CCACCATGTATCAGCAGCTT GTCGGGTTAATCGTTTGCAT	(TAGA)26	60	17	13	0.67

offspring: two alleles from the father and two alleles from the mother. To account for any genotyping errors due to mutation, unequal crossing over, or human error, we only accepted multiple paternity for a litter when two or more microsatellite loci exhibited more than four alleles. To determine the number of fathers contributing to each litter, we manually reconstructed male genotypes by splitting maternally related half-sib groups into full-sib groups (See Table 3 for a detailed example). This is easily done by inspection because, barring mutation, full sib groups will have no more than four alleles per locus. Male genotypes are then reconstructed based on shared, non-maternal alleles in the full-sib arrays (DeWoody et al. 2000; Feldheim et al. 2002). This gave the minimum number of males that contribute to each litter.

**RESULTS**

The loci used in this study exhibited a high number of alleles, 5 to 14 in *E. subtaeniata* and 10 to 20 in *E. enhydris*, and high levels of observed heterozygosity, 0.50 to 0.88 in *E. subtaeniata* and 0.67 to 1.00 in *E. enhydris* (Table 2). Manual reconstruction of male genotypes (Appendix 1) found multiple paternity for all litters in both species (Table 4). Manual reconstruction of genotypes indicated two aberrant results. At Esu24, female 33396 contains a null allele that she passed on to

several offspring. Furthermore, we found that Een 167 and Een 198 are linked (Appendix 1).

**DISCUSSION**

**Behavioral ecology.**—Molecular genetic studies are demonstrating that multiple paternity is a widespread feature of natural populations in diverse animal taxa. These studies are revealing the need to differentiate between “genetic” and “behavioral” descriptions of mating systems and reproductive success (Gibbs and Weatherhead 2001). Rivas and Burghardt (2005) note that, historically, polygyny has been accepted as the dominant mating system in snakes, despite the general lack of territorial systems, typically female-biased sexual dimorphism, and the relative rarity of male-male combat.

A general assumption in squamate behavioral studies was that a polygynous social system would result in males mating with multiple partners and females would produce litters or clutches sired by single males (Rivas and Burghardt 2005). The molecular advances of the last decade have revealed that in squamates and other taxa, polyandry, multiple mating by females, and polygynandry, in which both sexes engage in multiple matings, are common genetic mating systems, even in taxa that overtly appear to be socially polygynous or monogamous (e.g., Wesneat and Stewart 2003; Kissner et al. 2005; Madsen et al. 2005). These studies are

**TABLE 3.** The iterative process of male genotype reconstruction. An example of reconstructing male genotypes using a partial litter (13 of 18 embryos) and partial genotypes (3 of 9 microsatellite loci) from female snake 33395 (see Appendix 1 for the complete set of embryos and genotypes) is shown. The female's genotype and all known maternal alleles are shown in bold. The bold red allelic pairs indicate loci where the mother and offspring have identical genotypes and the identity of the maternal allele cannot be unequivocally determined. Note that at locus ESU 31, embryo 5, the maternal and the paternal allele are the same, and the maternal allele cannot be identified with certainty. To reconstruct male genotypes, we grouped shared paternal alleles together. We typically started with the most variable locus (in terms of number of alleles) because these loci are the most informative. At locus Esu54, embryos 2, 5, 7, 13, 16, and 18 share the 349 paternal allele (Table 3a). Grouping these embryos together (Table 3b) indicates that the paternal genotype (Male 1) at Esu53 is 179/228. Using this paternal genotype at Esu53, embryo 8 falls into this sib group, meaning the paternal genotype at Esu54 can be completed as 349/366. Finally, at locus Esu31, the paternal genotype for Male 1 is 226/230 for this sib group (Table 3b). With this knowledge, we can infer the maternal allele as 234 in embryos 2 and 18. Using this same logic, we can partially reconstruct genotypes for two other males in this example (Table 3c). An asterisk (\*) for these reconstructed genotypes indicates the paternal allele cannot be determined, but minimally, we can infer three sires in this example.

