Multiple paternity in the western terrestrial garter snake, *Thamnophis elegans*

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Abstract: Multiple paternity may be a widespread phenomenon in snakes, but studies to date are inadequate for assessing the effect that phylogeny may have on paternity. Hypothetical mechanisms responsible for polyandry in snakes include intersexual conflicts and avoidance of genetic incompatibilities due to inbreeding. We analysed the offspring of six litters of western terrestrial garter snakes (*Thamnophis elegans* (Baird and Girard, 1853)) using microsatellite DNA polymorphisms. We directly detected multiple paternity in half of the litters, one of which exhibited triple paternity, and substantial skew of paternal contributions in all multiply sired litters. Females producing multiply sired offspring were heavier postpartum and produced larger litters, suggesting that larger females that invest more in reproduction are more likely to be multiply mated, a result supporting the hypothesis that polyandry is due to intersexual conflict. Continued investigations of paternity patterns within this genus are under way, but if the factors driving polyandry in snakes are to be identified, controlled laboratory crosses are required.

Résumé : La paternité multiple peut être un phénomène répandu chez les couleuvres, mais jusqu'à maintenant les études n'ont pas réussi à évaluer l'effet possible de la phylogénie sur la paternité. Les mécanismes évoqués pour expliquer la polyandrie chez les couleuvres incluent les conflits entre les sexes et l'évitement d'incompatibilités génétiques dues à la consanguinité. Nous avons analysé l'ascendance dans six portées de la couleuvre de l'Ouest (*Thamnophis elegans* (Baird et Girard, 1853)) à l'aide de polymorphismes de l'ADN microsatellite. Nous avons détecté directement des paternités multiples dans la moitié des portées, dont l'une avec une triple paternité; dans toutes les portées à paternité multiple, la paternité est répartie bien inégalement. Les femelles qui produisent des portées à paternité multiple sont plus lourdes après la mise bas et produisent des portées plus grandes, ce qui laisse croire que les femelles plus grandes qui investissent plus dans leur reproduction ont plus de chances de s'accoupler plusieurs fois; ces données appuient l'hypothèse qui veut que la polyandrie s'explique par les conflits entre les sexes. Des études se poursuivent sur les patrons de paternité dans ce genre; cependant, pour réussir à élucider les facteurs qui expliquent la polyandrie chez les couleuvres, il faudra procéder à des croisements contrôlés en laboratoire.

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Introduction

Direct evidence for polyandry in snakes was first documented in the common garter snake, *Thamnophis sirtalis* (L., 1758), over 60 years ago (Blanchard and Blanchard 1941). Since then, morphological and (or) molecular evidence for multiple paternity has been published for *T. sirtalis* and at least four other snake species (*T. sirtalis*, Gibson and Falls 1975; *Lampropeltis getula* (L., 1766), Zweifel and Dessauer 1983; *Agkistrodon contortrix* (L., 1766), Schuett and Gillingham 1986; *Nerodia sipedon* (L., 1758), Barry et al. 1992; *Vipera berus* (L., 1758), Höggren

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and Tegelström 1995). Although the presence of multiple paternity in several colubrid and viperid lineages suggests that it may be widespread in snakes, the species studied to date preclude determination of the influence of phylogeny on paternity patterns. The effect of phylogeny is unlikely to be trivial: recent analyses of the patterns of bird extra-pair paternity ascribe up to 55% of variation to phylogeny (Arnold and Owens 2002; Westneat and Stewart 2003). Stearns (1984) showed that variation of relevant life-history traits in reptiles has a strong phylogenetic component at the levels of family and genus. This suggests that initial attempts to assess the influence of phylogeny on multiple paternity in snakes should focus on either of these scales. Members of the genus *Thamnophis* are good candidates for just such an examination. The phylogeny of the genus is both comprehensive and well resolved (de Queiroz et al. 2002); enormous morphological, ecological, and life history variations exist and these variations have been described for several species (reviewed in Rossman et al. 1996).

Comparative studies have shown that the frequency of multiple paternity can vary among populations as well as among species, with ecological factors such as population density, sex ratio, and the length of the breeding season correlating with paternity patterns (Garner et al. 2002; Prosser et al. 2002). In the two colubrid species for which systematic studies have been made (*N. sipedon* and *T. sirtalis*), larger

females that have presumably invested more in reproductive effort are more likely to produce litters fathered by multiple sires. This, along with a wealth of behavioural data collected on *T. sirtalis*, has led many researchers to conclude that snake polyandry is due to intersexual conflicts (Shine et al. 2000, 2003*a*, 2003*b*, 2003*c*, 2003*d*, 2004; Prosser et al. 2002; Hosken and Stockley 2003). However, in one viperid species multiple paternity correlated strongly with increased offspring viability (Madsen et al. 1992, 1996, 1999). The results of these latter studies suggest that polyandry in snakes is due instead to the avoidance of genetic incompatibilities associated with inbreeding (Zeh and Zeh 1996, 1997; Tregenza and Wedell 2000).

