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MATING, KINSHIP, AND POPULATION STRUCTURE IN ILLINOIS BEAVER POPULATIONS

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Mating, Kinship, and Population Structure in Illinois Beaver Populations

(TITLE)

BY

Joanne C. Crawford

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science in Biological Sciences

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

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INTRODUCTION

The North American beaver, *Castor canadensis*, is an important wildlife species for a variety of reasons. As a furbearer, it provides income and recreation to trappers throughout the state. As a wetland engineer, it helps maintain functioning ecosystems (Naiman et al. 1986, McKinstry and Anderson 2002). Finally, the beaver serves as a model organism for studies of mating and kinship, as it has beed reported to be almost exclusively monogamous (Sun 2003).

Monogamy in mammals is generally restricted to the primates, canids, and rodents, and is defined as a mating system in which a pair remains together for at least one breeding season (Kleiman 1977, Reichard 2003). Beavers most often are found living in discrete colonies composed of a mated adult pair and their offspring from the previous 2-3 breeding seasons (Bradt 1938, Novak 1977, Busher et al. 1983, Svendsen et al. 1980, Sun 2003). Svendsen (1989) has reported that beaver pairs remained together for an average of 2.5 years. Most pairs consisted of an older beaver paired with a younger one, and termination of the bond occurred upon the death of the older mate. The duration of pair bonds is highly variable among monogamous taxa. For example, the Malagasy giant jumping rat (*Hypogeomys antimena*) forms long-term associations that last until the death of a partner (Sommer 2003). The California mouse (*Peromyscus californicus*) and the prairie vole (*Microtus ochrogaster*) also form long-term pair-bonds and, like beavers, exhibit many behavioral characteristics associated with a monogamous mating system (Lonstein and De Vries 2000). In contrast, some avian species form pairs that last only one breeding season (Birkhead and Møller 1995).

Although monogamy is more frequently observed in avian species, molecular studies

in the past two decades have revealed that many socially monogamous birds are in fact not genetically monogamous and individuals will engage in extra-pair mating (Birkhead and Møller 1995). Assumptions regarding mating and parentage within beaver colonies have not been directly tested using molecular methods. Mating and dispersal behavior can have a significant influence on a population's genetic structure (Emlen and Oring 1977, Kleiman 1977, Bohonak 1999). For the most part, past research on beavers suggests equal dispersal rates between the sexes (Sun 2003). Such a pattern of dispersal is expected for monogamous species and may maintain within-population genetic variation, but limit overall variation among populations depending on dispersal ability (Bohonak 1999).

In addition to being an interesting and rare example of social monogamy, the beaver is also considered an ecosystem engineer because of its ability to shape wetland habitats through dam and lodge construction, and food acquisition. These activities have been shown to significantly modify wetlands; altering stream flow, water chemistry, sediment load, and vertebrate and invertebrate species composition (Naiman et al. 1986, Wright et al. 2002). Although beavers influence species diversity and ecosystem function and are used in habitat restoration, they also may be regarded as a nuisance species (Payne and Peterson 1986, McKinstry and Anderson 1999, Jensen et al. 2001, Wright et al. 2002). Beavers prefer deep pools of water around lodges (Havens 2006) and will attempt to dam free-flowing water to create these pools. This may result in blocked culverts, flooding of adjacent fields and roads, and crop destruction (Payne and Peterson 1986, Jensen et al. 2001). In an effort to limit human-wildlife conflicts, much of the research on the species to date has focused on its population structure and control (Peterson and Payne 1986,

Busher and Lyons 1999, Müller-Schwarze and Schulte 1999).

Beavers were trapped to near-extinction throughout the U.S. following the arrival of Europeans. Since the late 1800's when trapping pressure was reduced, populations had recovered to an estimated 20 million animals by the 1980's (Naiman et al.1986). Illinois beaver populations followed a similar pattern. They were nearly extirpated from the state by 1900 due to overharvest, with only a few remnant populations in the southernmost parts of the state (Pietsch 1956). Beavers were largely absent from the state by 1900, with only a few remnant populations in the southernmost parts of the state. Between 1929 and 1938, 46 beavers from Wisconsin were reintroduced to northern Illinois (Jo Daviess and Carroll Cos.) and southern Illinois (Union, Pope Cos.; Pietsch 1956). Since these reintroductions, populations have expanded and become established throughout the state (Pietsch 1956, Woolf et al. 2003).

During the past decade, Eastern Illinois University and Southern Illinois University partnered with the Illinois Department of Natural Resources to produce a large body of research on beaver ecology in Illinois. Beginning in 2000, Woolf et al. (2003) estimated colony density within southern watersheds using a block-sampling aerial survey method. Of 8 southern watersheds (Bay Creek, Big Muddy, Cache, Embarras, Kaskaskia, Little Wabash, Saline, and Vermilion) 43% of blocks surveyed were occupied. In addition, the highest density of colonies was reported in the Embarras watershed in central Illinois and Big Muddy watershed in southern Illinois (Woolf et al. 2003). In a second study on colony composition, McTaggart and Nelson (2003) found colonies in the Embarras watershed averaged 5.6 beavers/colony and suggested that colonies containing more than 2 adults (43%) may indicate high ecological densities that limit natal dispersal. Cox

(2005) also found the Embarras to have a high colony density (0.40 colony/km) and developed a multiple regession model using habitat characteristics to estimate colony density throughout the watershed.

Finally, three studies of movements and dispersal were completed in southern and central Illinois. In southern Illinois, research conducted in Union County in both landlocked lacustrine sites and wetland complexes showed that nearly 75% of 3-year-olds and 55% of 2-year-olds dispersed (McNew and Woolf 2005). Mean juvenile dispersal distances were lower in landlocked sites (1.7 km) than in wetland complexes (5.9 km). In contrast, dispersal behavior is different in the long, linear streams of the Embarras River watershed. Cleere (2005) and Havens (2006) reported much lower rates of dispersal for both 2-year-olds and 3-year-olds, but greater dispersal distances in this linear habitat. These results suggests that dispersal and population structure may be influenced by landscape characteristics such as the connectivity and spatial distribution of aquatic habitats.

While these studies have produced valuable insights into the ecology and population dynamics of beavers in Illinois, no previous genetic research has been conducted on these populations. These previous studies prompted questions regarding the social structure of beavers that only could be addressed using contemporary genetic techniques. Therefore, I chose to investigate genetic relatedness within and among beaver populations in Illinois to better elucidate the: 1) occurence of extra-pair matings within colonies through genetic parentage analysis, 2) average relatedness within colonies, 3) relationship between relatedness and geographic distance between colonies, and 4) genetic differentiation between populations in central and southern Illinois.

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CHAPTER 1. ISOLATION AND CHARACTERIZATION OF MICROSATELLITE LOCI NORTH AMERICAN BEAVERS.

ABSTRACT

Molecular markers are currently lacking for many species, including the North American beaver (*Castor canadensis*). Here, I describe the isolation and characterization of 9 polymorphic microsatellite markers in this species. Sixty individuals from southern and central Illinois were screened at each locus. All loci exhibited moderate levels of polymorphism, ranging from 5-13 alleles per locus, with average heterozygosity ranging from 0.317 to 0.867. Locus Cca5 deviated significantly from HWE ($p < 0.001$). The locus pair Cca4/Cca5 was shown to be in linkage disequilibrium in southern Illinois, but not in the central Illinois population. The remaining 8 loci will be useful in investigations of mating and kinship patterns in Illinois beaver populations.

INTRODUCTION

Following a dramatic population decline due to over-harvest in the 1800's, North American beaver (*Castor canadensis*) populations have recolonized many steams and wetlands in the U.S (Naiman et al. 1986). This important furbearer is considered a keystone species because of its ability to modify wetland habitats and alter species composition (Wright et al. 2002). Research on this species has primarily focused on the benefits to riparian ecosystems as a result of beaver activity and the associated damage to human environments (Payne and Peterson 1986). While ecologically important, the beaver can be a nuisance species and much of the research to date has focused on the species' social organization and reproductive potential in an effort to better understand

and control this animal in human-dominated landscapes (Schulte and Müller-Schwarze 1999).