3a. Individual Snake	MICROSATELLITE LOCI		
	Esu54	Esu53	Esu31
<b>33395 (mother)</b>	<b>296/313</b>	<b>202/224</b>	<b>230/234</b>
Embryo 1	281/ <b>313</b>	<b>202/224</b>	226/ <b>234</b>
Embryo 2	<b>313/349</b>	179/ <b>202</b>	<b>230/234</b>
Embryo 5	<b>313/349</b>	<b>224/228</b>	<b>230/230</b>
Embryo 7	<b>296/349</b>	<b>202/228</b>	226/ <b>230</b>
Embryo 8	<b>296/366</b>	179/ <b>224</b>	226/ <b>234</b>
Embryo 9	281/ <b>313</b>	<b>202/220</b>	226/ <b>234</b>
Embryo 11	<b>296/333</b>	220/ <b>224</b>	226/ <b>234</b>
Embryo 13	<b>313/349</b>	179/ <b>202</b>	226/ <b>230</b>
Embryo 14	281/ <b>296</b>	<b>202/224</b>	226/ <b>230</b>
Embryo 15	299/ <b>313</b>	187/ <b>224</b>	<b>234/268</b>
Embryo 16	<b>313/349</b>	179/ <b>224</b>	226/ <b>234</b>
Embryo 17	299/ <b>313</b>	187/ <b>202</b>	<b>234/268</b>
Embryo 18	<b>296/349</b>	179/ <b>202</b>	<b>230/234</b>
3b. Individual Snake	Esu54	Esu53	Esu31
<b>33395 (mother)</b>	<b>296/313</b>	<b>202/224</b>	<b>230/234</b>
Embryo 2	<b>313/349</b>	179/ <b>202</b>	230/ <b>234</b>
Embryo 5	<b>313/349</b>	<b>224/228</b>	<b>230/230</b>
Embryo 7	<b>296/349</b>	<b>202/228</b>	226/ <b>230</b>
Embryo 13	<b>313/349</b>	179/ <b>202</b>	226/ <b>230</b>
Embryo 16	<b>313/349</b>	179/ <b>224</b>	226/ <b>234</b>
Embryo 18	<b>296/349</b>	179/ <b>202</b>	<b>230/234</b>
Embryo 8	<b>296/366</b>	179/ <b>224</b>	226/ <b>234</b>
Male 1	349/366	179/228	226/230
Embryo 1	281/ <b>313</b>	<b>202/224</b>	226/ <b>234</b>
Embryo 9	281/ <b>313</b>	<b>202/220</b>	226/ <b>234</b>
Embryo 11	<b>296/333</b>	220/ <b>224</b>	226/ <b>234</b>
Embryo 14	281/ <b>296</b>	<b>202/224</b>	226/ <b>230</b>
Embryo 15	299/ <b>313</b>	187/ <b>224</b>	<b>234/268</b>
Embryo 17	299/ <b>313</b>	187/ <b>202</b>	<b>234/268</b>
3c. Individual Snake	Esu54	Esu53	Esu31
<b>3395 (mother)</b>	<b>296/313</b>	<b>202/224</b>	<b>230/234</b>
Embryo 2	<b>313/349</b>	179/ <b>202</b>	230/ <b>234</b>
Embryo 5	<b>313/349</b>	<b>224/228</b>	<b>230/230</b>
Embryo 7	<b>296/349</b>	<b>202/228</b>	226/ <b>230</b>
Embryo 13	<b>313/349</b>	179/ <b>202</b>	226/ <b>230</b>
Embryo 16	<b>313/349</b>	179/ <b>224</b>	226/ <b>234</b>
Embryo 18	<b>296/349</b>	179/ <b>202</b>	230/ <b>234</b>
Embryo 8	<b>296/366</b>	179/ <b>224</b>	226/ <b>234</b>
Male 1	349/366	179/228	226/230
Embryo 1	281/ <b>313</b>	<b>202/224</b>	226/ <b>234</b>
Embryo 9	281/ <b>313</b>	<b>202/220</b>	226/ <b>234</b>
Embryo 14	281/ <b>296</b>	<b>202/224</b>	226/ <b>230</b>
Embryo 11	<b>296/333</b>	220/ <b>224</b>	226/ <b>234</b>
Male 2	281/333	220/*	226/*
Embryo 15	299/ <b>313</b>	187/ <b>224</b>	<b>234/268</b>
Embryo 17	299/ <b>313</b>	187/ <b>202</b>	<b>234/268</b>
Male 3	299/*	187/*	268/*

revealing a lack of correlation between individual reproductive success measured by behavioral observations and reproductive success measured by genetic markers (e.g., Prosser et al. 2002). They are also showing that multiple paternity occurs in situations where it would seem likely to occur, such as with aggregated mating and highly male-skewed operational sex ratios that bring many males and females into close spatial proximity (e.g., *Thamnophis sirtalis* dens, Mason and Crews 1985), and in situations that would seem to be less than conducive to multiple mating with males and females dispersed during the mating season (e.g., *Elaphe obsoleta* males search for widely dispersed females, Blouin-Demers et al. 2005).

All published studies investigating multiple paternity in snakes have documented its occurrence in every species examined (Table 1). Table 1 also provides information on the frequency of multiple paternity within the species studied. Counting this study (and excluding Blanchard and Blanchard 1941), 277 litters or clutches from 11 different species have been analyzed. The number of litters or clutches investigated ranged from one to 46 per study. Twelve of the studies used wild-caught snakes, six of the studies used captive bred snakes. Of these 277 litters or clutches, 181 (65.0%) show multiple paternity and two to five fathers were recorded from the litters with multiple fathers; if captive breed snakes are excluded, 134/201 (67%) of wild caught litters exhibit multiple paternity. Kissner et al. (2005) notes that wild-caught and captive bred *Nerodia sipedon* show similar levels of multiple paternity (26/45 wild-caught litters and 25/46 captive-bred litters). Thus, multiple paternity appears to be a common ecological phenomenon in snakes.