The western terrestrial garter snake, *Thamnophis elegans* (Baird and Girard, 1853), is a member of the "widespread" clade of garter snakes, which includes *T. sirtalis* as a distant relative (de Queiroz et al. 2002). Local densities can be high and litter sizes are sufficient to allow for the reliable and quantitative detection of multiple paternity (Rossman et al. 1996). The species hibernates communally, females move away from den locations after mating, and males persist at den sites, presumably to continue to compete for mates (Farr 1988; Graves and Duvall 1990). Since obligatory postmating movement away from den sites by female, but not male, *T. sirtalis* is viewed as a key sex-specific behaviour associated with intersexual conflict, multiple paternity in *T. elegans*, if present, may also be caused by conflict. Alternatively, although inbreeding and inbreeding depression have not been documented in *T. elegans*, almost no migration events were recorded among a set of closely located populations of the species (Bronikowski 2000). Studies of several other snake species have documented inbreeding depression (Madsen et al. 1996; Gautschi et al. 2002; Újvári et al. 2002), and population genetic studies suggest that restricted gene flow may be common in snakes (Gibbs et al. 1997; Bushar et al. 1998). Furthermore, garter snakes frequently produce litters that include partially developed or stillborn offspring (Gregory 2001), a phenomenon that may be due to genetic incompatibility.

Here we contribute to the understanding of the distribution of polyandry in the genus *Thamnophis* by reporting the frequency and intensity of multiple paternity in a sample of *T. elegans* litters. We sampled six mothers and their offspring and assessed patterns of paternity using microsatellite loci developed for the species (Garner et al. 2004). We also investigated the relationships between number of fathers and various life-history characteristics of mothers and neonates. Specifically, we wanted to determine whether this garter snake species exhibits multiple paternity and, if so, whether paternity patterns suggest conflict or compatibility. If compatibility drives polyandry, then multiply sired litters should exhibit increased viability and paternity patterns should not correlate with maternal reproductive investment or body size. If conflict drives polyandry, then multiple paternity should not correlate with litter viability, but instead with female reproductive investment or body size.

Materials and methods

Six gravid *T. elegans* females were captured on 8 July 2002 near Chase, British Columbia, Canada. All females were subsequently held in captivity until parturition at the University College of the Cariboo, Kamloops, British Columbia. The animals were housed individually in Plexiglas® cages and provided with a source of heat and water. Cages were checked daily for evidence of parturition, and snakes were hand-fed fish (smelts) with vitamin supplements. *Thamnophis elegans* females maintained in captivity in this fashion do not produce anomalous litters (Gregory 2001). Females were measured for snout–vent length (SVL) to the nearest millimetre at time of capture and weighed to the nearest gram at capture and the nearest 0.1 g following parturition. We recorded the mass (to the nearest 0.01 g), SVL, and status (live versus dead) of each neonate from each litter. All snakes were sampled nondestructively by tail-clipping a small part of the distal tail segment. Tips were preserved in 99% ethanol until DNA extraction. Females and their live offspring were returned to the point of each female's capture once sampling was completed.

DNA was extracted from tail tips by grinding the tail tips under liquid nitrogen and then isolating the DNA using the QIAamp® DNA mini kit (QIAGEN). We used three microsatellite primer sets (Te1Ca2, Te1Ca3, Garner et al. 2004; 2Ts, Garner et al. 2002) for polymerase chain reaction amplification following the protocols included in the respective publications. Amplification products were electrophoresed using the SEA 2000® electrophoresis apparatus and Spreadex® gels (Elchrom Scientific, Switzerland), and microsatellite alleles were scored using the Q-EL™ 330 Digital Recording and Analysis System (Elchrom Scientific). Paternal alleles, genotypes, and the number of fathers were all estimated using GERUD1.0 (Jones 2001). Calculations were made without the benefit of an allele frequency file, as only maternal and offspring alleles were available; therefore, exclusion probabilities were not calculated. In all cases exhaustive searches were used to find all possible father combinations. The number of fathers and their relative contributions to progeny arrays were based on priority scores assuming 1:1 Mendelian segregation of paternal alleles from each father. In some cases offspring carried ambiguous paternal genotypes (see Appendix A). In these cases we assigned offspring to both fathers and calculated percent paternity (the percentage of offspring that could be attributed to each putative father) accordingly.

