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Molecular documentation of polyembryony and the micro-spatial dispersion of clonal sibships in the nine-banded armadillo, *Dasypus novemcinctus*

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SUMMARY

A battery of allelic markers at highly polymorphic microsatellite loci was developed and employed to confirm, genetically, the clonal nature of sibships in nine-banded armadillos. This phenomenon of consistent polyembryony, otherwise nearly unknown among the vertebrates, was capitalized upon to describe the micro-spatial distributions of numerous clonal sibships in a natural population of armadillos. Adult clone mates were significantly more dispersed than were juvenile sibs, suggesting limited opportunities for altruistic behavioural interactions among mature individuals. These results, and considerations of armadillo natural history, suggest that evolutionary explanations for polyembryony in this species may not reside in the kinds of ecological and kin selection theories relevant to some of the polyembryonic invertebrates. Rather, polyembryony in armadillos may be associated evolutionarily with other reproductive peculiarities of the species, including delayed uterine implantation of a single egg.

1. INTRODUCTION

Polyembryony, the production of two or more embryos from a single zygote, occurs sporadically in diverse animal taxa (including humans). Unlike other forms of organismal clonality such as ameiotic parthenogenesis, genetic identity under polyembryony is intra-generational rather than between parent and offspring. Polyembryony has been interpreted as one possible evolutionary resolution of inherent conflicts of interest between competing parental and offspring fitness strategies, or between those of monozygotic twins and non-twins within a sibship (Williams 1975; Grbic *et al.* 1992; Gleeson *et al.* 1994; Hardy 1995; Shaanker & Ganeshaiah 1996). However, rarely is polyembryony the primary reproductive mode within a species. Among the vertebrates, only armadillos of the genus *Dasypus* (Mammalia, Edentata, Dasypodidae) reportedly display 'monozygotic twinning' as a fixed reproductive feature (Galbreath 1985). The occurrence of fixed polyembryony in a vertebrate raises broader questions as to the evolutionary circumstances that occasionally promote this phenomenon (Craig *et al.* 1995; Ryland 1996).

Among the six species in the genus, the nine-banded armadillo *Dasypus novemcinctus* has been studied most thoroughly (McBee & Baker 1982). Polyembryony in this species was first suspected early in this century from the observation that litter mates are of the same sex, and from direct embryological examinations (Lane 1909; Newman & Patterson 1910; Newman 1913; Patterson 1913; Vanneman 1917; Hamlett 1929). The developmental studies indicated that after a post-fertilization delay of 2–4 months, a single blastocyst implants in the armadillo uterus and divides into four primordial buds that develop independently. About 18 weeks later, four offspring usually are born. Because these siblings appear to be derived from a single zygote, the supposition has been that they represent genetically identical quadruplets, and indeed this provided one of the motivations for use of the nine-banded armadillo as a replicatable, model animal system for the experimental study of leprosy (Kirchheimer & Storrs 1971; Storrs *et al.* 1974).

Nonetheless, the clonal nature of sibling armadillos is not entirely without doubt. Newman & Patterson (1910) noted variation among litter mates in size, scute numbers and banding patterns, although such variability could of course be non-genetic. Storrs & Williams (1968) provisionally attributed large inter-sibling variances in organ weights and in concentrations of metabolic compounds to differences in cytoplasmic factors including, perhaps, mitochondria. Based on details of primordial bud development, some speculation also exists that armadillo 'quadruplets' might represent two sets of twins (Storrs & Williams 1968). In any event, the genetic identity of armadillo siblings has not yet been critically documented in DNA- or protein-level genetic assays. One possible explanation for this is the paucity of polymorphic allozyme markers available in North American populations of *D. novemcinctus* (Ramsey & Grigsby 1985).

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Here, we develop and utilize molecular assays for polymorphic microsatellite genes in nine-banded armadillos. Our immediate goals were to: (i) generate a battery of variable DNA-level markers for *D. novemcinctus*; and (ii) apply these to validate or refute the genetic identity of siblings. After verifying the phenomenon of polyembryony in armadillos, we then capitalize upon the clonal nature of siblings to identify sibships in nature and empirically assess armadillo dispersion patterns in a population in northern Florida. The micro-spatial distributions of armadillo clones have implications for the opportunity for kin selection, one of the classes of hypotheses sometimes invoked to explain the evolution of polyembryony.