**Phylogenetic considerations.**— Multiple paternity studies in snakes to date have dealt primarily with the Colubroidea (Table 1), the large, monophyletic clade that includes the majority of extant snake species. Here, we report multiple paternity in two species of the Homalopsidae. Thus, to date, six genera (ten species) of colubroids (*Enhydris*,



**TABLE 4.** Paternity results for four *E. subtaeniata* and two *E. enhydris*. Litter number, litter size, number of loci used for each litter, and minimum number of fathers for each litter are given.

Species	Litter Number	Litter Size	Number of Loci Used	Minimum Number of Fathers
<i>E. subtaeniata</i>	33395	18	9	5
<i>E. subtaeniata</i>	33396	17	9	3
<i>E. subtaeniata</i>	33404	25	9	4
<i>E. subtaeniata</i>	33405	14	9	5
<i>E. enhydris</i>	33394	29	4	5
<i>E. enhydris</i>	33403	36	3	3

*Thamnophis*, *Nerodia*, *Lampropeltis*, *Vipera*, and *Agkistrodon*) representing three families (Colubridae, Homalopsidae, Viperidae,) have been found to exhibit multiple paternity (Table 1).

The recent phylogeny of the Colubroidea by Lawson et al. (2005) recognizes five colubroid families: Colubridae, Elapidae, Homalopsidae, Pareatidae, and Viperidae. The monophyly of the Pareatidae and Homalopsidae has yet to be fully evaluated, but the available evidence supports a basal position for these taxa with the Pareatidae indicated as the sister group to the Viperidae, and the Homalopsidae indicated as the sister group to the Colubridae plus Elapidae (Voris et al. 2002; Lawson et al. 2005). If this hypothesis is correct, multiple paternity has been demonstrated in three of the five colubroid families (Colubridae, Homalopsidae, Viperidae), and the vipers and homalopsines are basal lineages with respect to the majority of snake species that are found in the Colubridae and Elapidae. Every published study that has investigated paternity in snakes (19 papers, 11 species, including this study) has documented multiple paternity in the species studied, including “advanced” taxa (in the Colubroidea) and a “primitive” taxon, the Water Python (*Liasis fuscus*, Pythonidae, Alethinophidia). Also, as noted above, multiple paternity is a common occurrence in the species investigated (65% of the 277 litters or clutches studied). Thus, multiple paternity appears to be both phylogenetically widespread and an ecologically frequent occurrence; these observations support the hypothesis that multiple paternity is an ancestral behavior in snakes.

With respect to all reptiles, Olsson and Madsen (1998) demonstrated that in more than 80% of reptile species studied (33 of 41 species), females mate with multiple males, and molecular data has now confirmed that multiple paternity in reptiles is widespread. In addition to snakes, multiple paternity also has been documented within five of the 24 lizard families (Iguanidae, Agamidae, Lacertidae, Teiidae, and Scincidae; Morrison et al. 2002; Pianka and Vitt 2003; Laloï et al. 2004), and in both territorial and non-territorial species. Multiple paternity has been documented in five of the 13 families of turtles (Cheloniidae, Chelydridae, Emydidae, Podocnemididae, and Testudinidae.; Pearse and Avise 2001; Zug et al. 2001), and has been confirmed in the

American Alligator (*Alligator mississippiensis*) using genetic markers developed for other crocodylians (Alligatoridae, one of three families of crocodylians; Davis et al. 2001; Zug et al. 2001). Studies vary considerably with respect to the frequency of multiple paternity reported, but all reptilian taxa tested and reported to date, except the Leatherback Turtle (*Dermochelys coriacea*; but see Pearse and Avise 2001), have exhibited some level of multiple paternity.

**Behavioral ecology of homalopsids.**—There is relatively little known about the reproductive biology of homalopsids, from either laboratory or field studies; homalopsids are cryptic and infrequently observed, resulting in few observations of breeding behavior. The natural history literature contains scattered information on litter sizes, dates of birth, and anecdotal behavioral notes (Murphy et al. 1999). Radiotelemetry-based field studies of homalopsids (e.g., Voris and Karns 1996; Karns et al. 1999-2000; Karns et al. 2002; Karns et al. 2005) have documented aspects of diet, reproduction, habitat utilization, movements, and activity patterns. These studies show that homalopsids are typically associated with the mud-root-tangle found along aquatic edges.

Available information suggests that aggregated breeding behavior occurs in *E. enhydris*. We have studied *E. enhydris* in southern Thailand (Murphy et al. 1999; Karns et al. 1999-2000) and *E. enhydris* and *E. subtaeniata* in northeastern Thailand (Karns et al. 2005), and these studies provide some information on the behavioral ecology of these species. Local residents reported seeing breeding aggregations of *E. enhydris*, although we have not personally witnessed aggregations. We have also noted that snake-traps with females attract males and that male snakes vibrate their bodies in response to handling during data collection, possibly a response to female pheromones left on our hands during processing (Daryl Karns and Harold Voris, pers. obs.), suggesting a mechanism, similar to other snakes, for the formation of breeding aggregations.

