A Genetic Analysis of the Virginia Opossum Mating System: Evidence of Multiple Paternity in a Highly Fragmented Landscape

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Using molecular techniques, we examined patterns of paternity in Virginia opossums occupying a highly fragmented agricultural landscape in northern Indiana. During 2008, we collected tissue from 64 females and their pouch young in 34 forest patches distributed over a 1100-km^2 region. Using genotypes from 10 microsatellite loci, we determined the minimum number of fathers contributing to each litter using GERUD 1.0. Genotyped offspring with known mothers were then analyzed using CERVUS 3.0, incorporating genotypes from 317 males sampled from 2007–2008 to identify potential fathers. Our analyses revealed that promiscuity was common among females, with 26 (41%) litters having ≥2 sires. Despite the fact that we intensively sampled forest patches for potential fathers, we only were able to identify 13 fathers contributing to 14 litters, with an average Euclidean distance of 18.7 km between father–offspring pairs found in disparate patches (N = 6). Our inability to identify most (85%) fathers of sampled litters, coupled with the extensive distances observed between putative father–offspring pairs, suggests that opossums may not maintain explicit home ranges in highly fragmented landscapes.

Key words: CERVUS, GERUD, marsupial, microsatellite, movement, reproduction

Field studies investigating the mating strategies of mammals often result in ambiguous or erroneous assessments as copulations rarely are observed for most nocturnal or solitary species. However, the application of molecular techniques to the study of animal mating systems has facilitated our ability to elucidate the social and reproductive strategies of many wildlife species. One product of the application of molecular techniques to ecological research that has greatly informed our understanding of the ecology and evolution of animal mating systems is evidence of female promiscuity. Current research suggests that multiple paternity within litters is relatively common throughout the animal kingdom, occurring in species ranging from those that are polyandrous to species which are socially monogamous (Birkhead and Moller 1992; Kraaijeveld-Smit, Ward, and Temple-Smith 2002; Wolff and Macdonald 2004).

Among mammals, marsupials are particularly amenable to genetic parentage studies as entire litters can easily be sampled by capturing females with pouched young. For several species of marsupials, female promiscuity is known or suspected to occur (Kraaijeveld-Smit, Ward, and Temple-Smith 2002; Holleley et al. 2006; Alistair et al. 2009); however, multiple paternity in Virginia opossums (*Didelphis virginiana*), the only marsupial present in North America, has yet to be examined. Unlike many species of marsupials, Virginia opossums have thrived in a region almost exclusively dominated by placental mammals and currently range from southern Canada to northern Costa Rica (Gardner and Sunquist 2003). Despite their relative abundance, few data exist regarding the mating strategies employed by this species as opossums primarily are nocturnal and socially solitary (Ryser 1995; Gardner and Sunquist 2003).

Results of radiotelemetry studies suggest that Virginia opossums likely employ a promiscuous mating system, with males maximizing their reproductive success by mating with as many females as possible (Ryser 1992). Based on visual observations of interactions between radiocollared males proximal to estrous females, Ryser (1992) concluded that male mating success presumably was positively associated with body size for Virginia opossums. Indeed, within marsupials there appears to be a strong selective force on male size as male mating success is correlated with body size for many species (Kraaijeveld-Smit, Ward, and Temple-Smith 2002; Clinchy et al. 2004; Holleley et al. 2006; Alistair

et al. 2009). However, given that age is positively correlated with body size for many marsupials it is unclear whether increased mating success in large individuals ultimately is an effect of body mass, age, or the interaction of these characteristics. Female opossum mating strategies are more ambiguous, however, as copulations rarely are observed in wild populations. It is thought that females mate only once during a single estrous cycle (Reynolds 1952), although up to 3 males have been observed simultaneously accompanying a single female during the reproductive season (Ryser 1992).