2. MATERIALS AND METHODS

(*a*) *Armadillo samples*

Tall Timbers Research Station (near Tallahassee, Florida) is home to a large, natural population of nine-banded armadillos that is the subject of ongoing observational and experimental study (Loughry & McDonough 1994, 1996*a–b*). Armadillos were captured using a large dip net attached to a 1.5 m pole, and were aged, sexed, measured, and marked. The spatial location of each animal was obtained using the Trimble Pathfinder Basic+ Global Positioning System. Small notches of ear tissue were preserved in 70–99% ethanol. Essentially all animals (196 adults and 114 juveniles, the latter defined as less than one-year-old) caught during extensive sampling in the summers of 1992–1995 were examined here genetically. Attempts to obtain pregnant females at this site and from other sources were unsuccessful, so all assessments of clonality were based on field-caught post-partum animals (see Discussion).

(*b*) *Library construction and screening*

Total genomic DNA was isolated from ear clips using a simplified phenol/chloroform method (Taggart *et al.* 1992). For library construction, 10μg of DNA (pooled from three adults) was fully digested with *Mbo*I and size-fractionated by gel electrophoresis (1.5% agarose 1X TBE). A slice containing 200–700 bp DNA fragments was cut from the gel, and the fragments purified using the Prep-A-Gene DNA Purification Matrix Kit (Bio-Rad). A total of 60 ng of the DNA fraction was ligated to 100 ng of dephosphorylated *Bam*HI digested pBluescript II KS(-) phagemid vector (Stratagene) in a 10 μl reaction volume.

Two approaches to library construction were utilized: standard and enriched. In the standard approach, one-fifth of the ligation reaction above was used to transform 100 μl of supercompetent XL1-Blue host cells (Stratagene). Transformed cells were grown overnight at 37 °C on LB agar plates containing ampicillin (100 μg ml^{-1}) and spread with both 100 μl of 2% X-gal and 40 μl of 100 mM IPTG for 30 min before plating transformants. Positive colonies were hand-picked and transferred to new 'gridded' LB/AMP plates which again were incubated overnight. Colonies were blotted onto Hybond-N membranes (Amersham). Following blotting, the LB plates were reincubated at 37 °C to allow colony recovery, and then stored at 4 °C.

Colony blots were alternately hybridized with two 'cocktails' (1 and 2) containing the end-labeled (γ^{32}PdATP) oligonucleotide probes: 1- $(GAA)_6$, $(TCC)_5$, $(CAC)_5$, $(CCG)_5$; and 2- $(GT)_{10}$, $(AGC)_5$, $(GGA)_5$, $(GGAT)_4$, $(GATA)_4$, $(GACA)_4$. Pre-hybridization (2 h) and hybridization (overnight) were done at 42 °C in 6X SSC (20X stock: 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 0.1% SDS, and 6X Denhart's solution (50X stock: 1% Ficoll, 1% PVP, 1% BSA Fraction V). Two post-hybridization washes were carried out in 6X SSC (0.1% SDS for 30 min each). After 2–4 h of autoradiographic exposure, positive recombinants were identified and isolated from the gridded LB plates. Positive recombinants were grown in 4 ml LB medium containing ampicillin (100 μg ml^{-1}) and the phagemid construct was purified using the QIAprep Spin Plasmid Kit (Qiagen). Manual sequencing of both ends (primers T3 and T7) was accomplished on 6% acrylamide gels using the *fmol* DNA cycle sequencing system (Promega).