High population density may influence multiple paternity by increasing frequency of contact between males and females. Our field studies indicate that homalopsid snakes can exhibit very high population densities; at a field site in southern Thailand we

estimated a density of 0.5 *E. enhydris* per meter of shoreline (Murphy et al. 1999). Jayne et al. (1988) estimated a density of 1-3 subadult snakes/m of shoreline for *Cerberus rynchops*, a coastal marine homalopsid. There may also be a correlation between the operational sex ratio (OSR) and multiple paternity because multiple mating may be influenced by the number of males encountered by receptive females (Prosser et al. 2002). We have found considerable variation in OSR among sites and between years for *E. enhydris*. For example, in 1997 we recorded a male-biased OSR of 3.3:1 (n = 111) in a southern Thailand wetland (Murphy et al. 1999), and in 2004 we recorded an OSR of 1.01:1 (n = 280) and 0.61:1 (n = 29) at two sites in northeastern Thailand (Karns et al. 2005). Thus, multiple paternity in *Enhydris* may be associated with a large number of males mating with fewer females, a situation in which females need to weigh the costs of resisting matings with several males (precopulatory female choice) versus engaging in multiple matings. Such “convenience polyandry” has been suggested in insects (Rowe 1992; Weigensberg and Fairbairn 1994), sharks (Portnoy et al. 2007; DiBattista et al. *in press*), and marine turtles (Lee and Hays 2004).

**Future work.**—We encourage other investigators to expand the phylogenetic scope of multiple paternity investigations. In serpents, this would include the Elapidae, other non-colubroid Alethinophians, and the basal Scolecophidians. Habitat correlates would also be of interest. Studies thus far have focused on terrestrial and aquatic species, but fossorial and arboreal species have not been investigated. Furthermore, only two of the studies noted in Table 1 involve tropical species where the potential for year-round breeding may influence mating systems.

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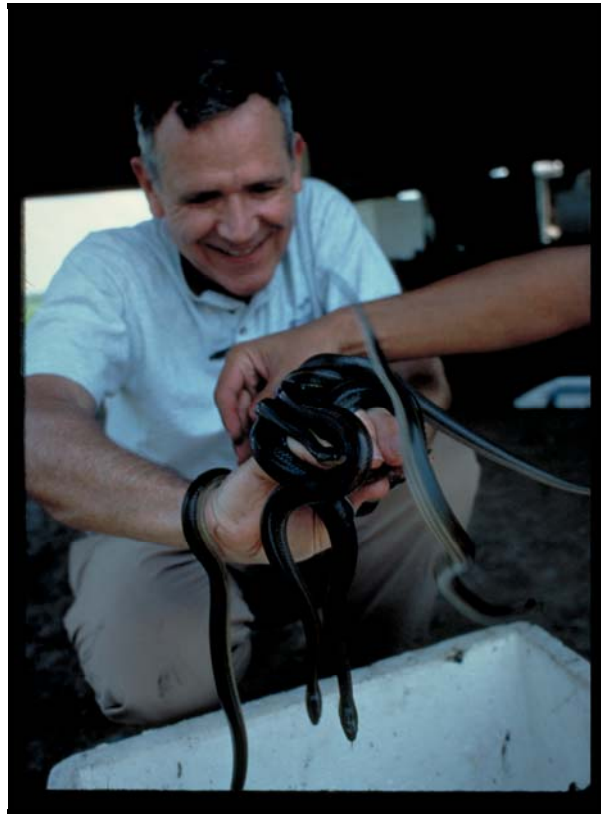
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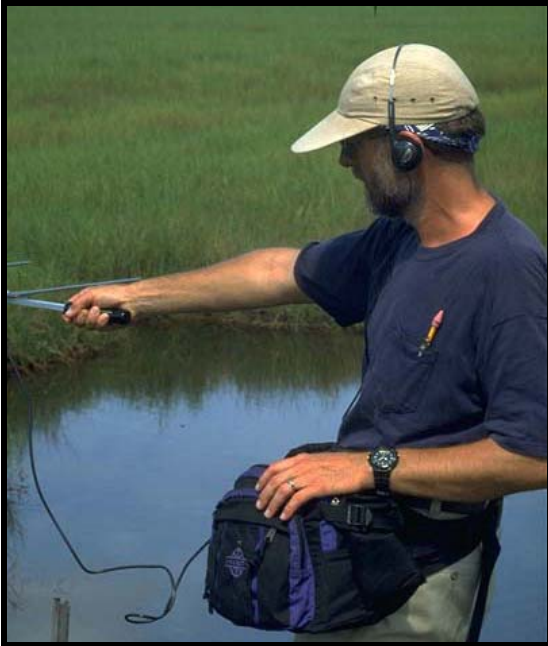


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**DARYL R. KARNs** is a Professor of Biology at Hanover College, Hanover, Indiana, USA and a Research Associate at the Field Museum of Natural History. He is an ecologist with special interests in the ecology, biogeography, systematics, and conservation of the aquatic snakes of Southeast Asia. He is shown tracking *Enhydris enhydris* in southern Thailand. Photographed by John C. Murphy.