Beavers have historically been reported to be monogamous, inhabiting discrete colonies consisting of a mated adult pair and their offspring (Sun 2003). However, this characterization of mating behavior has been based solely on observational field studies (Sun 2003). To date, DNA-based parentage methods, although widely used in other species, have not been utilized to confirm the observed mating system. As one of the few non-primate monogamous mammals, molecular studies of parentage and kinship would provide insights into the social and environmental factors under which monogamy is favored in this species. Prior to this project, microsatellite markers had not been identified in the North American beaver; however, primers were obtained for the Eurasian beaver (*C. fiber*) (H. Ellegren, Uppsala University, Sweden). Eight markers (loci B3, B4, B12, B16, B18, B20, B34, B134) were tested in *C. canadensis*, but all failed to produce a PCR product under a variety of amplification conditions. Because of this, I produced an enriched library of microsatellite DNAs from beaver populations in Illinois and developed microsatellite primers that would be useful for parentage testing in the North American beaver.

MATERIALS AND METHODS

Whole genomic DNA from a male beaver was isolated using a commercial kit (Promega Inc) and used to construct an enriched genomic library following the protocol of Glenn and Schable (2005). Briefly, 200 ng of whole genomic DNA was digested with *Rsa*I, and SNX linkers were ligated to DNA fragments. Enrichment of microsatellite fragments was carried out using streptavidin-coated beads (Dynal Biotech) and a mix of

biotin-labeled probes, including $(TG)_n$, $(AG)_n$, $(ACT)_n$, and $(AAG)_n$. A total of 10 μl of linker-ligated DNA was hybridized to 10 μl of oligonucleotide probe (1 μM each) in 25 μl 2 x Hyb solution. Following hybridization, 50 μl of washed Dynabeads were added to the DNA-probe mixture and incubated sideways on an orbital shaker for 30 min at room temperature. To remove unbound DNA, beads were washed four times using 400 μl 2x SSC, 0.1% SDS, and two additional times using 400 μl 1x SSC, 0.1% SDS. Bound, single-stranded fragments were isolated by ethanol precipitation and amplified to double-stranded form. Amplified fragments were inserted into pCR 2.1 vector and transformed into cells using a TOPO TA Cloning Kit (Invitrogen). Positive colonies were amplified using M13 forward and reverse primers in colony PCR and sequenced on an ABI 3730XL using Big Dye 3.1 cycle sequencing chemistry (Applied Biosystems) at Purdue Genomics Core Research Facility (http://www.genomics.purdue.edu/~core/).

Fifty sequences were chosen for primer design. Primer pairs were designed using the program Primer3 (http://frodo.wi.mit.edu/). Of the 50 primer pairs designed, only 20 unlabeled pairs were tested by PCR due to time constraints. Reactions were carried out in 25 μl volumes containing 50-100 ng DNA, 1x PCR buffer, 200 μM each dNTP, 0.3 μM each primer, 3.5 mM $MgCl_2$ and 1U *Taq*. Following a 5 min initial denaturation at 95°C, amplification consisted of 36 cycles at 95°C for 30 s, 30 s at the annealing temperature (Table 1), 2 min at 72°C, followed by a final extension step at 72°C for 1 hr. Of the twenty pairs tested, six failed to amplify a product and four amplified multiple products. Forward primers for the remaining ten loci were labeled with Well-Red fluorescent tags (Sigma-Aldrich) and screened on a CEQ8800 (Beckman Coulter).

RESULTS AND DISCUSSION

A total of 60 beavers from southern (n = 30) and central Illinois (n = 30) were screened for polymorphism at each locus (Table 1). All loci were polymorphic, ranging from 5-13 alleles/locus and observed heterozygosities ranging from 0.317 to 0.867. The program CERVUS was used to test for Hardy-Weinberg equilibrium and null alleles (Marshall et al. 1998). Most loci were in HWE; however, locus Cca5 deviated significantly due to heterozygote deficiency and an estimated null allele frequency of 0.340 ($p < 0.001$). Linkage disequilibrium tests with Bonferroni correction were conducted using Genepop version 3.4 and identified disequilibrium in the locus pair Cca4/Cca5 in the southern Illinois population, but this pattern was not observed in the central Illinois population. This may be attributed to both a paucity of homozygotes at locus Cca5 and an excess of closely related individuals. Mother-fetus controls also indicated the presence of null alleles at Cca4 and this could affect relatedness estimates and parentage assignment. Finally, another locus, Cca14 (GenBank Accession no. EF524507), was difficult to score without ambiguity and therefore was removed from this analysis. The remaining 7 loci show moderate levels of polymorphism and are appropriate for use in population level studies.

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Wright JP, Jones CG, Flecker AS (2002) An ecosystem engineer, the beaver, increases species richness at the landscape scale. *Oecologia*, **132**, 96-101.

Table 1 Characterization of loci isolated from *Castor canadensis*; T_a is the reaction-specific annealing temperature; A is the number of alleles; H_o is the observed heterozygosity; H_E is the expected heterozygosity; ASR indicates the approximate size range. Asterisks indicate statistically significant differences from Hardy-Weinberg equilibrium at $\alpha = 0.05$.

Locus	Primer sequence (5'- 3')	Repeat motif	T_a	A	ASR	H_o	H_E	GenBank Accession no.
Cca4	GATTTCAGACACAGCCACCA AGTGATGGGATTGAACTCCAG	$AC_{(17)}$	61	10	362-364	0.700	0.772	EF524501
Cca5	TGCTTTCATCTGCTCTATGAAAAT CAGTGATGAAGGGAAGAGGAA	$CT_{(21)}$	61	11	157-185	0.317	0.621*	EF524502
Cca8	GGGCTCAGAGGAAAAAGGAG GATCAGGCAAAAGGCTGGTA	$GATA_{(12)}$	61	10	356-426	0.800	0.837	EF524503
Cca9	TCTTTCTTGTTGGTCCTGGAA TGGGAGAGTGGTTGCCTATC	$TG_{(21)}$	60	10	136-156	0.767	0.753	EF524504
Cca10	TTTTGTTGGGAAATATGCTGTT TGCAGAACAAAGAAAATATTGAAAG	$TC_{(19)}$	60	13	120-154	0.833	0.862	EF524505
Cca13	CCCTAGACTTTGATTATACGG AGGTTGCCTAGAGAGAGGTGTG	$GT_{(11)}GT_{(7)}$	60	6	277-295	0.450	0.481	EF524506
Cca15	TCTGCCTTATGTGATGGTCAA CTCAAAGCACACAGGTCAGC	$AG_{(6)}AG_{(7)}$	59	5	177-185	0.650	0.583	EF524508
Cca18	CTGCTGTGGGATCTTGGATT TGGTATGTGCTACACAGAAAACAA	$CT_{(10)}$	59	5	205-220	0.500	0.513	EF5245010
Cca19	TTGAGGTCAACCTGTGGCTA TTAGACATGCACCGCCATAC	$TG_{(12)}AG_{(10)}$	59	12	220-266	0.867	0.815	EF5245011

CHAPTER 2. MATING AND KINHSIP WITHIN AND AMONG BEAVER COLONIES.

ABSTRACT

Monogamy is rare among mammals, and molecular investigations have revealed that many socially monogamous species participate in extra-pair mating. The North American beaver (*Castor canadensis*) is a socially monogamous species that exhibits classic monogamous behavior, living in discrete colonies composed of a mated pair and their offspring. I examined the genetic relationships within and among colonies for two populations in central and southern Illinois to investigate 1) average relatedness within colonies, 2) occurrences of extra-pair mating within or between colonies and 3) the influence of geographic distance on intercolony relatedness. Seven microsatellite loci developed for the beaver were used to estimate relatedness and parentage for 46 beavers from 12 colonies in central Illinois and 49 from 3 colonies in southern Illinois. Average within-colony relatedness varied widely in both populations, ranging from 0.037 to 0.636 in central Illinois and from 0.155 to 0.406 in southern Illinois. Colonies were composed primarily of first- and second-order relatives, but included unrelated individuals. Paternity analysis found that 5 of 9 (56%) litters had been sired by at least 2 males. Extra-pair mating frequently occurred between members of neighboring colonies in southern Illinois. Distance between colonies was not found to be a strong predictor of relatedness in either population. Our results suggest that beavers are not strictly monogamous and colonies are not necessarily discrete family units, but may vary widely in composition.