Table 1. *pcr primers and optimized running conditions for the nine microsatellite loci employed*

locus[1]		primers	allele cloned[2]	repeat motif	T(°C)	$MgCl_2$ (mM)
Dnov-1	**F**:	CATAACTTCCACCCCTGATAC	233 bp	$(GACA)_2$ $(GATA)_{14}$	57	1.0
	R:	AGTGATTATCCTCGTTCTTAG				
Dnov-2	F:	TGCCAATTTGCCATATTATTC	254 bp	(GATA) $(GATT)_2$	58	1.0
	R:	GGACACAGAGAACAGACAACTG		$(GATA)_{15}$		
Dnov-3	**F**:	TTAAAGTATGAAAGGAGTGACAA	167 bp	$(CA)_4$ (TATG)	57	0.9
	R:	GATCTTCTCACCCAGAGATACAT		$(CA)_5$ (AT) $(TA)_4$		
Dnov-6	**F**:	TGCTGCATTTGTCATATCATTC	189 bp	(GATA) $(GACA)_3$	59	0.9
	R:	CAGCCTACACTCAAGTATTCGG		$(GATA)_5$ (GGTA)		
				$(GATA)_{10}$		
Dnov-7	F:	TTTCAGTAGAACCATTATTCTTG	217 bp	$(GT)_2$ (CA) $(GT)_{14}$	58	1.0
	R:	TTCAAGTCTGCCTCTACTGAG				
Dnov-16	**F**:	GAAAGGAGGGGATAAATAAAG	226 bp	$(AT)_{13}$ $(GT)_2$ (AT)	59	0.9
	R:	TTCCAGTTTTTGAATGTTCTAG		$(GT)_6$ (AG) $(AT)_4$		
Dnov-24	F:	TTTTAACATTTTTCCACTTTTCC	261 bp	$(GATA)_{17}$	57	1.0
	R:	AATACATGGCAACGGACTTTAGT				
Dnov-51	F:	TTTAGACATATTGTAGAGACAGG	251 bp	$(GATA)_4$ (AATA)	51	1.0
	R:	AGCCTTCCTCTTTGGTTTAG		$(GATA)_8$		
Dnov-65	**F**:	GATCAACCAATTAAAACTTT	186 bp	$(GATA)_3(GACA)_2$	57	0.9
	R:	ATATAAACAATCCACACTCA		(GATA) $(GACA)_6$		
				$(GATA)_{15}$		

[1] The end-labeled primer (F or R) is indicated in bold.
[2] Distance in base pairs (bp) between microsatellite primers.

Table 2. *Levels of genetic variation and agreement of genotypic frequencies with Hardy-Weinberg expectations (HWE) in the microsatellite survey of 196 adult and 114 juvenile (juvs.) armadillos from Tall Timbers Research Station*[1]

		heterozygosity								
		adults		juvs.		pooled		HWE (probability)		
locus	no. alleles	obs.	exp.	obs.	exp.	obs.	exp.	adults	juvs.	pooled
Dnov-1	7	0.678	0.719	0.715	0.744	0.695	0.727	0.144	0.102	0.075
Dnov-2	3	0.500	0.502	0.529	0.535	0.516	0.514	0.446	0.337	0.421
Dnov-6	4	0.639	0.612	0.654	0.653	0.649	0.628	0.822	0.425	0.732
Donv-7	3	0.392	0.409	0.564	0.557	0.445	0.465	0.183	0.512	0.156
Dnov-16	3	0.253	0.252	0.235	0.238	0.249	0.247	0.548	0.670	0.670
Dnov-24	3	0.528	0.544	0.608	0.620	0.553	0.573	0.381	0.562	0.380
Dnov-65[2]	7	0.314	0.740	0.427	0.658	0.355	0.661	< 0.0001	< 0.0001	< 0.0001

[1] The outcomes of all statistical tests were virtually identical when duplicate, genetically-identified polyembryonic individuals were excluded from the calculations.

[2] As described in the text, the apparent departures from HWE at this locus proved to be attributable to X-linkage, and to the fact that genotypes in males that initially appeared 'homozygous' as tabulated here are actually hemizygous.

The strategy for preparing an enriched microsatellite library followed Kijas *et al.* (1994). Part of the resulting pBluescript/insert ligation described above (≈ 50 ng) was used as a template to generate single-stranded DNA in an asymmetric polymerase chain reaction (PCR) employing the primers T3 and T7. $(GATA)_6$ was the biotinylated probe used to enrich the library. The ligation of GATA-rich fragments into the vector – transformation, plating, blotting, screening with a $(GATA)_4$ probe and sequencing of positives – followed procedures described above.