This is the first paper to evaluate the mating system of Virginia opossums using molecular techniques. Using microsatellite markers developed in our laboratory (Fike et al. 2009), our primary goal was to determine whether multiple sires existed within single opossum litters. Second, we attempted to assign paternity of sampled offspring based on extensive sampling of male opossums present within forest patches where female–offspring pairs were captured and to evaluate whether male reproductive success was associated with body mass. Finally, we used data on assigned paternity to make inferences regarding the movement behavior of this species in a highly fragmented agricultural landscape.

Materials and Methods

Study Area

Our 1165-km^2 study area was located in the upper Wabash River basin (UWB) in northcentral Indiana, USA. Agriculture (primarily corn and soybeans) was the dominant land use in the UWB, comprising 71% of the available land area. Only 13% of the basin was forested, with all contiguous forest tracts confined to major drainages where frequent flooding or locally steep topography made the land unsuitable for crop production. The remaining native forests (predominantly oak–hickory–maple [*Quercus–Carya–Acer*]) in the basin were highly fragmented, with the distribution of forest patch sizes dominated by patches <5 ha (75%; Moore and Swihart 2005).

Sample Collection and Laboratory Methods

Trapping was conducted from mid March through early June (2007 and 2008) in 60 forest patches distributed throughout the study area. A detailed outline of the trapping methods are described elsewhere (Beasley and Rhodes 2008). Briefly, opossums were captured using Tomahawk live traps (Tomahawk Live Trap Co, Tomahawk, WI) baited with commercial cat food. Traps were placed in a grid (50-m spacing) and maintained for 10 consecutive nights. Captured opossums ≥10-months old were ear-tagged (Monel no. 3, National Band and Tag Company, Newport, Kentucky), sexed, weighed, and a tissue sample (ear biopsy) was taken for genetic analysis. In addition, during 2008, all female opossums with young >2″ in length present within their marsupium were immobilized with an intramuscular injection of Telazol at a rate of 5 mg/kg of estimated body mass, and tissue was collected (tail biopsy) from each offspring. Maternity was thus assumed unambiguous because offspring tissue samples were collected prior to pouch emersion. Offspring <2″ in length were not sampled to minimize any impacts of tissue collection on future development. Only offspring sired during the early (first) breeding season were sampled in this study, as offspring sired during the late (second) breeding season are not present within the marsupium until mid June in northern Indiana. Tissue samples were preserved in 95% ethanol in the field and then transferred to an ultra low freezer (−80 °C) for long-term storage. All trapping and handling methods conformed to Purdue University Animal Care and Use Committee policies under Protocol 01-079.

DNA was extracted from tissue samples using an ammonium acetate protocol (modified from the purgene kit; Gentra Systems). Thirteen microsatellite loci (OP03, OP08, OP14, OP16, OP18, OP19, OP30, OP36, OP39, OP41, OP42, OP46, and OP48; Fike et al. 2009) were amplified via multiplex polymerase chain reaction and electrophoresed on an ABI 3730 (Applied Biosystems, Foster City, CA). Specific amplification conditions and annealing temperatures for each locus are provided in Fike et al. (2009). Genotypes were manually assigned with GeneMapper v3.7 (Applied Biosystems). All laboratory techniques were employed using quality control measures, standard for our laboratory, and we ensured that the overall genotyping error rate and overall levels of missing genotypic data were <1% (see Latch and Rhodes 2005 for quality control details).

We used CERVUS 3.0 (Kalinowski et al. 2007) to calculate observed and expected heterozygosities, numbers of alleles/locus, and allele frequencies for our study population. Based on the constraints of our quality control measures and tests for Hardy–Weinberg equilibrium (HWE), the loci OP30 and OP41 were removed from all paternity analyses. In the case of OP41, we detected a significant deficit of heterozygotes, indicating a violation of the assumption of HWE, whereas genotyping error rates exceeded 3% for OP30. The remaining 11 loci (OP03, OP08, OP14, OP16, OP18, OP19, OP36, OP39, OP42, OP46, and OP48) were used in subsequent statistical analyses.