We explored the relationship between number of fathers (single paternity versus multiple paternity) and life-history traits using Pearson product–moment correlation coefficients and Mann–Whitney *U* tests on untransformed data. First, we sought correlations between female SVL and both pre- and post-partum mass. We then used Mann–Whitney *U* tests to determine whether litters exhibiting single versus multiple paternity differed in terms of maternal body size (SVL, pre- and post-partum mass), characteristics of maternal reproductive investment (litter size, average neonate SVL, average neonate mass), and offspring viability (proportion of offspring stillborn).

Results

Multiple paternity was detected in 50% of the litters, and three fathers best explained the variation detected in litter 6 (Table 1; see Appendix A for allelic information for three

Table 1. Life-history characteristics of six female *Thamnophis elegans* and their litters, including best estimates of the numbers of fathers contributing to each litter.

Mother ID	SVL (mm)	Prepartum mass (g)	Postpartum mass (g)	Litter size	Average litter SVL (mm)	Average litter mass (g)	No. of fathers	No. dead in litter
1	495	88	36.2	12	272.2 (3.099)	2.134 (0.151)	1	0
2	575	118	57.8	11	186.1 (2.809)	2.556 (0.117)	1	0
3	680	207	117	24	182.1 (7.500)	2.344 (0.260)	2	3
4[a]	585	144	78	14	192.3 (2.614)	2.739 (0.120)	2	1
5	555	97	44.3	8	189.5 (3.251)	2.383 (0.167)	1	0
6	Missing	113	72	14	175.2 (4.870)	2.055 (0.146)	3	0

Note: Paternal estimate for litter 6 is from the two best estimates. Standard deviations for averages are in parentheses after the mean value.
[a]Paternity was estimated using only two loci owing to the presence of a null allele at locus Te1Ca3.

multiply sired litters). Paternity was skewed in favour of one father in all multiply sired litters. Twenty of 24 offspring in litter 3 were sired by one of the two fathers, and up to 10 of 14 offspring in litter 4 were sired by one of two fathers. Paternity assignment for litter 6 did not arrive at a single best estimate of paternal contributions, but the two best estimates assigned either 50% or 57% to the most successful of three fathers. The other two fathers of this litter shared equally the remaining offspring in both of the estimates. Neonate mortality was detected in only two litters (7% of litter 4, 12.5% of litter 3, see Table 1) and the proportion of offspring that were stillborn did not differ significantly among singly and multiply sired litters (Mann–Whitney $U = 1.5$, $p = 0.121$). Both prepartum and postpartum mass were strongly correlated with female SVL (Pearson coefficients, respectively, 0.958 and 0.959). Maternal SVL ($p = 0.083$), prepartum mass ($p = 0.127$), average neonate SVL ($p = 0.275$), and average neonate mass ($p = 0.827$) did not differ significantly between the two paternity classes, while postpartum mass ($p = 0.050$) and litter size ($p = 0.046$) were significantly different. Multiply mated females were heavier postpartum, and multiply sired litters were larger (Table 1, Fig. 1).

Discussion

The proportion of *T. elegans* litters exhibiting direct evidence of multiple paternity in this study is comparable to the proportions previously detected in *N. sipedon* (Prosser et al. 2002; but see Barry et al. 1992) and falls within the range previously detected in *T. sirtalis* (Schwartz et al. 1989; McCracken et al. 1999; Garner et al. 2002). Whereas up to five fathers were detected in *N. sipedon*, to date a maximum of three fathers have been detected in any *Thamnophis* litter (this study; McCracken et al. 1999). Here we report the first estimates of paternal skew in *Thamnophis* sibships. Skew is substantial, ranging from 71.4% to 83.3% in half sibships and being either 50% or 57% in the one litter fathered by three males. We have no way of determining whether these patterns are due to first- or last-male sperm precedence or to random variation and sperm mixing.