(*c*) *PCR*

After sequencing, clones containing the larger microsatellite regions were selected, and primers complementary to unique DNA sequences flanking these regions were designed. pcr for armadillo microsatellite screening was done in 12 µl volumes, each containing 1X Promega Taq buffer, 1–1.5 mM $MgCl_2$, 100 mM dNTPs, 1 pM each of the end-labelled and unlabelled primers, 100 ng DNA template, 1U Taq, and H_2O to 12 µl. The best conditions for each set of primers are given in table 1. Following a 2 min denaturation period at 94 °C, 27 cycles were performed, each consisting of 50 s at 94 °C, 50 s at the ideal temperature for each primer set (see table 1), and 50 s at 72 °C. The pcr amplification product was mixed with 4 µl of sequencing stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF), heated to 80 °C for 2 min, iced and loaded (3–4 µl) onto 6% (1X TBE) polyacrylamide gels. Original clones were run as size standards. Gels were run at 56W (2.5–3 h), dried, and autoradiographed (1–2 h).

Totals of 1000 and 600 clones were screened in the standard and enriched libraries. Among these, ten positives (1%) and 114 positives (19%), respectively, were identified. A total of 56 of the positive clones were sequenced (ten from the standard library, 46 from the enriched). Among these, 26 (57%) had adequate flanking regions for the design of pcr primers, and nine were chosen for development and assay (table 1).

(*d*) *Computation and statistics*

Allelic frequencies and heterozygosities were determined by direct count. Expected heterozygosities were calculated using the unbiased estimates of Nei (1978). Exact tests for Hardy-Weinberg equilibrium (HWE) (Rousset & Raymond 1995) employed the program GENEPOP (Raymond & Rousset 1995).

3. RESULTS

(*a*) *Microsatellite variation*

Seven microsatellite loci proved moderately variable, with three to seven alleles segregating per locus and expected per-locus heterozygosities ranging from 25–74% in adults (see table 2). These values accord with reports for microsatellite loci in other mammals (see, for example, Roy *et al.* 1994; Allen *et al.* 1995; Deka *et al.* 1995; Paetkau *et al.* 1995).

Except for one locus, no significant deviations of genotypic frequencies from HWE were observed (table 2). However, a highly significant deficiency of heterozygotes was present at the *Dnov*-65 locus. Further examination revealed that all males displayed single alleles, whereas genotype frequencies in adult females were in accord with HWE ($p = 0.86$). These findings indicate that the *Dnov*-65 locus is X-linked, and that males are hemizygous with respect to the X chromosome.

(*b*) *Genetic confirmation of polyembryony*

The microsatellite polymorphisms offer considerable power for examining the suspected clonal nature of armadillo litter mates. First-approximation probabilities of each observed multilocus genotype were calculated using the product rule from the data available for adults. These probabilities ranged from 2.3×10^{-9} to 9.4×10^{-4} (table 3). Such calculations assume random mating and linkage equilibrium, properties that may not be strictly true. However, because the samples were in agreement with HWE (table 2), and because the genetic examinations involved litters each with four individuals, realistic violations of these assumptions should not affect the clonal identity tests for sibships.

Before the genetic assays, it was strongly suspected from field observations that each one of five cohorts of

Table 3. *Expected probabilities (frequencies) of particular multilocus genotypes at the assayed microsatellite loci in female armadillos*[1]

probability	proportion of composite genotypes
$< 10^{-7}$	3.5%
10^{-7}–10^{-6}	9.8%
10^{-6}–10^{-5}	25.1%
10^{-5}–10^{-4}	40.9%
10^{-4}–10^{-3}	20.7%
$> 10^{-3}$	0%

[1] As calculated by multiplying the expected HWE genotypic frequencies across the seven polymorphic microsatellite loci scored. Values for males are only slightly different, due to the X-linked nature of one of the microsatellite loci.

four young individuals belonged to single complete litters (Loughry & McDonough 1994): members of each cohort were of the same size and sex, and were captured at the same time in a micro-spatial area. As indicated in figure 1, all four members of each putative sibship proved to be genetically identical at all surveyed loci, but clearly were distinct from individuals of other litters and from all other individuals in the study. Given the low probabilities for particular multilocus genotypes, and appreciating that the joint appearance of identical genotypes across multiple individuals is a functional product of such probabilities (e.g. for four individuals, on the order of 10^{-14}), it is unambiguously clear that the assayed sibships are clonal. Thus, our results confirm the polyembryonic nature of assayed armadillo siblings, and also help validate the criteria employed by Loughry & McDonough (1994) to identify siblings in the field.