Statistical Analyses

We used the computer program GERUD 1.0 (Jones 2001) to determine the minimum number of males contributing to each mother–offspring array. Because GERUD uses exclusion to estimate the number of male genotypes contributing to a given progeny array, estimates using this program are considered conservative and should never overestimate the number of sires for a litter (Jones 2001). Using known maternal genotypes, GERUD calculates the minimum number of fathers contributing to a given progeny array by subtracting the known maternal alleles from offspring genotypes, simulating all possible paternal genotypes, and determining the combinations of the remaining
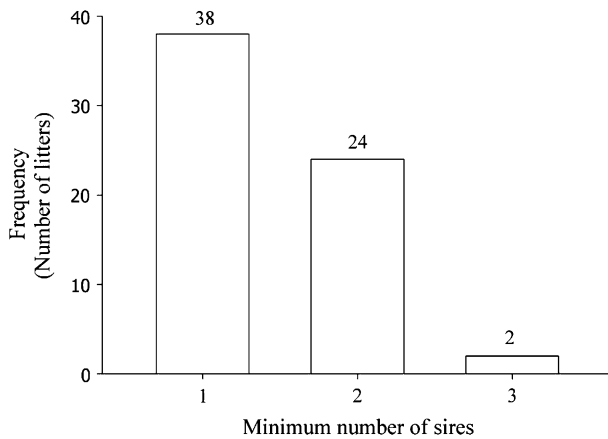
Figure 1. Frequency of multiple paternity in litters of Virginia opossums sampled in northern Indiana, USA, 2008. The mean number of sires within litters was 1.44, SE = 0.070.

alleles that yield the fewest possible sires (Jones 2001). Currently, GERUD is only capable of using data from a maximum of 10 microsatellite loci, and in some instances, analyses employing >6 loci can exceed the computational limitations of this program, depending on the allele frequencies of mother–offspring arrays.

To maximize our ability to identify multiple fathers within litters, we used the 10 most polymorphic loci or the maximum number of loci GERUD would allow for each mother–offspring array (range: 6–10, $\bar{X}$ = 9.23). For example, if a given mother–offspring array exceeded the computational capacity of GERUD using 10 loci, we systematically excluded the least polymorphic loci until a result was achieved. Ultimately, 50 (78%) mother–offspring arrays were run using ≥9 loci in our paternity analyses and only 1 array was run using only 6 loci. Within these constraints, we performed an exhaustive search of the number of possible combinations of fathers that could explain each progeny array.

To evaluate the power of our microsatellite loci to detect multiple paternity, we used the program GERUDsim 1.0 (Jones 2001). Using the populations observed allele frequencies, GERUDsim simulates sets of offspring genotypes based on user specified litter sizes (in our case 10), draws a sample of offspring, and then estimates the number of sires present in each litter. We ran 1000 iterations of the simulation, each using a single multilocus maternal genotype and up to 3 randomly generated paternal multilocus genotypes (based on observed levels of multiple paternity) to evaluate the probability of correctly determining the number of fathers within litters. Simulations were conducted using the 5 most polymorphic loci as simulations using >5 loci exceeded the computational limitations of GERUDsim.

Genotyped offspring with known mothers were then analyzed using CERVUS 3.0 (Kalinowski et al. 2007) to identify potential fathers from within our sampled patches. All males sampled during 2007 (162) and 2008 (155) were incorporated into CERVUS as potential sires. Male opossums are capable of reproducing at ∼8 months of age (Reynolds 1952; Biggers 1966). Thus, given that sampling occurred during the spring when only individuals ≥10-months old were present within the population, all sampled males were considered as potential fathers.