INTRODUCTION

The study of mating systems is central to understanding population dynamics and conservation biology, and molecular studies of parentage have become commonplace in wildlife research (Jones and Arden 2003). A growing body of literature suggests that, for many species, social monogamy does not equate to genetic monogamy and extra-pair matings are not rare (Birkhead and Møller 1995; Westneat and Sherman 1997). Monogamy generally refers to a mating system in which a mated pair remains together for at least one breeding season (Kleiman 1977; Reichard 2003). The North American beaver (*Castor canadensis*) has typically been described as a socially monogamous species, living in discrete colonies that consist of an adult male and female, young of the year and juveniles from the previous breeding season (Bradt 1938; Svendsen et al. 1980; Busher et al. 1983; Sun 2003).

Field studies have revealed that beavers exhibit many of the behaviors considered characteristic of monogamous mating systems (Kleiman 1977), including long-term pair bonding, biparental care, and territorial defense by both adults (Svendsen 1980; Busher et al. 1983; Svendsen 1989; Sharpe and Rosell 2003). Furthermore, analysis of anal gland secretion compounds used in territorial scent-marking has revealed similar chemical composition among individuals from the same colony (Sun and Müller-Schwarze 1998).

Biparental care is considered an important component of monogamy. Kleiman (1977) contends that the need for food resources for offspring is sufficiently high to require male participation in food acquisition. In beavers, both adults participate in dam and lodge construction, food acquisition and territorial defense (Svendsen 1989; Sharpe and Rosell 2003; Sun 2003). Offspring typically disperse at 2-3 years of age (Svendsen 1980; Van

Deelen and Pletscher 1996; Sun et. al. 2000). Accordingly, both parents must remain with the family long enough to provide for the young. However, because adult pair-bonds may only last 2-3 years (Svendsen 1989), it is probable that some colonies contain half-siblings and any subsequent adult males tolerate unrelated subadults sired from the previous male.

Monogamous pairings appear to be the dominant pattern in beavers, yet colony composition does deviate from the expected pattern. Some studies have reported colonies with at least 3 adults present and it is thought that "extra" adults are older offspring that have not dispersed (Busher et al. 1983; Müller-Schwarze and Schulte 1999; McTaggart and Nelson 2003). A few studies have found more than one lactating or pregnant female in a colony (Bergerud and Miller 1977; Busher et al. 1983; Wheatley 1993). These findings raise questions about the reported social organization and assumed familial relationships of individuals living in a colony.

In spite of these occassional deviations, colony members usally are assumed to be first-order relatives. However, to date no genetic studies have been conducted to investigate parentage or kinship within colonies. As one of the few non-primate monogamous mammals, the beaver offers an opportunity to investigate the social and environmental factors under which monogamy is favored. Therefore, I used microsatellite loci developed for the beaver to describe genetic relationships within and among beavers colonies in central and southern Illinois. I was particularly interested in examining: 1) the average relatedness among colony members, 2) the occurence of extra-pair matings within or between colonies, and 3) the influence of geographic distance on relatedness.

MATERIALS AND METHODS

Study Area.— This study was conducted at two locations in Illinois during the 2005-06 and 2006-07 trapping seasons. Beavers were trapped in central Illinois within the Embarras River watershed (ERW). Habitat here consists of linear streams in Coles and Cumberland counties. Beavers also were collected from southern Illinois in the Union County Conservation Area (UCCA). This 2,510 ha refuge is managed by the Illinois Department of Natural Resources as wetland complex consisting of interconnected wetlands, including 3 large lakes.

Sample Collection.— Beavers were trapped using Conibear 330 traps placed around active lodges. Trappers attempted to remove all colony members over a 2-week period. The location of each lodge was recorded in UTM coordinates. Animals were sexed by primary sex organs viewed during dissection, weighed, and categorized as kits, yearlings, 2-year-olds, or adults based on body mass (McTaggart and Nelson 2003). A small section of muscle tissue from each animal was removed using a biopsy punch and stored in 95% ethanol or aluminum foil at –20°C. Tissues were collected from pregnant females and their fetuses when possible.

During the 2005-06 trapping season, additional animals were live-trapped using cable snares (McNew et al. 2007), and using protocols approved by Eastern Illinois University's Institutional Animal Care and Use Committee (protocol 06-001) and Southern Illinois University Carbondale's Institutional Animal Care and Use Committee (protocol 01-020). Live-trapped animals were anesthetized, weighed, and aged, and sexed by palpation (Osborn 1955). A biopsy punch of ear tissue was collected for genetic analysis. Sex was later confirmed using the SRY marker (Kühn et al. 2002).

Microsatellite Analysis. ——DNA was extracted using a DNeasy Extraction Kit (Qiagen, Inc., Valencia, California) and amplified using a PTC-100 thermocycler (MJ Research, Inc., Waltham, Massachusetts, USA). Polymerase chain reactions (PCR) were carried out separately in 25 μl volumes for each of 7 microsatellite loci (Cca8, Cca9, Cca10, Cca13, Cca15, Cca18, Cca19) as described by Crawford (2007). Forward primers for each locus were labeled with Well-Red fluorescent tags D3 or D4 (Sigma-Aldrich, St. Louis, Missouri). Reactions included 50-100 ng DNA, 1X PCR buffer, 200 μM each dNTP, 0.3 μM each primer, 3.5 mM $MgCl_2$ and 1U *Taq*. Amplifications consisted of an initial 5 min denaturation at 95°C, followed by 36 cycles at 95°C for 30 s, 30 s at the locus-specific annealing temperature (Crawford et al. 2007), and extension for 2 min at 72°C, and a final extension step at 72°C for 1 hr. PCR products were screened by capillary electrophoresis and scored using Fragment Analysis on a CEQ8800 automated sequencer (Beckman Coulter, Fullerton, California).

Statistical Analysis. ——Deviations from Hardy-Weinberg Equilibrium (HWE) and the presence of null alleles were tested in both populations using CERVUS software version 3.0 (Marshall et al. 1999). Linkage disequilibrium tests with Bonferroni correction were conducted using Genepop version 3.4 (Raymond and Rousset 1995). For both populations, the average relatedness (R) within each colony was calculated with jackknife resampling over all loci using the computer program Relatedness 5.0.8 (Queller and Goodnight 1989). For colonies containing ≥3 kits, I calculated average relatedness among kits within each colony. The average relatedness of adult females within colonies also was calculated to examine female philopatry. The likelihood based software Kinship version 1.3.1 (Goodnight and Queller 1999) was used to test hypotheses of kinship

among colonies. This software tests the likelihood ratio of a primary hypothesis of relatedness (such as full sibship) over the null hypothesis of non-relatedness for all pairs. Statistical confidence was tested at the 95% and 99% levels using 9,999 simulated pairs for each hypothesis. A primary hypothesis of relatedness was accepted if the likelihood ratio exceeded that required for confidence at the 95% level.

Parentage was assigned by a likelihood approach using CERVUS. This program assigns parentage by calculating the difference, Δ, in likelihood scores between the most-likely parent and the second-most-likely parent. The cutoff in Δ scores for candidate parents is determined for both the 80% and 95% levels of confidence following a parentage simulation. CERVUS is most appropriate for this study for several reasons. As with all genetic analyses, scoring errors, mutation, and null alleles can reduce the statistical confidence in parentage and kinship assignments (Pemberton et al. 1995, Jones and Arden 2003). Marshall et al. (1998) has included corrections for null alleles, scoring errors, and mutations in CERVUS, whereas other potentially useful programs do not accommodate all types of error consistently (Jones and Arden 2003). The statistical confidence in assignments is also more robust because the program calculates an expected distribution of Δ based on a simulated data set. A critical value of Δ is established so that the significance of Δ values calculated from the study population can be determined (Marshall et al. 1998, Jones and Arden 2003).

Critical values of Δ were separately determined from 10,000 simulations for maternity, paternity, and parental pairs in each population. Simulations allowed for a genotyping error of 0.02 estimated from mother-fetal controls. Based on trapping efforts involved and size of colonies, a 50% sampling efficiency for candidate parents was

assumed for the population in central Illinois, and 80% for colonies from the southern Illinois population. Occurrences of extra-pair mating within the same litter or breeding season were investigated using CERVUS and confirmed, when possible, by examining allelic variation among offspring. Percentages and means ± 1 SE are reported throughout the text.