Thirteen other sets of juveniles similarly shared unique multilocus genotypes. A total of five such sets involved identical triplets, and eight involved identical twins. From field experience, each of these cohorts also had been suspected of constituting a sibship. On the other hand, eight other putative twinships similarly suspected of being litter mates proved not to be clone mates based on the microsatellite evidence. The most likely explanation is that spatially associated juveniles of the same sex and similar age do not invariably belong to one litter. (Note: (i) evidence for these juveniles as sibs was less strong (e.g. they were not caught together); and (ii) separate litters do overlap spatially (Kalmbach, 1943; W. J. Loughry & C. M. McDonough, personal observations)) For the adults, seven sets of clonal sibships were identified in the microsatellite assays: two unique sets of triplets, and five unique sets of twins. All seven adult sibships were comprised of males.

(*c*) *Spatial distribution of armadillo clones*

Overall, 25 armadillo sibships (18 of juveniles, seven of adults, equal to 22% of individuals in the total collection) were documented. The mean spatial distance separating clone mates at their time of capture was 54.8 m for the juveniles and 455.9 m for the adults. This age-related difference in dispersion pattern is significant (one-way ANOVA: $F_{1,23} = 18.5$, $p < 0.001$). Among the juveniles, mean spatial separations within female sibships and male sibships (50.2 and