To generate a distribution of logarithm of the odds (LOD) scores for use in discriminating among nonexcluded males, we ran simulations in CERVUS using 10 candidate males and estimated the proportion of candidate fathers sampled as 25%; although additional analyses in which these parameters where altered did not alter our results. Parameter estimates for use in the simulation were based on the average number of males captured per forest patch in a concomitant study occurring in the same landscape (i.e., average of 2.5 per patch), and the number of patches (4) we assumed male opossums likely utilized in our landscape based on previous mark–recapture experiments (Beasley JC, unpublished data). We ran 100 000 simulations using 1 known parent (mother), assuming a 1% (observed) genotyping error rate, and assigned paternity at both 95% and 99% confidence levels (Marshall et al. 1998). For each offspring, paternity was assigned based on the candidate father with the highest LOD score. However, as a conservative estimate of the number of fathers identifiable within our forest patches, only fathers assigned to offspring with no mismatches at all 11 loci were considered true fathers. In all instances where candidate fathers were assigned offspring mismatching at 1 locus, we regenotyped assigned fathers, mothers, and offspring to confirm genotypes.

Results

We captured and successfully genotyped 317 adult male opossums during 2007 (162) and 2008 (155) from within our 60 study patches. During 2008, we captured and sampled 67 mother–offspring litters consisting of 596 offspring. Of these, we successfully genotyped 64 mothers and 572 offspring from 34 forest patches. Mean litter size of females was 8.71 (standard error [SE] = 0.25).

The 11 loci used in our analyses exhibited high levels of polymorphism (11.18 alleles/locus; range: 7–16), with mean observed and expected heterozygosities of 0.793 and 0.796, respectively. Our paternity analyses revealed that female promiscuity was common across sampled patches, with 26 (41%) litters having ≥2 sires ($\bar{X}$ = 1.44, SE = 0.07; Figure 1). Of those litters identified as having >1 sire, 24 had a minimum of 2 sires and 2 had at least 3 sires.

In 100% and 99.5% of the 2 and 3 father simulations performed in GERUDsim, respectively, the reconstructed number of sires equaled the number assigned by the program and in no circumstances was the number of real fathers overestimated by the heuristic search algorithm employed by GERUDsim. Given that our GERUDsim simulations revealed ∼100% accuracy in assignment of the minimum number of potential fathers using only 5 loci and
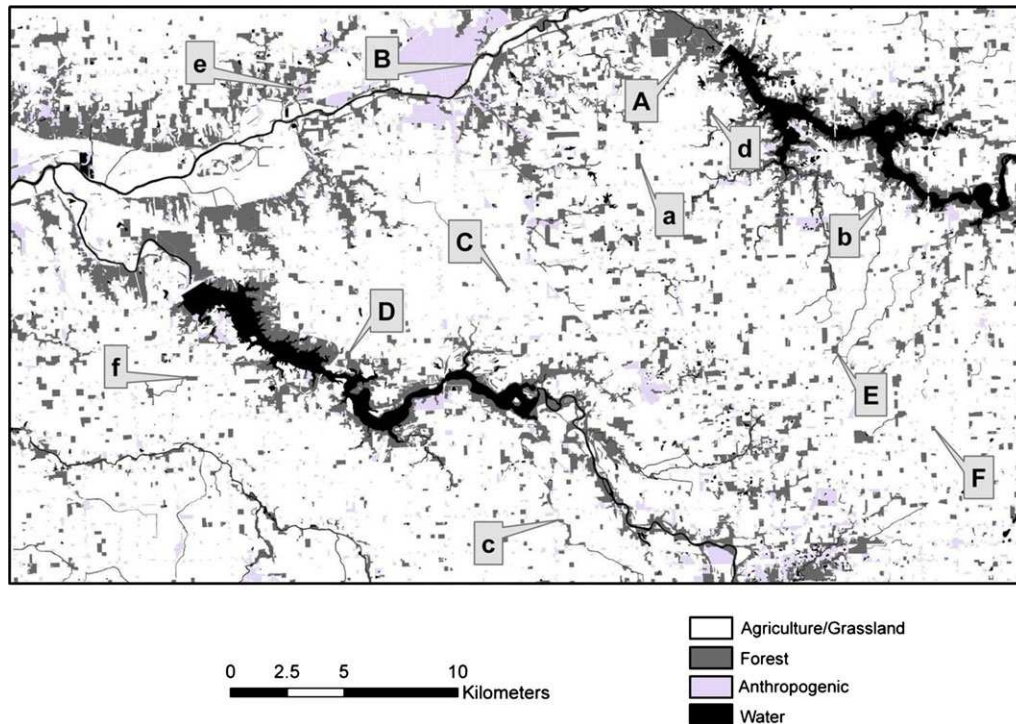
Figure 2. Distribution of all father–offspring pairs identified using Cervus 3.0 that were not present within the same forest patch. Matching letters represent father–offspring pairs, with capital letters representing the location of sires and lowercase letters representing mother–offspring locations, for individuals sampled in northern Indiana, USA in 2007–2008.