**KEVIN FELDHEIM** is Lab Manager of the Pritzker Laboratory for Molecular Systematics and Evolution at the Field Museum of Natural History in Chicago, USA. He is pictured here in Marquesas Key, Florida with a Lemon Shark. He received his Ph.D. from the University of Illinois at Chicago working under Drs. Mary Ashley and Samuel Gruber. His research includes using microsatellite genetic markers to examine parentage and population genetics in a variety of organisms. Photographed by Samuel H. Gruber.



**BOBAK KECHAVARZI** is a graduate of Hanover College where he received B.A. degree in Biology and minored in Computer Science. He interned in collaboration with Megan Rinehart at the Field Museum of Natural History during the summer of 2006, and developed lab activities to introduce students to botany. He lives in Louisville, Kentucky, USA. He is shown analyzing results from a capillary electrophoresis. Photographed by Daryl R. Karns.



**MEGAN RINEHART** is a graduate from Hanover College where she received her B.A. degree in Biology. She interned at the Field Museum of Natural History during the summer of 2006. She lives in Sullivan, Indiana, USA. She is pictured here preparing DNA samples for PCR. Photographed by Daryl R. Karns.

# Voris et al.—Multiple Paternity in Homalopsid Watersnakes

**APPENDIX 1.** Maternal, paternal (manually reconstructed), and embryo genotypes for four *E. subtaeniata* litters and two *E. enhydris* litters. Female genotypes are in bold pink and maternal alleles in offspring are highlighted in pink. Where no offspring allele is highlighted in pink, it is impossible to determine which is the maternal allele. Paternal genotypes were reconstructed manually (see Table 3) and are shown in bold blue. Some paternal genotypes are incomplete due to small numbers of offspring. A \* indicates that we are unsure of a male's allele.

## *Enhydris subtaeniata* litters

Individual	Locus								
	Esu17	Esu24	Esu31	Esu51	Esu53	Esu74	Esu54	Esu57	Esu70
<b>33395</b>	<b>161/173</b>	<b>218/218</b>	<b>230/234</b>	<b>240/255</b>	<b>202/224</b>	<b>199/203</b>	<b>296/313</b>	<b>168/172</b>	<b>172/172</b>
<b>Male 1</b>	<b>165/177</b>	<b>206/*</b>	<b>226/230</b>	<b>306/*</b>	<b>179/228</b>	<b>193/199</b>	<b>349/366</b>	<b>168/176</b>	<b>172/176</b>
Embryo 2	161/177	206/218	230/234	240/306	179/202	193/199	313/349	172/176	172/176
Embryo 8	165/173	206/218	226/234	240/255	179/224	193/203	296/366	168/168	172/172
Embryo 13	173/177	206/218	226/230	240/306	179/202	193/203	313/349	168/172	172/176
Embryo 3	161/177	206/218	226/230	255/306		193/199	296/349	168/176	172/176
Embryo 5	161/165	206/218	230/230	240/306	224/228	193/203	313/349	172/176	172/172
Embryo 6	173/177	206/218	230/234	240/306	179/224	199/199	313/349	168/176	172/176
Embryo 7	173/177	206/218	226/230	240/306	202/228	193/199	296/349	168/176	172/176
Embryo 18	161/177	206/218	230/234	255/306	179/202	199/199	296/349	168/168	172/172
Embryo 16	173/177	206/218	226/234	240/306	179/224	193/203	313/349	168/168	172/176
<b>Male 2</b>	<b>161/*</b>	<b>218/*</b>	<b>226/*</b>	<b>247/255</b>	<b>220/*</b>	<b>196/199</b>	<b>281/333</b>	<b>164/*</b>	<b>172/180</b>
Embryo 1	161/161	218/218	226/234	255/255	202/224	196/199	281/313	164/172	172/172
Embryo 11	161/173	218/218	226/234	247/255	220/224	196/203	296/333	164/172	172/180
Embryo 14	161/173	218/218	226/230	255/255	202/224	199/199	281/296	164/172	172/172
Embryo 9	161/173	218/218	226/234	240/255	202/220	196/203	281/313	164/168	172/172
<b>Male 3</b>	<b>161/*</b>	<b>206/*</b>	<b>268/*</b>	<b>247/250</b>	<b>187/*</b>	<b>*/*</b>	<b>299/*</b>	<b>168/*</b>	<b>172/180</b>
Embryo 15	161/161	206/218	234/268	247/255	187/224	199/203	299/313	168/168	172/180
Embryo 17	161/161	206/218	234/268	240/250	187/202	199/203	299/313	168/172	172/172
<b>Male 4</b>	<b>165/*</b>	<b>210/235</b>	<b>230/*</b>	<b>247/306</b>	<b>209/*</b>	<b>196/*</b>	<b>265/341</b>	<b>180/*</b>	<b>176/*</b>
Embryo 4	161/165	210/218	230/230	240/247	202/209	196/199	296/341	172/180	172/176
Embryo 12	161/173	218/235	230/234	240/306	209/224	199/203	265/296	168/180	172/176
<b>Male 5</b>	<b>161/*</b>	<b>222/*</b>	<b>238/*</b>	<b>*/*</b>	<b>205/*</b>	<b>*/*</b>	<b>337/*</b>	<b>*/*</b>	<b>172/*</b>
Embryo 10	161/161	218/222	234/238	240/255	205/224	199/203	296/337	168/172	172/172