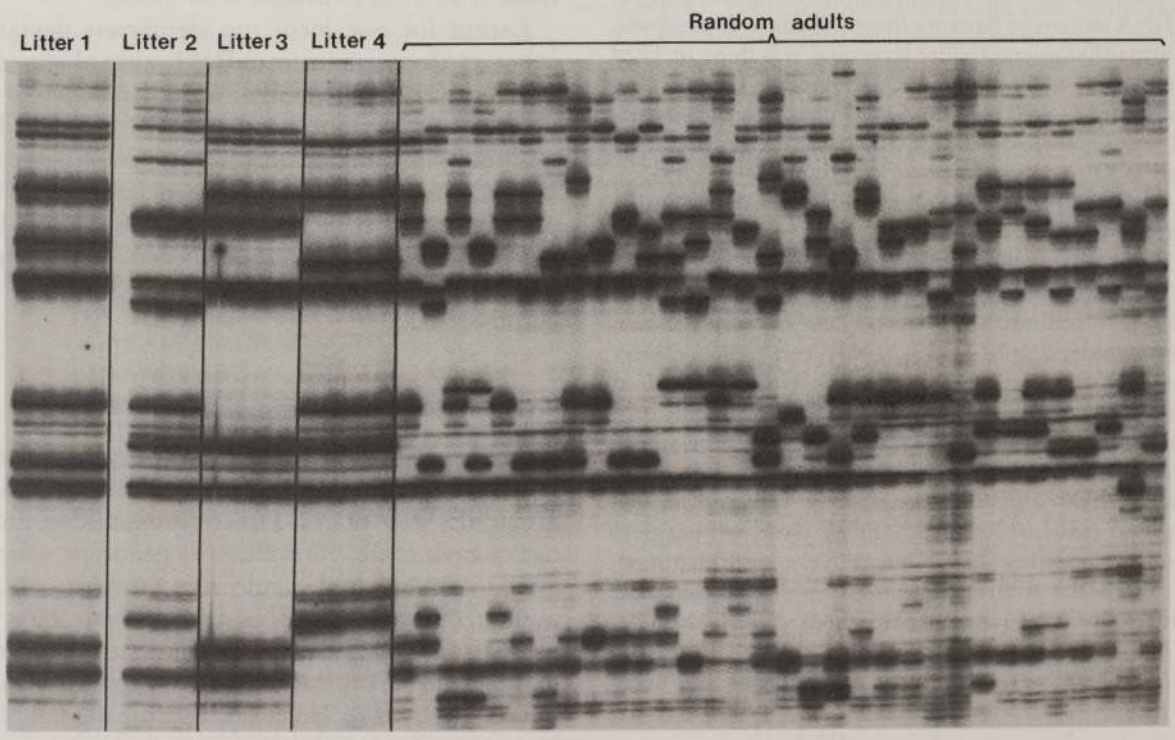

Figure 1. Autoradiograph illustrating the microsatellite profiles of four confirmed clonal sibships of armadillos (leftmost 16 lanes, one of which failed to amplify), and of other non-clonal adults randomly sampled from the Tall Timbers population (rightmost 32 lanes). Actually, this gel is a composite of all seven polymorphic microsatellite loci assayed simultaneously, using a pcr cocktail that included multiple pcr primers. This particular gel was run in this fashion for the express purpose of providing a visually convincing documentation of the polyembryony phenomenon. However, all data in the study otherwise were gathered by assay of single microsatellite loci, one at a time.