the fact that accuracy in GERUDsim is positively associated with the number of loci used (Jones 2001), it is likely that our estimates of the minimum number of males contributing to litters in GERUD are conservative (given that an average of >9 loci were used for these analyses and no litters were run with <6 loci).

Of the 572 offspring evaluated for evidence of paternity, CERVUS identified 13 unique fathers for 14 litters (1 male sired 2 separate litters) with no mismatches at any loci from within our sampled population of 317 males. All candidate fathers were assigned parentage with 99% confidence. Of those fathers identified as having contributed to litters, 8 (62%) were captured in the same patch as their assigned mother–offspring pair, all of which were captured in the same year as their assigned offspring. For those fathers not captured in the same patch as their putative offspring, the average Euclidean distance between the original capture sites for known father–offspring pairs was 18.7 km (range 5–33 km; Figure 2). However, of the 6 sires which were not captured in the same patch as their assigned offspring, 4 were initially tagged during 2007 (the year prior to sampling of mother–offspring pairs) but were not captured in 2008.

Due to the fact that we only were able to identify 14 fathers of sampled litters, we felt it was inappropriate to conduct further statistical analyses on our paternity data to explore the relationship between paternity success and male body size.

Discussion

The results of this study indicate that multiple paternity is common in litters of wild Virginia opossums and that both sexes employ a promiscuous mating strategy. Over the last decade multiple paternity has been reported in several species of marsupials, suggesting that female promiscuity is an important aspect of the reproductive ecology for this infraclass of mammals (Taylor et al. 2000; Wooller et al. 2000; Kraaijeveld-Smit, Ward, and Temple-Smith 2002; Holleley et al. 2006; Alistair et al. 2009). For many species of mammals promiscuity in females likely evolved as a means of confusing paternity in order to minimize infanticide by males (Wolff and Macdonald 2004). However, in marsupials male infanticide likely is uncommon as young emerge from the marsupium as precocial and relatively mobile infants (Wolff and Macdonald 2004). Nonetheless, many species of marsupials clearly exhibit female promiscuity and thus, alternative evolutionary benefits (e.g., increased litter sizes) or proximal behaviors (e.g., sexual coercion) likely exist for promiscuity to be maintained in this group.

For Virginia opossums it is unknown if alternative benefits explain the development and maintenance of female promiscuity; however, this mating strategy can facilitate increased litter sizes, (Hoogland 1998; Kraaijeveld-Smit, Ward, and Temple-Smith 2002), increased genetic diversity within litters (Madsen et al. 1992; Tregenza and Wedell 2000), and increased genetic compatibility between maternal

and paternal gametes (Kraaijeveld-Smit, Ward, and Temple-Smith 2002; Kraaijeveld-Smit, Ward, Temple-Smith, and Paetkau 2002). Alternatively, female promiscuity may simply be a function of male sexual coercion, with no direct benefit to females and potential fitness or evolutionary costs to those individuals who mate with multiple males (Clutton-Brock and Parker 1995; Head and Brooks 2006). Nonetheless, our finding that females commonly mate with multiple males suggests that sexual selection could be occurring within opossum populations at multiple biological scales: 1) direct competition among males for access to females and 2) competition among sperm from disparate males within the reproductive tract of females.