Individual	Locus								
	Esu17	Esu24	Esu31	Esu51	Esu53	Esu74	Esu54	Esu57	Esu70
<b>33396</b>	<b>165/165</b>	<b>218/null</b>	<b>242/242</b>	<b>251/255</b>	<b>190/197</b>	<b>196/196</b>	<b>328/345</b>	<b>168/176</b>	<b>184/184</b>
<b>Male 1</b>	<b>157/161</b>	<b>218/222</b>	<b>222/242</b>	<b>243/247</b>	<b>198/205</b>	<b>179/203</b>	<b>295/341</b>	<b>168/172</b>	<b>176/180</b>
Embryo 6	161/165	218/218 <sup>a</sup>	222/242	247/255	190/205	179/196	341/345	172/176	180/184
Embryo 7	161/165	218/218 <sup>a</sup>	242/242	247/251	190/205	196/203	295/345	168/168	176/184
Embryo 9	161/165	218/218 <sup>a</sup>	222/242	243/255	190/205	179/196	295/345	168/172	176/184
Embryo 10	161/165	222/null	222/242	247/255	190/205	196/203	295/328	168/172	176/184
Embryo 11	161/165	218/218 <sup>a</sup>	222/242	247/251	197/198	196/203	295/345	168/168	176/184
Embryo 12	157/165	218/218 <sup>a</sup>	242/242	247/255	197/198	179/196	295/328	172/176	180/184
Embryo 13	161/165	218/222	242/242	243/251	190/198	196/203	341/345	168/176	176/184
Embryo 14	161/165	218/218	222/242	247/255	190/198	179/196	295/328	168/172	180/184
<b>Male 2</b>	<b>161/165</b>	<b>214/218</b>	<b>230/234</b>	<b>247/255</b>	<b>187/228</b>	<b>199/*</b>	<b>350/365</b>	<b>176/184</b>	<b>168/172</b>
Embryo 1	165/165	218/218 <sup>a</sup>	230/242	247/251	187/197	196/199	328/350	168/168 <sup>b</sup>	168/184
Embryo 4	161/165	214/218	230/242	247/255	190/228	196/199	328/350	168/176	172/184
Embryo 5	165/165	214/null	230/242	251/255	187/190	196/199	328/365	168/184	168/184
Embryo 8	165/165	218/218 <sup>a</sup>	234/242	251/255	190/228	196/199	345/365	176/184	168/184
Embryo 15	165/165	218/218 <sup>a</sup>	230/242	247/255	187/190	196/199	345/365	176/184	172/184
Embryo 16	165/165	218/218 <sup>a</sup>	230/242	255/255	187/190	196/199	328/365	168/184	168/184
Embryo 17	165/165	214/218	234/242	255/255	197/228	196/199	345/350	168/176	168/184
<b>Male 3</b>	<b>165/177</b>	<b>222/*</b>	<b>226/*</b>	<b>251/*</b>	<b>211/236</b>	<b>199/*</b>	<b>307/*</b>	<b>172/*</b>	<b>176/*</b>
Embryo 2	165/165	222/null	226/242	251/255	197/211	196/199	307/328	168/172	176/184
Embryo 3	165/177	222/null	226/242	251/251	190/236	196/199	307/328	172/176	176/184