Finally, the computer program SPAGeDi version 1.2 (Hardy and Vekemans 2002) was used to examine the relationship between geographic distance and genetic relatedness. SPAGeDi calculates pairwise relatedness (R) according to Queller and Goodnight (1989) and regresses these values against pairwise, straight-line distances between individuals. Numerical resampling is performed to assess the significance of the regression. Because the program permutes spatial locations, the regression analysis is equivalent to performing a Mantel test (Hardy and Vekemans 2002). I calculated pairwise R values for all pairs within populations and used UTM coordinates of colony locations to calculated pairwise distance between individuals.

RESULTS

A total of 55 beavers were trapped from central Illinois and 72 from southern Illinois. Colonies in central Illinois contained a mean of 3.8 ± 2.4 beavers/colony (n = 46 in 12 colonies), whereas colonies in southern Illinois averaged 9.0 ± 2.0 beavers/colony (n = 27 in 3 colonies) (Table 1). Most animals in southern Illinois colonies were classified as adults. These colonies were trapped late in the season during March; age classification based on body mass may have misidentified subadults. Each colony in southern Illinois had at least one pregnant female, providing an additional 22 fetal samples from 6 litters. The remaining 32 beavers (9 from central Illinois, 23 from

southern Illinois) were live-trapped, but were not part of completely sampled colonies. These animals were genotyped and included in regression analysis, but were omitted from further analyses. All microsatellite loci were moderately polymorphic in both populations and none were identified as linked after Bonferroni correction at $\alpha = 0.05$ (Table 2). However, Cca8 and Cca10 deviated significantly from HWE due to an excess of heterozygotes.

Colony Kinship.——Colonies in both study areas varied widely in average relatedness (Table 3), ranging from 0.037 to 0.64 in central Illinois and from 0.16 to 0.41 in southern Illinois. Of 6 fetal litters collected in southern Illinois, 2 (33%) were composed of half-siblings, though the most-likely fathers could not be identified. In central Illinois, kits occupying the same colony were identified as full-siblings in 4 of 6 (67%) colonies with *R*-values near 0.50. In larger colonies containing ≥7 individuals (n = 4), adult females were shown to be first-order relatives; however, mother-daughter pairs could not be distinguished from full-sibling pairs based on genetic relatedness. My sample included 3 colonies with 2 or more adult males. In each case, these males were either unrelated to other colony members or second-order relatives.

Parentage Assignments. ——Microsatellite loci showed moderate levels of polymorphism in both populations, giving a combined total exclusionary power of 0.987 for the 1st parent and 0.917 for the 2nd parent in central Illinois and 0.990 and 0.933 respectively in southern Illinois. In central Illinois, CERVUS identified ≥1 parent with 95% confidence in 23 of 31 (74%) young, including parental pairs (16%) for 3 kits. CERVUS identified a colony in which 1 male sired the kits of 2 females. Allele counts

and likelihood analysis of 3 kits from another colony also showed extra-pair mating with 2 kits being full-siblings and the other a half-sibling.

In southern Illinois, 16 of 26 (61%) young were assigned to 10 parental pairs. All fetal specimens were correctly assigned to their mothers with 95% confidence, but only 5 father-offspring pairs could be identified with 95% confidence. When relaxing the confidence level to 80%, 6 males were identified as the sires of 13 of 22 (59%) fetuses. Males from different colonies were identified as the most-likely fathers for 6 of these 13 fetuses (46%). As noted above, 2 litters were shown to contain half-siblings. This was supported by allele counts; 4 paternal alleles were detected among fetuses, indicating that the litter had been sired by $\geq$2 males. All kits and yearlings (n = 4) were assigned to one or both parents. A half-sibling pair was found occupying the paternal colony of one of the kits, while the most-likely mother occupied a separate colony with another mate. This male was identified as the sire of the subadult offspring still occupying the natal colony, as well as the sire of her current unborn litter. The second most-likely parental pair was also from a different colony and was not identified as likely parents for the kit's half-sibling. In total, I found 3 occurrences of within-season extra-pair mating and 7 intercolony mating.

Spatio-genetic Analysis. ——I found no relationship between relatedness and distance between colonies (n = 1,380 pairs, $r^2 = 0.001$, $P = 0.283$) (Figure 1). Pairwise distances ranged from 0.037 to 68.1 km. Genetic relatedness also was not significantly correlated with distance in southern Illinois (n = 2,120, $r^2 = 0.002$, $P = 0.06$) (Figure 2). Distance between individuals spanned a smaller range than in central Illinois, with a range of pairwise distances from 0.050 to 4.33 km.

DISCUSSION

This research was aimed primarily at examining the degree to which beavers fit the model of monogamy, living in discrete, first-order family groups. Indeed, multiple empirical studies on behavior, dispersal, and pheromones support the view that this species is strictly monogamous (Sun 2003). In this study, colonies in both populations showed a wide range of average relatedness, including unrelated groups, as well as combinations of first- and second-order relatives. Only one colony in central Illinois could be shown to contain an adult mated pair and their two offspring, although failure to detect other such single-family colonies may be due to incomplete sampling. McTaggart and Nelson (2003) reported an average colony size of 5.6 beavers/colony within the Embarras River Watershed. In comparison, an average of 3.8 beavers/colony were trapped in this study. Given that many parents remained unidentified, it is hard to assume with certainty that the other colonies were not single-families. For example, 2 other colonies were shown to contain one parent and its pair of full-sibling offspring, but the other parent could not be positively identified, perhaps due to incomplete sampling rather than single parenthood.

Large colonies tended to be composed of extended relatives; 3 of 4 colonies contained more than one pregnant female. While female beavers can become sexually mature by their second year, reproduction among these subadults is thought to be suppressed by the presence of dominant adults in the den (Brooks et al. 1980; McTaggert and Nelson 2003). Sterilization of either adult in a colony has been shown to inhibit colony reproduction, suggesting that one or both dominant adults may prevent mating, either through behavior or physiology (Brooks et al. 1980). McTaggart and Nelson (2003) reported 3 colonies in

central Illinois in which ovulation had occured in subordinate females when the pregnant adult female had been removed >2 weeks earlier. In contrast, subadult females that were trapped within a week of the removal of the pregnant female had yet to ovulate. Several studies have documented the presence of more than one pregnant or lactating female within a colony (Bergerud and Miller 1977; Busher et al. 1983; Wheatley 1993). It is possible that the ability of the dominant pair to restrict matings by other colony members may be limited in large colonies.

Adult females within the same colony were always identified as first-order relatives, whereas adult males always were unrelated mates of females in the colony. This was unexpected, as previous research on my study areas suggested that natal dispersal rates are nearly equal between the sexes, suggesting that females are not strongly philopatric (Cleere 2005; McNew and Woolf 2005; Havens 2006). It has been reported that natal dispersal is delayed in high density populations (Brooks et al. 1980; Müller-Schwarze and Shulte 1999; Havens 2006). This has been observed in central Illinois (Cleere 2005, Havens 2006), where colony density was estimated at 0.40 colonies/km of stream (Cox 2005). McTaggart and Nelson (2003) reported that 43% of colonies on the Embarras River contained more than 2 adults, indicating delayed dispersal. McNew and Woolf (2005) also reported relatively high colony density at the UCCA study area, yet nearly 75% of subadults dispersed from their natal colonies. In contrast, I found that large colonies contained multiple related females and most of these females had reproduced.

I identified 3 of 15 colonies (20%) that contained at least one individual who was unrelated to others; two of these were kits. Beavers use anal gland secretions in territorial marking and have been observed agressively defending territory and expelling intruders

(Rosell and Bjørkøyli 2002; Sun 2003). However, members of adjacent or nearby colonies may be tolerated. The Eurasian beaver (*C. fiber*) has been reported to spend less time investigating and responds less aggressively to neighbors' scent-mounds than strangers' scent-mounds (Rosell and Bjørkøyli 2002). Under high population densities, dispersing individuals from neighboring colonies, may reside periodically in non-natal colonies before establishing breeding territories (Svendsen 1980). Busher et al. (1983) observed frequent intercolony movement of subadults and adults of both sexes in a dense population in Nevada. Sun et. al. (2000) frequently observed natal or secondary adult dispersals to neighboring sites in an unexploited New York population. In Illinois, unrelated colony members may represent dispersers, or, in the case of kits, refugeed orphans.