Previous research indicates that competition among male opossums is extensive with male mating success presumably tied to body mass (Ryser 1992). Given the brief period of fertility (12 h) within the estrous cycle of female opossums (Reynolds 1952), competition among males for access to receptive females undoubtedly is extensive and thus, sexual selection favoring large body mass would not be surprising for this species. In highly fragmented landscapes such as the UWB, competition among males likely is magnified as a result of the brief female receptivity period coupled with the patchy distribution of females. Consequently, our estimates of the rate of multiple paternity for opossums occupying a fragmented agricultural landscape may be an underestimate when compared with populations in more contiguous habitats (Banks et al. 2005).

A rather interesting finding of this study was that despite our intensive sampling regime within local patches, we only were able to capture 14 fathers (out of the possible 92 fathers estimated to have contributed to our 64 litters). Although the lack of identifiable fathers could be due to an inability to capture males present within sampled patches, we believe this is unlikely due to our intensive trapping effort (i.e., traps were placed at a density of 6 traps/ha and maintained for 10 consecutive nights). Moreover, opossums are highly susceptible to trapping during the spring when our sampling occurred, and population estimates for our study patches indicate that at least 69% of each local population is captured during a 10-day sampling period (Beasley JC, unpublished data).

Due to the large size of our study area and logistical constraints of trapping, a number of habitat patches were not sampled which, combined with opossum movements extending beyond the effective area of our trapping grids, likely contributed significantly to the limited number of fathers detected. High levels of male mortality prior to sampling also could have contributed to our inability to identify fathers of litters. However, this is highly unlikely as all sampling occurred <2 months postcopulation, male capture success remained unchanged throughout the trapping season, and no evidence of male mortality was observed within sampled patches while trapping occurred.

Thus, our inability to identify most (85%) fathers of sampled litters, coupled with the extensive distances observed between putative father–offspring pairs, suggests that opossums may not maintain spatially explicit home ranges in highly fragmented landscapes such as the UWB as they do in other landscapes, presumably due to the patchy distribution of resources (e.g., food, water, den sites, females, etc.). However, these extensive ranging patterns actually may reflect ultimate rather than proximate movement behaviors as we observed evidence of short-term home range fidelity given that 8 of the 14 assigned fathers were still present within the same patch as their assigned mother–offspring pair 1–2 months postcopulation.

Nonetheless, long-term mark–recapture data from our study area support our supposition of extensive ranging behavior as movements of up to 24 km within a 2-week period have been observed for individuals ≥11-months old, and only a fraction (<1%) of the 579 opossums marked in long-term control patches in our landscape have been captured in the same patch during subsequent year(s). Dispersal, which is male biased in opossums, occurs from late summer up until the onset of the breeding season in February as both sexes are sexually mature by 8 months of age (Gardner and Sunquist 2003). Although 4 of the 6 identified fathers (captured outside the patch where their assigned offspring were sampled) were initially captured during the year prior to the capture of their putative offspring, it is unlikely that the extensive distances observed between putative father–offspring pairs resulted from male dispersal during the year prior to offspring sampling as all males were sampled subsequent to their first breeding season and thus already should have dispersed (Wright et al. 1995; Gardner and Sunquist 2003).

Previous research on Virginia opossums is ambiguous with regards to the movement behavior of this species. Early researchers proposed that opossums were nomadic or solitary wanderers with unstable home ranges (Lay 1942; Reynolds 1945), whereas recent studies using radio transmitters have identified well-defined home range boundaries (Gillette 1980; Ryser 1995; Gipson and Kamler 2001). Differences in resource distribution, opossum density, and abundance of predators/competitors undoubtedly exist among all the aforementioned studies. Thus, it is possible that incongruities among previous studies on this species may be a product of varying ecological attributes among study sites rather than advances in animal monitoring technology alone. Future research should evaluate whether opossums indeed have adopted unique movement and reproductive behaviors in disparate landscapes, and if so, what underlying ecological factors are driving these incongruent behaviors (e.g., patchy distribution of resources, competition with other mesopredators, and distribution of females).

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