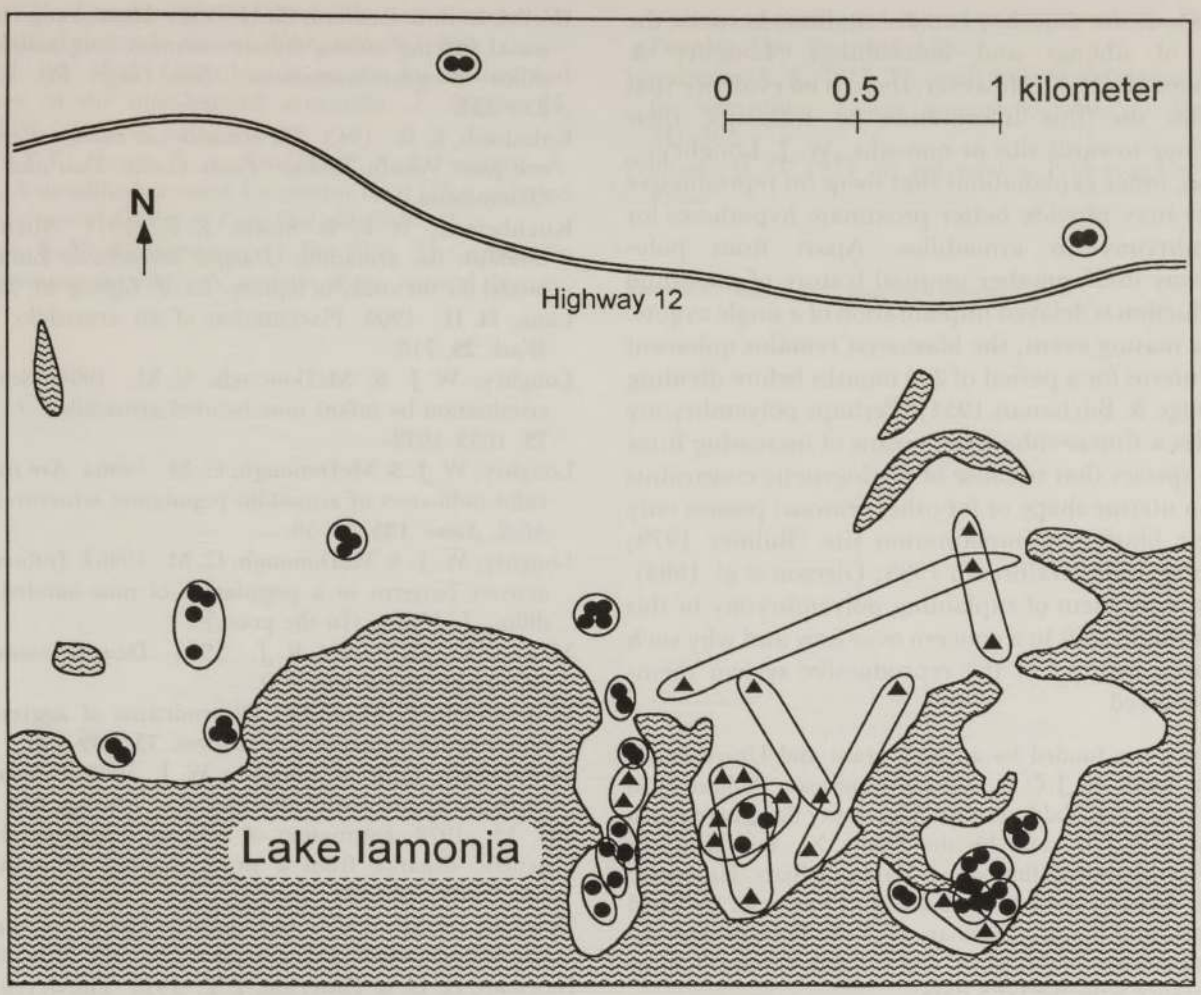

Figure 2. Capture sites for clonal siblings of nine-banded armadillos, as identified by microsatellite markers, at the Tall Timbers Research Station in northern Florida. Enclosed by ellipses are 18 juvenile sibships (dots) and seven adult sibships (triangles) represented among the total of 310 armadillos genetically characterized in the study. Other non-sibling armadillos (not shown) were distributed across many of the same areas, sometimes spatially interspersed with the clonal siblings (particularly so for the more widely-dispersed adults).

61.2 m respectively) were not statistically different ($F_{1,16} = 0.22$, $p = 0.64$). Spatial distributions of all sibling cohorts are summarized in figure 2.

4. DISCUSSION

These microsatellite analyses provide the first molecular–genetic documentation of regular polyembryony in the nine-banded armadillo, and indeed in any vertebrate species. Of course, our data (or any other) cannot prove that all sibships of *D. novencinctus* are clonal, but they do confirm a prevalence of polyembryony in this species.

Constitutive polyembryony is unusual in the animal world, and perhaps best known in several taxonomic families of parasitoid Hymenoptera (Ode & Strand 1995). In some polyembryonic species in the wasp family Encyrtidae, asexual clone mates exhibit extreme altruism, foregoing their own reproduction and devoting their short lives (they die before pupation) to defending sexual but genetically identical sibs (Cruz 1981). Such parasitoids illustrate two classes of evolutionary considerations, potentially independent but perhaps sometimes operating in conjunction, that might be entertained for the origin and maintenance of constitutive polyembryony: (i) a special ecological opportunity for single zygotes to profit from polyembryonic divisions (in this case, a single parasitoid egg is laid into a small host egg that later develops into a larger caterpillar food source); and (ii) an opportunity for kin selection via collaborative efforts among the genetically identical sibs (early-developing polyembryonic offspring defend the food supply against invasion from unrelated parasitoids).

For armadillos, it remains unclear as to how the ecological setting or natural history of this relatively asocial omnivore might favour polyembryony more so than for other terrestrial mammals. It also remains unclear how potential altruism among sibs might be expressed. Our data on the spatial dispersion of clone mates as a function of age suggest that armadillo siblings do not remain together throughout life, nor generally maintain especially tight spatial associations from which they theoretically might profit (e.g. from collaborative foraging, predator avoidance or territorial defense). Furthermore, post-nursing individuals display no obvious cooperative behaviours (McDonough 1994; McDonough & Loughry 1995) although burrow sharing has been observed (Kalmbach 1943; Taber 1945; Herbst & Redford 1991). Our data suggest that sibling nine-banded armadillos remain spatially close as juveniles, and earlier exper-

iments indicate that they can discriminate between the scents of siblings and non-siblings (Loughry & McDonough 1994). However, there is no evidence that juveniles use this information to influence their behaviour towards sibs or non-sibs (W. J. Loughry).

Thus, other explanations that focus on reproductive biology may provide better proximate hypotheses for polyembryony in armadillos. Apart from polyembryony itself, another unusual feature of armadillo reproduction is delayed implantation of a single zygote. After a mating event, the blastocyst remains quiescent in the uterus for a period of 2–4 months before dividing (Talmage & Buchanan 1954). Perhaps polyembryony provides a fitness-enhancing means of increasing litter size in species that because of phylogenetic constraints (e.g. on uterine shape or for other reasons) possess only a single blastocyst implantation site (Bulmer 1979; Eisenberg 1983; Galbreath 1985; Gleeson *et al.* 1994). If so, the problem of explaining polyembryony in this species would shift to a concern over how and why such associated features of the reproductive system themselves evolved.

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