Extra-pair matings occurred in over 50% of litters and these were often the result of intercolony matings. Although biparental care is necessary in this species, cooperative activities among colony members may afford males the opportunity to seek out additional mates (Emlen and Oring 1977). My results suggest that outbreeding is the rule in beavers; intercolony matings are fairly common and intracolony mates are not close relatives. By accepting mates from outside of their colony, females avoid inbreeding depression and may secure additional resources for their offspring. I observed that kits that are from intercolony matings may reside in either parent's colony, and this may effectively double their available territory and resources. Anal gland secretions are thought to be under genetic control and beavers have been shown to respond less aggressively to scent mounds from unfamiliar relatives than to unrelated strangers (Sun and Müller-Schwarze 1997, 1998). Dense populations, limited resources, and warmer

winters may alter dispersal patterns, increasing the likelihood of extra-pair and intercolony matings (Emlen and Oring 1977; Sun 2003; McNew and Woolf 2005).

Geographic distance was not a significant predictor of average relatedness among individuals in either population. This is expected in a mongamous mating system in which dispersal should be similar between the sexes (Sun 2003). However, previous studies reporting fewer aggressive interactions between neighboring colonies led me to hypthesize that adjacent colonies may be more closely related than distant ones (Svendsen 1980; Sun et al. 2000; Rosell and Bjørkøyli 2002). In central Illinois, our study area spanned more than 2 counties and the longest pairwise distance between colonies was nearly 70 km. Hence, my large-scale, coarse-grain sampling scheme did not proviede data for a series of neighboring colonies and I may have missed fine-scale patterns of intercolony relatedness. Nonetheless, in central Illinois, the median natal dispersal distance among juveniles is 12.2 km, indicating that offspring do not necessarily establish territories near their parents, but routinely disperse considerable distances prior to settling (Havens 2006).

Alternatively, in southern Illinois, where dispersal distances are shorter, I found a weak relationship between close neighbors. Although this association was not statistically significant, intercolony mating documented in this study would suggest that neighboring colonies do contain related individuals. This sampling area was considerably smaller than that of central Illinois, with the longest pairwise distance between colonies at 4.3 km. McNew and Woolf (2005) reported a mean dispersal distance among juveniles of only 5.9 km in the UCCA population. Therefore, it is speculative but consistant with these data to suggest that the long, linear stream habitats of central Illinois may lead to

longer dispersals and more genetic mixing in this population. In contrast, the interconnected wetland complex of southern Illinois is associated with shorter dispersals and more genetic relatedness among adjacent colonies.

This study is the first molecular investigation of mating and kinship in beavers. In contrast to long-held views that beavers are monogamous and colonies are typically first-order relatives, I documented a wide range of relationships among colony members and mulitple paternity in over 50% of litters. This was most evident in large colonies containing multiple mating adults. Multiple paternity has also been observed in other rodents, such as the prairie vole (*Microtus ochrogaster*) (Solomon et al. 2004), striped field mouse (*Apodemus agrarius*), and wood mouse (*A. sylvaticus*) (Baker et al. 1999). Additional research is now needed to describe more fully the mating system and spatial genetic patterns in beavers including investigations designed to elucidate environmental factors that may influence these.

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Table 1. Age classes of beavers trapped from 12 colonies in central Illinois and 3 colonies in southern Illinois during 2005-2007.

	Central Illinois	Southern Illinois
Fetal samples	0 (0.0%)	22 (44.8%)
Kits	22 (47.8%)	3 (6.1%)
Yearlings	1 (2.1%)	1 (2.0%)
Subadults	13 (28.3%)	2 (0.04%)
Adults	10 (21.7%)	21 (42.9%)
Total	46 (100.0%)	49 (100.0%)

Table 2. Microsatellite loci used to examine relatedness in central (ERW) and southern (UCCA) Illinois populations of *Castor canadensis*.

Locus	No. of Alleles (ERW)	No. of Alleles (UCCA)
Cca8	9	7
Cca9	8	9
Cca10	15	12
Cca13	4	5
Cca15	4	5
Cca18	3	3
Cca19	10	8

Table 3. Average relatedness values for age and sex classes within beaver colonies in central and southern Illinois.

	Average pairwise relatedness ($R \pm 1$ SE)	No. of pairwise comparisons (no. colonies)
All members		
Central Illinois	0.33 ± 0.19	97 (12)
Southern Illinois	0.24 ± 0.14	420 (3)
Fetal littermates		
Southern Illinois	0.45 ± 0.13	33 (3)
Kits		
Central Illinois	0.50 ± 0.19	19 (6)
Adult females		
Central Illinois	0.55	1 (1)
Southern Illinois	0.45 ± 0.09	30 (3)
Adult males		
Southern Illinois	0.03 ± 0.31	10 (3)

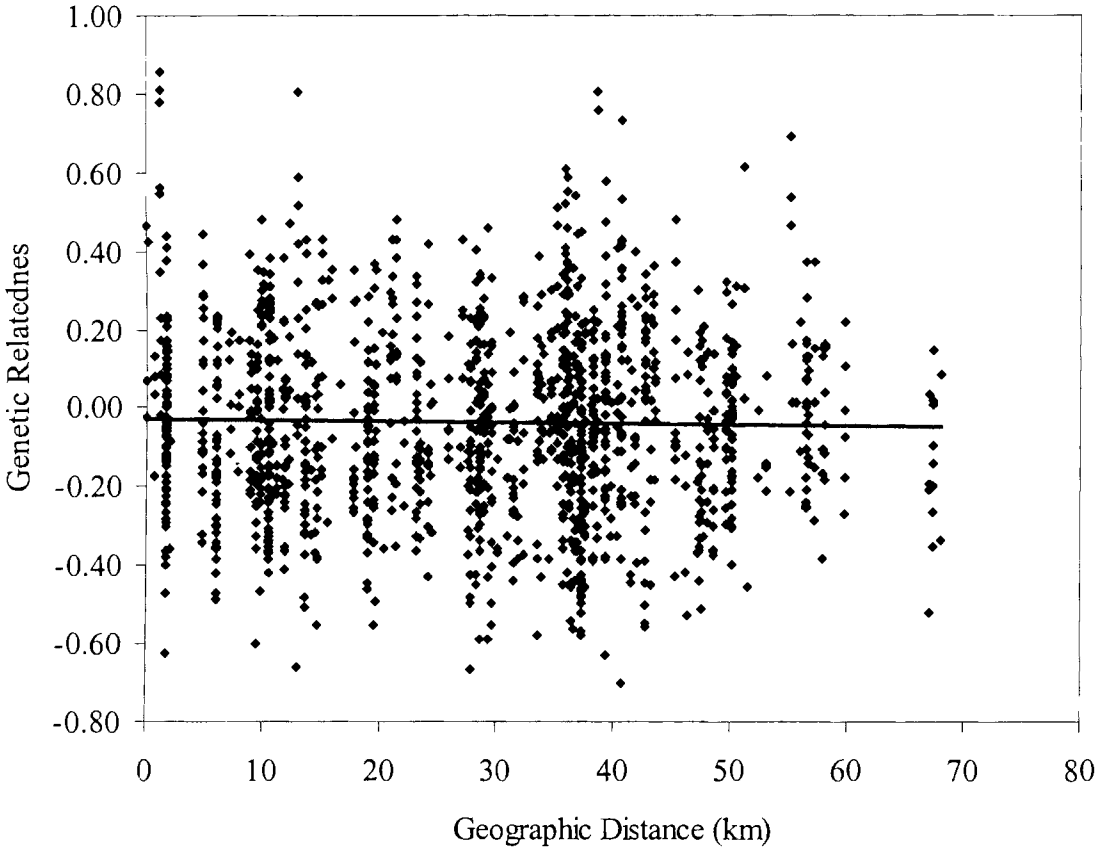

Figure 1. Regression analysis of genetic relatedness and geographic distance between beavers in the Embarras River Watershed of central Illinois. Genetic relatedness is not significantly related to distance ($r^2 = 0.001$; $P = 0.283$).