Heavier females producing more offspring were more likely to be multiply inseminated than lighter females producing, on average, smaller litters. These results are compatible with previous hypotheses regarding female reproductive condition, the intensity of male courtship, and multiple inseminations in common garter snakes due to intersexual conflict. Larger common garter snake females generally invest

Fig. 1. (*a*) Box plot of maternal postpartum mass of mothers producing *Thamnophis elegans* litters exhibiting direct evidence for single (1) or multiple (≥2) paternity. Boxes show sample median (center vertice), central 50% of the sample range (edge of box), and overall sample range (whiskers outside box). (*b*) Box plot of litter size of singly (1) and multiply (≥2) fathered litters of *T. elegans*. Boxes as in Fig. 1*a*.
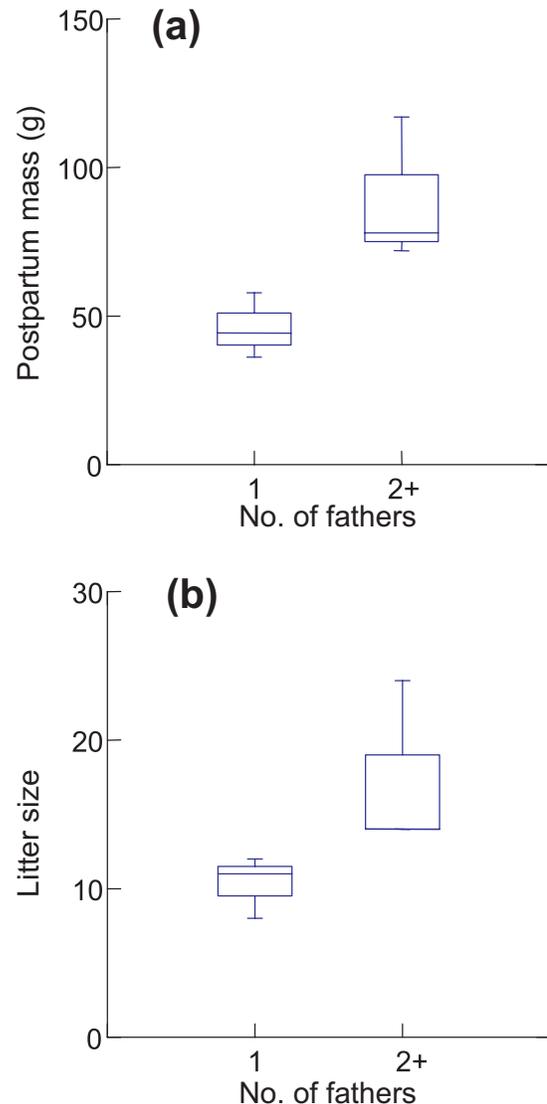
Table 2. Published evidence for multiple paternity in snakes.

Species	Type of study	Sample size	Marker	No. of litters multiply sired	Paternal skew	Within/among season pattern	Reference
Thamnophis sirtalis	Laboratory	2	Melanism[a]	—	—	Within	Blanchard and Blanchard 1941[b]
Thamnophis sirtalis	Field	22	Melanism	13	—	—	Gibson and Falls 1975
Thamnophis sirtalis	Field	32	Allozymes	16	—	—	Schwartz et al. 1989
Thamnophis sirtalis	Field	8	Microsatellites	6	—	—	McCracken et al. 1999
Thamnophis sirtalis	Field	4	Microsatellites	4	Yes (weak)	—	King et al. 2000[c]
Thamnophis sirtalis	Field	16	Microsatellites	6	—	—	Garner et al. 2002
Thamnophis elegans	Field	6	Microsatellites	3	Yes	—	This study
Nerodia sipedon	Field	14	Allozymes	8	—	—	Barry et al. 1992
Nerodia sipedon	Field	45	Microsatellites	26	Yes	—	Prosser et al. 2002
Lampropeltis getula	Laboratory	1	Allozymes	1	No	Within	Zweifel and Dessauer 1983
Vipera berus	Laboratory	9	Allozymes	5	Yes	Both	Stille et al. 1986
Vipera berus	Laboratory	6	Fingerprinting	5	Yes	Within (possibly both)	Höggren and Tegelström 1995
Vipera berus	Laboratory	8	Fingerprinting	6	Yes (first-male advantage[d])	Within	Höggren and Tegelström 2002
Agkistrodon contortrix	Laboratory	5	Subspecific pattern polymorphism	2	Yes	Among	Schuett and Gillingham 1986

Note: Type of study refers to either laboratory crosses or patterns from field-captured and inseminated females. Number of litters multiply sired is litters or clutches with direct evidence of more than one father. Paternity skew is provided when stated or when data are shown in the relevant publication. Within- or among-season multiple paternity is also provided only if stated in the relevant publication.