APPENDIX 1. *Continued.*

Individual	Locus								
	Esu17	Esu24	Esu31	Esu51	Esu53	Esu74	Esu54	Esu57	Esu70
33404	161/165	214/230	222/234	251/298	193/226	199/268	296/303	172/176	176/184
<b>Male 1</b>	<b>157/*</b>	<b>226/230</b>	<b>222/*</b>	<b>255/263</b>	<b>187/224</b>	<b>203/*</b>	<b>345/353</b>	<b>172/*</b>	<b>172/184</b>
Embryo 12	157/161	214/226	222/234	251/263	193/224	203/268	296/353	172/176	176/184
Embryo 18	157/165	230/230	222/222	263/298	224/226	199/203	303/353		172/176
Embryo 20	157/161	214/230	222/234	255/298	193/224	203/268	303/345	172/172	184/184
Embryo 13	157/161	226/230	222/234	263/298	187/193	203/268	303/345	172/172	172/176
Embryo 22		214/230		263/298	187/193	199/203		172/176	172/176
Embryo 23	157/165	214/226	222/234	255/298	224/226		296/353	172/176	172/176
Embryo 15	157/161	214/226	222/222	251/263	224/226	203/268	296/353	172/176	172/184
Embryo 16	157/165	214/226	222/234	251/263	187/226	199/203	303/353	172/172	176/184
Embryo 3	157/161	214/226	222/222	263/298	187/226	203/268	303/345	172/172	176/184
Embryo 5	157/161	214/226	222/234	255/298	224/226	203/268	296/345	172/176	176/184
Embryo 8	157/165	214/230	222/222	251/255	224/226	199/203	296/353	172/176	176/184
Embryo 2	157/165	226/230	222/234	255/298	224/226	199/203	303/345	172/176	172/176
Embryo 7	157/165	226/230	222/234	263/298	187/193	203/268	303/345	172/176	184/184
<b>Male 2</b>	<b>161/165</b>	<b>214/*</b>	<b>230/*</b>	<b>247/*</b>	<b>221/236</b>	<b>203/*</b>	<b>321/341</b>	<b>172/*</b>	<b>176/184</b>
Embryo 1	161/161	214/230	230/234	247/298	221/226	199/203	303/321	172/172	176/176
Embryo 6	161/165	214/230	222/230	247/251	221/226	199/203	296/341	172/172	176/184
Embryo 11	161/165	214/214	230/234	247/251	193/236	203/268	303/321	172/176	176/176
Embryo 4	161/165	214/230	222/230	247/298	193/221	199/203	303/321	172/172	184/184
Embryo 14	161/161	214/230	222/230	247/298	226/236	203/268	296/321	172/176	176/184
Embryo 24	161/165	214/230	230/234	247/251	221/226	199/203	303/321	172/176	176/184
Embryo 25	161/161	214/230	230/234	247/298	193/236	199/203	303/341	172/172	176/184
Embryo 17	165/165	214/230	230/234	247/298	221/226	203/268	296/321	172/172	176/176
<b>Male 3</b>	<b>169/*</b>	<b>207/226</b>	<b>230/*</b>	<b>251/302</b>	<b>183/197</b>	<b>193/*</b>	<b>315/341</b>	<b>176/*</b>	<b>176/180</b>
Embryo 9	161/165	214/226	230/234	251/298	193/197	193/268	296/341	172/176	176/180
Embryo 10	165/169	207/230	222/234	251/251	183/226	193/268	303/315	172/176	176/176
Embryo 19	165/169	207/230	222/234	251/302	193/197	193/268	303/315	176/176	180/184
<b>Male 4</b>	<b>*/*</b>	<b>214/*</b>	<b>230/*</b>	<b>251/*</b>	<b>236/*</b>	<b>203/*</b>	<b>345/*</b>	<b>168/*</b>	<b>*/*</b>
Embryo 21	161/165	214/214	222/230	251/251	226/236	199/203	296/345	168/172	176/184

Individual	Locus								
	Esu17	Esu24	Esu31	Esu51	Esu53	Esu74	Esu54	Esu57	Esu70
33405	161/169	203/230	230/234	251/255	193/220	196/199	328/366	168/168	168/176
<b>Male 1</b>	<b>157/169</b>	<b>222/230</b>	<b>222/*</b>	<b>247/255</b>	<b>228/*</b>	<b>193/*</b>	<b>345/365</b>	<b>176/180</b>	<b>160/*</b>
Embryo 1	157/169	222/230	222/230	247/255	193/228		328/345	168/176	168/176
Embryo 8	157/169	203/230		247/255	193/228	193/199	328/365	168/176	168/176
Embryo 9	157/161	203/222	230/234	247/255	220/228	193/199	328/345	168/176	160/176
Embryo 5	169/169	222/230	230/234	247/251	193/228	193/199	345/366	168/180	168/176
Embryo 11	157/169	203/230	222/230	251/255	220/228	193/193 <sup>c</sup>	328/365	168/180	168/176
Embryo 14	161/169	230/230	230/234	251/255	193/228	193/193 <sup>c</sup>	365/366	168/180	160/168
<b>Male 2</b>	<b>157/161</b>	<b>226/*</b>	<b>226/238</b>	<b>247/255</b>	<b>173/228</b>	<b>199/*</b>	<b>323/345</b>	<b>168/*</b>	<b>168/180</b>
Embryo 3	161/161	226/230	226/230	255/255	220/228	199/199	323/366	168/168	168/168
Embryo 2	161/161	226/230	230/238	247/251	173/220	199/199	345/366	168/168	168/168
Embryo 13	157/169	226/230		255/255	173/220	199/199	323/328	168/168	176/180
<b>Male 3</b>	<b>157/173</b>	<b>238/*</b>	<b>226/*</b>	<b>298/*</b>	<b>205/*</b>	<b>190/*</b>	<b>311/350</b>	<b>176/*</b>	<b>180/192</b>
Embryo 10	161/173	203/238		255/298	205/220	190/196	311/328	168/176	176/180
Embryo 6	157/161	230/238	226/230	255/298	205/220	190/196	350/366	168/176	176/192
Embryo 4	157/169	203/218	230/234	251/255	209/220	196/196	328/350	168/168	168/176
Embryo 7	161/173	203/230	230/234	255/298	220/232	196/196	328/329	168/180	168/172
Embryo 12	169/173	203/214	226/234	251/290	193/198	199/199	328/328	168/168	168/172

NOTE: The remaining three offspring can be explained by two paternal genotypes for a minimum of five males for this litter.