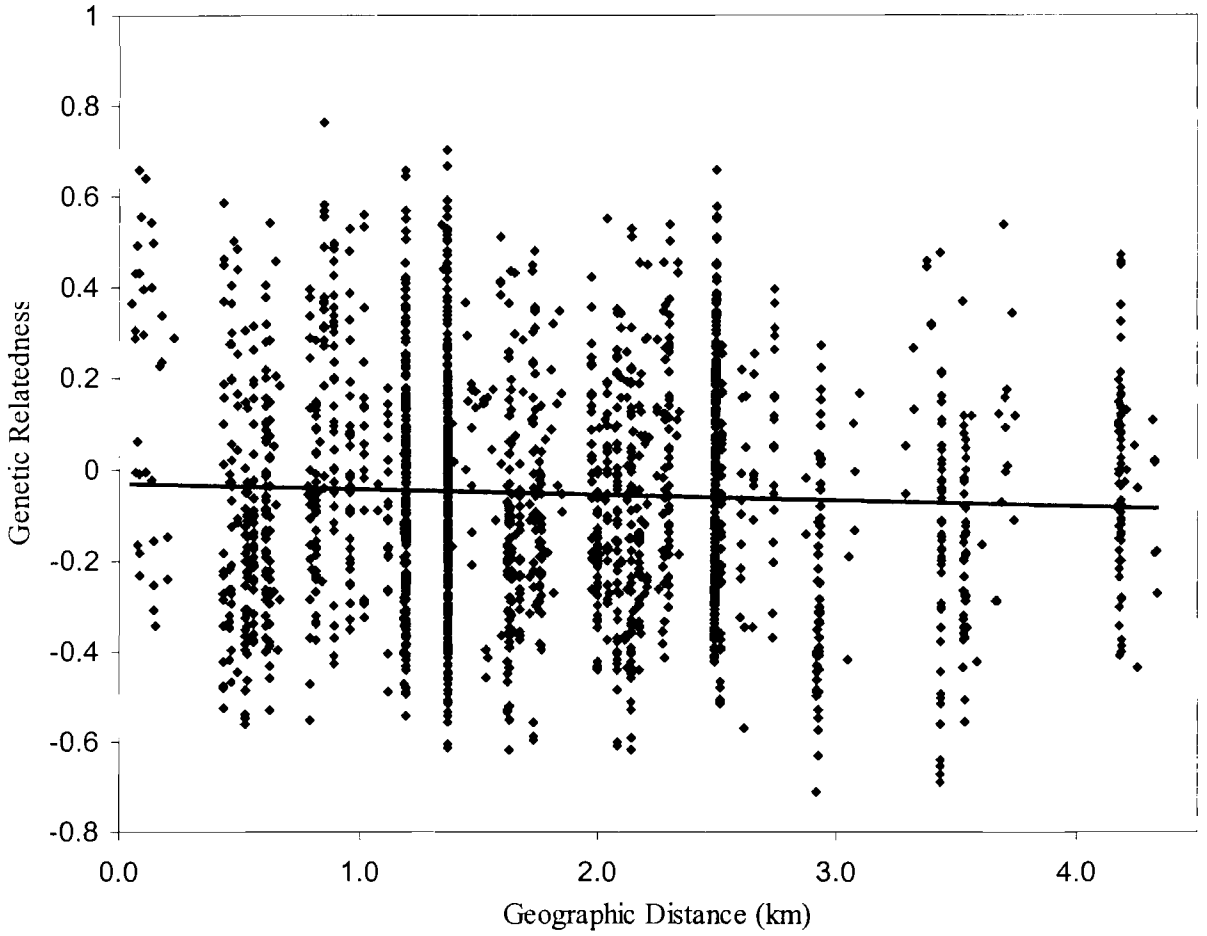

Figure 2. Regression analysis of genetic relatedness and geographic distance between beavers from Union County Conservation Area in southern Illinois. Relatedness is not significantly related to distance ($r^2 = 0.002$; $P = 0.06$).

CHAPTER 3. GENETIC VARIATION WITHIN AND BETWEEN ILLINOIS BEAVER POPULATIONS

ABSTRACT

North American beaver (*Castor canadensis*) populations experienced dramatic declines following the arrival of Europeans. Reintroductions began in the early 20th century from remnant populations throughout the U.S. Beavers were reintroduced to Illinois beginning in 1929 and spread quickly through most of the state. Populations in southern Illinois may be the descendents of translocated beavers from Wisconsin, but the origin of the central Illinois population is unknown. I used 7 microsatellite loci to quantify genetic differences between southern and central Illinois populations. Fisher's Exact tests revealed significant differences in allelic distribution for all but one locus, Cca18 ($P = 0.772$). Individual F_{ST} values ranged from 0.052 – 0.149 and were significantly different from zero for 6 of 7 loci ($P < 0.001$). Overall F_{ST} was also significant (0.0676 ± 0.0119). Further research incorporating mitochondrial DNA and beavers from throughout the state should provide further insights into the origin Illinois populations.

INTRODUCTION

Genetic differentiation can be a useful measure of movement between populations and may be applied to studies investigating dispersal, population isolation, and patterns of colonization (Bohonak 1999). Research on population differentiation among rodents has focused on the effects of habitat fragmentation at fine- and broad-scales (Dobson 1994, Mossman and Waser 2001, Shulte-Hostedde et al. 2001). The North American beaver, *Castor canadensis*, is an important wetland species and represents a model organism for

the study of molecular ecology, yet genetic studies are lacking for this species. Prior to European settlement, beavers were estimated to number 20 million, but were extirpated throughout most of the U.S. by the mid-1800's due to overharvest. Following a reduction in trapping pressure, populations have recovered to an estimated 6-12 million over the past century (Naiman et al. 1986). This species has been reported to be socially monogamous based on behavior and colony composition (Sun 2003). Colonies are primarily composed of first- and second-order relatives, but often contain unrelated individuals (Crawford 2007a). All members of a colony have been observed to work cooperatively in dam and lodge construction, food acquisition, and territory defense (Svendsen 1989, Sun 2003). As one of the few mammals known to exhibit social monogamy, beavers represent an interesting mammalian model for molecular investigations of mating patterns and population structure.

Recognized as an important wetland species and wildlife resource, beavers were reintroduced in Illinois in 1929 and spread quickly throughout most of the state (Pietsch 1956). Reintroductions of 46 beavers occurred in several northwestern and southern counties, including Union County; the focus of this study. However, a remnant population was documented in Alexander Co., adjacent to Union Co, and dispersing juveniles may have contributed to the current Union Co. population. Less is known about the founders of the population in central Illinois. Beavers were not reported in these counties for several decades following reintroductions (Pietsch 1956). The current population in central Illinois has become established during the past 50 years and may be the descendents of reintroduced animals from anywhere in the state or immigrants from surrounding states, particularly dispersers from reintroduced populations in northwestern

Indiana that were noted to travel long distances down the Kankakee River in northeastern Illinois (Pietsch 1956). In addition to questions regarding patterns of recolonization, southern and central Illinois populations have not been identified as genetically discrete populations and may be connected by infrequent dispersal.

Beavers currently represent a wildlife resource for fur trappers as well as a nuisance species to landowners in Illinois, and current populations in both central and southern regions are considered to be at high ecological densities (Woolf et al. 2003, McNew and Woolf 2005). Over the past decade, the Illinois Department of Natural Resources has funded several research projects aimed at describing beaver ecology and social structure in these populations. These studies have focused on population surveys (Woolf et al. 2003), habitat suitability models (Cox 2005), colony composition (McTaggart and Nelson 2003), and dispersal (Cleere 2005, McNew and Woolf 2005, Havens 2006, Bloomquist 2007). Given the wealth of information that has accumulated from field observations, research on population genetics seemed the next logical step in describing beaver ecology in Illinois. Specifically, I wanted to begin an examination of population genetic structure by describing genetic differentiation between southern and central Illinos beavers using microsatellite markers.

MATERIALS AND METHODS

I collected DNA samples from beavers live-trapped between September and February 2004-2006 in central (39° N, 88° W) and southern (37° N, 89° W) Illinois. Colonies in central Illinois were located in 2nd, 3rd, and 4th order streams within the Embarras River Watershed (ERW) (Havens 2006). In southern Illinois, beavers were trapped from the Union County Conservation Area (UCCA) (Bloomquist 2007), a wetland and waterfowl

refuge along the Mississippi River. Beavers were trapped using cable snares placed near active lodges (McNew et al. 2007). Following capture, animals were anesthetized, sexed by palpation in the field, weighed, and categorized as kits, yearlings, subadults, or adults based on body mass (McTaggart and Nelson 2003). A biopsy punch of ear tissue was collected and stored in 95% ethanol or aluminum foil at -20°C. Sex was later confirmed by molecular sex diagnosis using the SRY marker located on the Y-chromosome (Kühn et al. 2002, Crawford 2007a). Additional tissue samples were collected from removal-trapped beavers harvested during the 2005-06 and 2006-07 trapping seasons at both study locations. Tissue samples were collected and stored in the manner described above. Beavers were assigned to age-classes based on mass and sex was determined by primary sex organs viewed during dissection.