[a]Melanism inherited though single-locus homozygous expression of a recessive allele has been debated (Zweifel 1998), but most recent research shows clear Mendelian inheritance via this mechanism (King 2003).

[b]Multiple mating observed, evidence for multiple paternity equivocal.

[c]Sample is probably selective and frequency of multiple paternity is unlikely to be representative of the population that females were sampled from.

[d]Crosses were three male matings.

more in reproduction and are effective at signalling their increased investment to males chemically (Gartska and Crews 1981; Gartska et al. 1982; Mason et al. 1989; Shine et al. 2003*c*) and directly through visual cues (Shine et al. 2003*b*, 2003*c*). Large-sized and high-investment females are courted more intensely than smaller females (Aleksiuk and Gregory 1974; Shine et al. 2003*a*, 2001) and by more males of all sizes (Aleksiuk and Gregory 1974; Shine et al. 2001, 2003*b*). Intense courtship increases the probability of forced insemination, as does an increase in the number of courting males (Shine et al. 2003*a*, 2003*d*). This leads to larger, high-investment females being inseminated by more males, and a correlation between female body size or reproductive condition and multiple paternity has been detected in both *T. sirtalis* and *N. sipedon* (Garner et al. 2002; Prosser et al. 2002). Prosser et al. (2002) noted that a correlation between increased litter size or female body size and multiple paternity could be driven purely by a statistical effect. Their data suggested that bigger females do copulate more frequently but that chance determined paternity patterns after females had been multiply inseminated. Our sample size is too limited for a definitive conclusion on the relative importance of chance versus postcopulatory sexual selection in our study species, but it is notable that all singly sired litters were large enough to show evidence of multiple paternity had it been present and given the paternity skews we detected. The presence of such skews may itself be evidence that chance is not the best explanation for mixed paternity patterns in this species, since consistently skewed paternity should not be evident if sperm are randomly mixed and ejaculates do not vary substantially in size, composition, and sperm quality.

Offspring quality was not correlated with multiple paternity in *T. elegans*, in terms of either offspring size characteristics or the number of stillborn offspring. While polyandry contributed greatly to offspring quality in an isolated population of adders, this has not been found for any other snake species studied to date (Madsen et al. 1992; Garner et al. 2002; Prosser et al. 2002). Studies of multiple mating in other adder populations have shown no such clear fitness benefit associated with mating rate (Capula and Luiselli 1994; Luiselli 1995). Capula and Luiselli (1994) and Luiselli (1995) indirectly suggested that the effect described by Madsen et al. (1992) may be specific to isolated and thus inbred populations (but see Olsson et al. 1996). This conclusion was further supported by subsequent research performed by Madsen and coworkers. The introduction of foreign and genetically diverse males further reduced the incidence of stillborn offspring (Madsen et al. 1996), and introgression of MHC alleles carried by these introduced males was strongly correlated with the demographic recovery of the population (Madsen et al. 1999). The snakes in our study were obtained from a reasonably pristine area where anthropogenic habitat fragmentation is minimal. Snakes in this area are relatively abundant, and connectivity with other snake populations is likely quite high. All this suggests that geographic isolation and genetic incompatibility are likely having minimal influences. Furthermore, only multiply inseminated females produced stillborn offspring in our study.

Of course, the patterns of paternity we have detected may not accurately reflect female mating patterns. Instead, it is conceivable that all singly sired litters are the result of multiply mated females selecting the most compatible sperm, as may be the case for Swedish sand lizards (*Lacerta agilis* L., 1758; Olsson et al. 1996). However, we doubt this, as the prevalence of multiple paternity in all species studied to date is high (Table 2), an unlikely situation if selection favoured females that cryptically selected sperm. In the one species in which genetic benefits appear to matter the most (*V. berus*), controlled matings revealed no such sperm selection and instead showed a first-male mating advantage, presumably due to fertility assurance (Höggren and Tegelström 2002). We await controlled laboratory crosses that will directly address this issue.