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APPENDIX 1. *Continued.*

Individual	Locus			
	Een162	Een167	Een198	Een166
<b>33394</b>	<b>175/183</b>	<b>263/267</b>	<b>195/199</b>	<b>262/281</b>
<b>Male 1</b>	<b>167/191</b>	<b>271/*</b>	<b>175/199</b>	<b>258/302</b>
Embryo 2	167/183	267/271	175/199	281/302
Embryo 3	175/191	267/271	199/199	262/302
Embryo 5	167/183	267/271	175/199	258/281
Embryo 7	167/183	267/271	175/199	281/302
Embryo 8	167/175	267/271	175/199	262/302
Embryo 9	175/191	267/271	199/199	281/302
Embryo 10	183/191	263/271	175/195	281/302
Embryo 11	175/191	263/271	195/199	258/281
Embryo 13	175/191	267/271	199/199	258/262
Embryo 15	183/191	263/271	175/195	281/302
Embryo 19	183/191	267/271	199/199	281/302
Embryo 22	167/183	267/271	199/199	262/302
Embryo 23	167/183	263/271	175/195	262/302
Embryo 26	167/183	267/271	199/199	281/302
Embryo 27	167/175	267/271	175/199	262/302
Embryo 28	175/191	263/271	195/199	258/262
Embryo 29	167/175	267/271	175/199	262/302
<b>Male 2</b>	<b>179/186</b>	<b>275/283</b>	<b>175/199</b>	<b>244/*</b>
Embryo 14	183/186	267/283	199/199	244/281
Embryo 20	183/186	267/275	175/199	244/262
Embryo 21	179/183	263/283	195/199	244/281
Embryo 24	175/186	267/283	199/199	244/262
<b>Male 3</b>	<b>175/179</b>	<b>256/259</b>	<b>187/207</b>	<b>306/*</b>
Embryo 16	175/175	259/263	187/195	281/306
Embryo 18	175/179	256/267	187/199	262/306
Embryo 25	175/179	259/267	199/207	281/306
Embryo 6	175/182	263/267	183/195	281/311
Embryo 12	182/183	267/287	179/199	273/281
Embryo 4	167/175	267/275	199/199	262/332
Embryo 17	171/175	259/263	175/195	244/262

Note: remaining four offspring can be explained by two paternal genotypes for a minimum of five males for this litter.



APPENDIX 1. *Continued.*

Individual	Locus			
	Een162	Een167	Een198	Een166
33403	147/160	259/267	167/171	
<b>Male 1</b>	<b>164/172</b>	<b>236/263</b>	<b>163/175</b>	
Embryo 1	160/164	236/267	171/175	
Embryo 2	160/164	259/263	163/167	
Embryo 3	160/172	259/263	163/167	
Embryo 5	160/164	259/263	163/167	
Embryo 7	147/164	263/267	163/171	
Embryo 8	160/164	236/259	167/175	
Embryo 9	160/164	236/259	167/175	
Embryo 10	147/164	263/267	163/171	
Embryo 11	147/164	236/267	171/175	
Embryo 12	147/172	236/259	167/175	
Embryo 13	160/172	263/267	163/171	
Embryo 15	147/164	236/259	167/175	
Embryo 16	160/164	263/267	163/167	
Embryo 17	147/172	236/267	171/175	
Embryo 18	147/164	236/267	171/175	
Embryo 19	147/172	263/267	163/167	
Embryo 20	147/172	236/259	167/175	
Embryo 21	147/172	236/259	167/175	
Embryo 22	147/172	259/263	163/167	
Embryo 24	147/164	259/263	163/167	
Embryo 25	147/164	236/267	171/175	
Embryo 26	147/164	263/267	163/171	
Embryo 27	147/172	236/259	167/175	
Embryo 28	160/172	236/267	171/175	
Embryo 30	160/172	236/259	167/175	
Embryo 31	147/172	236/267	171/175	
Embryo 33	160/164	263/267	163/171	
Embryo 35	147/164	263/267	163/171	
<b>Male 2</b>	<b>164/180</b>	<b>247/251</b>	<b>171/187</b>	
Embryo 4	147/164	251/267	171/187	
Embryo 6	160/180	251/259	167/187	
Embryo 23	147/180	251/267	171/187	
Embryo 14	147/164	247/267	167/171	
Embryo 29	160/164	247/267	167/171	
Embryo 32	147/164	247/267	167/171	
<b>Male 3</b>	<b>180/*</b>	<b>247/*</b>	<b>163/*</b>	
Embryo 34	147/180	247/259	163/167	

<sup>a</sup> The 218/218 genotype in the offspring can also be 218/null.

<sup>b</sup> indicates either a mutation to the 168 allele or a mutation to a null allele from father to offspring.

<sup>c</sup> The maternal allele either mutated to 193 or is a null allele in these individuals.