DNA was extracted using a DNeasy Extraction Kit (Qiagen, Inc., Valencia, California) and 7 microsatellite loci were amplified as described by Crawford (2007b). Forward primers for each locus were labeled with Well-Red fluorescent tags D3 or D4 (Sigma-Aldrich, St. Louis, Missouri). I used GENEPOP 3.4 (Raymond and Rousset 1995) to conduct Fisher's exact tests of Hardy-Weinberg equilibrium (HW) across all loci and both populations using the Markov chain method (Guo and Thompson 1992). Genepop was also used to determine differences in allele frequencies between populations using Fisher's Exact tests. Significance of multiple tests was assessed after *P*-values were adjusted using a sequential Bonferroni correction as described by Rice (1989), where k was defined as the number of microsatellites. The program SPAGeDi version 1.2 (Hardy and Vekemans 2002) was used to calculate F_{ST} as described by Weir

and Cockerham (1984) and significant *P*-values were determined by permutation tests. All tests were conducted at $\alpha = 0.05$.

RESULTS

Loci showed moderate polymorphism in both populations (Table 1). Exact tests showed all loci to be in Hardy-Weinberg equilibrium in the ERW population; however, the UCCA population deviated significantly from HWE ($p < 0.007$) due to an excess of heterozygotes at 2 loci (Cca8 and Cca10). The overall exact test showed the UCCA population to significantly deviate from HW equilibrium. The UCCA and ERW populations differed significantly in allele frequencies at 6 of 7 loci ($P < 0.001$). However, allele frequencies at locus Cca18 were not significantly different between populations ($P = 0.772$). The overall F_{ST} value (0.068 ± 0.012) between populations was significantly different from zero ($P < 0.001$). Locus Cca18 was not shown to be significantly different from zero between populations ($F_{ST} = -0.008$, $P = 0.250$). All other F_{ST} values for single loci were significantly different from zero and ranged from $0.052 - 0.15$ ($P < 0.001$).

DISCUSSION

Allelic heterogeneity is an important measure of genetic variation in populations and can be used to estimate inbreeding within a population or differentiate between populations. F_{ST}, measures population subdivision in populations due to random genetic drift. F_{ST} ranges from 0 to 1, with 0 indicating that no fixation has occurred and 1 indicating that both populations have become fixed for different alleles (Hartl 1988). Given the geographic distance between these populations, as well as mean dispersal distances, I expected F_{ST} estimates to reflect moderate levels of subdivision. Overall and

single-locus F_{ST} values indicate that there are moderate levels of genetic differentiation between central and southern Illinois beaver populations (Wright 1978). Significant differences in allelic distribution also illustrate that both populations contain unique alleles at all but one locus. The biological significance of such measures is difficult to compare across taxa and habitats, and the influence of social structure in mammals on F_{ST} has yet to be resolved (Bohonak 1999, Storz 1999). Highly kin-structured mammals such as black-tailed prairie dogs (*Cynomys ludovicianus*) (Chesser 1983, Dobson et al. 1998) and red howler monkeys (*Alouatta seniculus*) (Pope 1998) show between-group F_{ST} values ranging from 0.142 to 0.227. Conversely, Schwartz and Armitage (1980) found that yellow-bellied marmots (*Marmota flaviventris*), despite moderate female philopatry, had enough dispersal of both sexes between colonies to limit genetic structuring, resulting in a much lower estimate of between-colony F_{ST} (0.07). Local population subdivision among yellow-pine chipmunk (*Tamias amoenus*) populations led to F_{ST} values between 0.019-0.036 and showed evidence of isolation-by-distance (Shulte-Hostedde et al. 2001). Alternatively, Dobson (1994) reported a lower average F_{ST} value (0.026) for Columbian ground squirrel (*Spermophilus columbianus*) populations separated by at least 25 km where dispersal between populations was unlikely.

Few genetic surveys have been conducted to compare regional differences among populations of monogamous mammals. Using microsatellite markers, researchers found that the monogamous shrew (*Crocidura russula*) exhibited F_{ST} values between populations that mirrored the estimate found in this study at 5-6% (Balloux et al. 1998). Sommer (2003) showed significant differences among fragmented populations of the Malagasy giant jumping rat (*Hypogeomys antimena*). In her study, differences in F_{ST}

values were evident for coding versus noncoding DNA. Values were much lower for coding MHC genes (0.02) than noncoding mitochondrial DNA (0.77). These differences are expected because mitochondrial DNA mutates faster than nuclear genes (Sommer 2003). However, this difference highlights an additional constraint on making comparisons of F_{ST} across studies.

Although the relationship is difficult to assess, there is some consensus that F_{ST} values and dispersal ability are negatively correlated (Bohonak 1999, Neigel 2002). I expected to find significant genetic differences between the ERW and UCCA beaver populations, in part because they were separated by > 200 km. Although a few individual beavers have been shown to make long-distance dispersal movements, average a long-distance dispersal of 286 km has been recorded in the ERW population (Havens 2006), average dispersal distances in both populations are too short to allow for frequent immigration (McNew and Woolf 2005, Havens 2006). Still, the relatively low F_{ST} value does indicate limited subdivision and it is plausible that these two populations are part of a larger population inhabiting the southern portion of the state.

Given the limitations of my data, I cannot draw further conclusions regarding the founders of either population. The observed level of genetic subdivision between these populations may reflect divergence following recolonization by the same founding population in both areas. A more thorough study of the phylogeography utilizing mitochondrial DNA from individuals throughout the state may lead to insights regarding patterns of recolonization over the past century.

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Table 1 Number of alleles (A), observed heterozygosity (H_o), expected heterozygosity (H_e), and associated *P*-values for beaver populations in central (ERW) and southern (UCCA) Illinois. Asterisks indicate significant deviation from HWE after Bonferroni correction at $\alpha = 0.05$.

Population/Locus	A	H_o	H_e	**P**
EMB				
Cca8	9	0.740	0.750	0.068
Cca9	8	0.727	0.707	0.533
Cca10	15	0.764	0.818	0.021
Cca13	4	0.389	0.350	1
Cca15	4	0.655	0.630	0.297
Cca18	3	0.527	0.504	0.570
Cca19	10	0.788	0.837	0.093
Overall				0.065
UCCA				
Cca8	7	0.886	0.842	0.001*
Cca9	9	0.806	0.772	0.342
Cca10	12	0.847	0.852	0.002*
Cca13	5	0.542	0.522	0.978
Cca15	5	0.514	0.476	0.113
Cca18	3	0.472	0.498	0.528
Cca19	8	0.817	0.731	0.441
Overall				0.001*

CHAPTER 4. SEX DETERMINATION IN BEAVERS: A COMPARISON OF TRADITIONAL AND MOLECULAR TECHNIQUES.

ABSTRACT

The traditional method of sex identification in beavers (*Castor canadensis*) by external palpation can be inaccurate. I tested 2 genetic methods for determining sex in beavers, the zinc-finger DNA marker and the Y-chromosome specific SRY marker. This paper describes the results of this comparison for 102 beavers, as well as an assessment of the accuracy of the traditional palpation technique for sexing 62 animals. The SRY marker identified sex correctly in 92 of 102 beavers (90%), while the zinc-finger technique was successful less often ($P < 0.001$) in only 71 of 102 (70%) animals. Sex was correctly assigned by palpation for 53 of 62 animals (85%). Beaver studies in which accurate sex identification is critical may benefit by verifying the sex of individuals using one or both of these molecular markers.

INTRODUCTION

Sex identification is essential to research projects in mammal conservation and management. Accurate sexing is required for studies that examine demographics, dispersal patterns, and mating behavior. For sexually dimorphic species, sex determination is straightforward and can be conveniently conducted based on external characteristics. However, some species lack such characteristics, thereby making accurate sex identification difficult. In these species, minimally invasive molecular methods of sex determination can be useful (Woods et al. 1999, Kühn et al. 2002, Williams et al. 2004). These methods use PCR-based techniques to amplify selective

regions of the X and/or Y chromosomes to differentiate the sexes with high accuracy (Kuhn et al. 2002; Shaw et al. 2003; Williams et al. 2004).