Within the *Thamnophis* "widespread" clade, multiple paternity has now been detected directly in two species from different ends of the phylogenetic distribution. Further effort might be best expended seeking broods with more than one father from species intermediate within the "widespread" clade. *Thamnophis radix* (Baird and Girard, 1853), *Thamnophis marcianus* (Baird and Girard, 1853), and *Thamnophis cyrtopsis* (Kennicott, 1860) are all species that exhibit large body size and a large brood size range that includes litter sizes unlikely to mask sperm usage patterns due to sampling error (Rossman et al. 1996; de Queiroz et al. 2002). A survey of the literature on snake polyandry (Table 2) shows clearly that *T. sirtalis* is the best-studied species to date, both in terms of number of studies and number of locations sampled. What is also clear is that the majority of studies identify the presence of multiple paternity but do not document the proportions of offspring that can be attributed to the different fathers of multiply sired broods. Furthermore, since almost all studies have been field-based, it is impossible to say whether multiple paternity is driven by within-season or among-season mating patterns. If the proximate and ultimate causes of polyandry in snakes are to be identified, patterns of paternal contributions need to be quantified with respect to order of insemination and time between copulations. These patterns can be determined only through controlled laboratory crosses, preferably using reproductively naïve females.

In summary, we have shown that *T. elegans* litters exhibit multiple paternity and that paternal contributions are skewed towards a single male. We have shown that female condition and litter size are both significantly and positively correlated with paternity rates, suggesting that the mating patterns of this species are the result of intersexual conflict. Our data do not support the hypothesis that polyandry in this species is due to the avoidance of genetic incompatibilities, but a larger sample size would address this issue more comprehensively. The genus *Thamnophis* would be an appropriate complex of species for investigating polyandry in snakes within a phylogenetic framework, but continued research on snake multiple paternity would benefit greatly from the addition of controlled laboratory crosses designed to address how skews in paternal contributions are mediated.

Acknowledgments

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Appendix A

Table A1. Maternal genotype, allele scores, and putative paternal genotypes for three multiply sired *Thamnophis elegans* litters.

Litter	Individual[a]	Te1Ca2	Te1Ca3	2Ts
3	Mother	92/92	94/88	317/311
	1	92/88	94/88	317/307
	2	114/92	94/94	317/307
	3	92/88	94/94	317/313
	4	92/88	94/88	311/307
	5	92/92	94/88	317/313
	6	114/92	94/88	317/307
	7	92/92	94/94	313/311
	8	92/92	94/88	311/307
	9	92/88	94/88	311/307
	10	92/92	94/94	317/307
	11	92/88	94/88	313/311
	12	92/92	94/94	311/307
	13	92/88	94/88	317/313
	14	92/92	94/94	311/307
	15	92/88	94/94	317/313
	16	92/92	94/88	311/307
	17	92/90	88/88	317/313
	18	92/90	94/94	317/313
	19	92/88	94/94	317/307
	20	92/88	94/94	311/307
	21	92/92	94/88	317/313
	22	92/92	94/94	313/311
	23	92/88	94/88	317/307
	24	92/92	94/88	313/311
	Father1	92/88	94/94	313/307
	Father2	114/90	94/88	313/307
4	Mother	98/92		321/301
	1	92/92		315/301
	2	92/92		315/301
	3	98/90		321/315
	4	98/92		321/309
	5	98/98		301/297
	6[b]	98/92		315/301
	7	98/90		321/315
	8	92/90		309/301
	9	98/90		315/301
	10	98/98		301/297
	11	92/92		315/301
	12	98/98		321/297
	13	98/90		321/315
	14	118/92		301/297
	Father1	92/90		315/309
	Father2	118/98		315/297
6	Mother	92/90	94/88	309/291
	1	114/92	94/94	291/277
	2	98/90	94/88	309/309
	3	114/92	94/94	291/267
	4	114/90	94/94	291/267
	5	114/90	94/94	291/277
	6	92/92	94/94	309/291
	7[b]	98/92	94/94	291/291
	8	114/92	94/94	309/277
	9	114/90	94/94	309/277

Table A1 (*concluded*).

Litter	Individual[a]	Te1Ca2	Te1Ca3	2Ts
6	10[b,c]	98/92	94/88	309/291
	11	114/92	94/94	291/277
	12	94/92	94/88	309/291
	13[b]	98/92	94/88	309/309
	Father1a	114/114	94/94	277/267
	Father2a	98/92	94/94	309/291
	Father3a	98/94	94/94	309/291
	Father1b	114/98	94/94	277/309
	Father2b	114/98	94/94	267/291
	Father3b	94/92	94/94	309/309

Note: Allele scores are absolute sizes of polymerase chain reaction products. Two sets of paternal genotypes are provided for litter 6.

[a]Numbers refer to offspring.

[b]Offspring with genotype attributable to more than one father (in the case of litter 6, for the first paternal array).

[c]Offspring with genotype attributable to more than one father (in the second paternal array for litter 6).