The beaver (*Castor canadensis*) is difficult to sex using traditional field methods because the species does not exhibit external sex characteristics, with the exception of lactating females. Rasmussen and West (1943) and Osborn (1955) described the external palpation method in which the abdomen is palpated for the presence or absence of an os penis. Although commonly used, the accuracy of this technique may vary depending on the experience level of researchers (Osborn 1955). Williams et al. (2004) reported 95% accuracy in sex identification when experienced researchers used external palpation. Shulte and Müller-Schwarz (1995) described the use of anal gland secretions to determine sex based on differences in color and viscosity. This technique has been shown to be accurate, but requires the extraction of fluid from an anal gland (Shulte and Müller-Schwarze 1995, Williams et al. 2004). Furthermore, sexing by this method requires a high level of experience and may cause discomfort to the animal.

Using molecular techniques to determine sex has become more common in mammal studies due to the availability of PCR-based techniques. Kühn et al. (2002) used PCR techniques to amplify a 157-base pair fragment of the SRY gene on the Y chromosome to identify male beavers. This method uses primers designed specifically for the North American beaver and shows a positive result when the tested animal is a male. To verify the presence of DNA and successful PCR for samples that show no SRY band, the mitochondrial cytochrome *b* gene is co-amplified using universal primers. If a sample shows no SRY band but is positive for the mtDNA band, the individual is identified as a female. However, because mtDNA is more abundant in a standard genomic DNA

extraction than any single-copy nuclear gene or sex chromosome-specific gene, the amplification of mtDNA does not necessarily ensure the presence of high quality nuclear DNA in the extraction nor its amplification. As such, this method may lead to an over-estimate of females due to a false negative for amplification of the SRY band.

In order to develop a molecular method that results in sex-specific banding patterns from the amplification of a single genome, Williams et al. (2004) amplified a portion of the zinc-finger protein genes located on the X and Y chromosomes (Zfx, Zfy) using primers designed for *Odocoileus* species (Cathey et al. 1998). These primers amplify a 1350-bp fragment on the X chromosome and a 1200-bp fragment located on the Y chromosome. In electrophoresis of PCR products, a female will have a single band representing the X chromosome fragment, whereas a male will show double bands: the X chromosome fragment and the shorter Y chromosome fragment. Although this approach provides clear sex-specific expectations, the large fragment sizes necessitate the use of high molecular weight DNA and may not be appropriate for samples with degraded or low quality DNA (Shaw et al. 2003; Williams et al. 2004).

The zinc-finger DNA marker, SRY marker, and traditional palpation have not been compared regarding their accuracy in determining sex of individual beavers. I wanted to add to the work by Williams et al. (2004) and compare the accuracy of these two molecular methods along with the palpation technique for live-trapped and trapper-harvested beavers from Illinois, USA.

MATERIALS AND METHODS

Samples were collected from 102 beavers (62 live-trapped for research purposes, 40 salvaged from carcasses collected by commercial trappers during this study. Sex was

identified by palpation for 62 live-trapped beavers during September 2005-February 2006 in central and southern Illinois. Animals were live-trapped using cable snares (McNew et al. 2007), using protocols approved by Eastern Illinois University's Institutional Animal Care and Use Committee (protocol 06-001) and Southern Illinois University Carbondale's Institutional Animal Care and Use Committee (protocol 01-020). Individuals were categorized as kits, subadults, or adults based on body mass (McTaggart and Nelson 2003). Sex was assigned to each animal in the field by external palpation (Osborn 1955), and was later confirmed upon necropsy for 27 live-trapped and radiotagged animals. In addition, a small sample of tissue was removed from the tail or ear of each animal using a biopsy punch. Tissues from 40 additional beavers were salvaged from carcasses harvested by licensed trappers in 2006: sex was verified for these individuals during necropsies. All tissues were stored in 95% ethanol at -20°C, or at -20°C in aluminum foil.

DNA was extracted from all samples using a DNeasy Extraction Kit (Qiagen, Inc., Valencia, California) and quantified using uncut lambda DNA (λ50 ng/μl, λ100 ng/μl, λ200 ng/μl) as size standards. Sex was determined for each sample following the SRY protocol of Kühn et al. (2002) and the zinc-finger protocol of Williams et al. (2004). All polymerase chain reactions were performed on a PTC-100 thermocycler (MJ Research, Inc., Waltham, Massachusetts, USA). For the SRY protocol, 25 μl reactions consisted of 0.2 μM cytochrome *b* primer (forward: L1484; reverse: H15149; Kocher et al. 1989), and 0.2 μM SRY primer (Kühn et al. 2002), 100 μM dNTPs, 10 mM Tris, 50 mM KCl, 3.0 mM $MgCl_2$, 0.5 U Taq polymerase and 2 μl template DNA (50-100 ng). Amplification consisted of a 3-min pre-denaturation cycle at 94°C, followed by 35 cycles

of 94°C denaturation, 60°C annealing, and 72°C elongation for 45 s each. To amplify Zfy and Zfx regions of the zinc-finger protein genes, 25 μl reactions consisted of 2.5 μg BSA (New England Biolabs, Ipswich, Massachusetts), 0.2 mM dNTPs, 0.2 μM of primers LGL331 and LGL335 (Cathey et al. 1998), 1 U Taq polymerase, and 2 μl template DNA (25-50 ng). PCR cycling conditions for the zinc-finger marker consisted of 7 min at 95°C, then 37 cycles of 30 s at 94°C, 30 s at 47°C, and 45 s at 72°C. A final elongation step was performed at 72°C for 2 min. PCR products were visualized under UV light following electrophoresis at 100v for 45 min at room temperature through a 2% 1:3 Genepure (ISC BioExpress, Kaysville, Utah) agarose gel.

Accuracy of each marker was assessed based on the number of animals correctly classified using the 67 animals of known sex. For each marker, samples that were correctly identified were categorized as correct, while samples that were misidentified or for which PCR failed repeatedly were categorized as failed. Significant differences (α = 0.05 throughout) in performance between these markers were tested using chi-square goodness-of-fit tests in SPSS software.

RESULTS

Of the 67 beavers for which sex was known, the SRY marker successfully identified sex in 57 animals (85%), whereas the zinc-finger marker identified sex correctly for only 48 beavers (72%); the difference in success rates between the two molecular markers approached significance (χ^2= 3.565, df = 1, P = 0.059). The SRY marker incorrectly identified 4 males as female and failed to amplify DNA in another 6 samples. The zinc-finger marker misidentified 3 individuals: 2 females were identified as males and 1 male

was misidentified as a female. This marker could not identify sex in 16 additional samples due to repeated PCR failure.

Of the 35 live-trapped animals for which sex was not confirmed by necropsy, the zinc-finger marker failed to amplify DNA in 12 samples (34%), and this may have been due to a lack of high quality nuclear DNA. In such cases, streaking bands were often observed down the lane of the gel and therefore sex could only be verified for live-trapped animals using the SRY method. Based on the results of one or both molecular markers, I correctly identified sex in 53 of 62 beavers (85%) using external palpation. Of the 9 misidentified, sex had been confirmed by necropsy for 6 beavers. Accurate sexing of subadults using palpation proved problematic, as 8 of 9 misidentified animals were in these younger age-classes. Misidentification was equally likely between the sexes; 4 males were misidentified as females, whereas 5 females were incorrectly sexed.

Overall, the SRY marker accurately assigned sex in a significantly greater frequency of samples than the zinc-finger marker ($\chi^2 = 13.462$, df = 1, $P < 0.001$), assigning sex correctly in 92 of 102 samples, whereas the zinc-finger marker identified sex correctly in only 71 of 102 (70%) samples. Of the 31 samples for which sex could not be determined using the zinc-finger marker, 19 (61%) were from ear tissues that had been subjected to long-term frozen storage in aluminum foil.

DISCUSSION

Sex identification using traditional field techniques can be inaccurate and may vary with the experience of the researcher and the age of the beaver. Williams et al. (2004) reported 95% accuracy sexing beavers by palpation for researchers that had range of experience. In our study, researchers with 2-3 years of experience identified sex

correctly in 85% of animals. Although the palpation technique is relatively accurate and simple, some ecological and behavioral studies of beavers may require greater accuracy. This is especially true in field studies, where sample sizes may be small. In these studies, when accurate sex identification may be critical, verifying the sex of beavers using molecular methods will be most useful.

Molecular methods of sex identification will also be useful when intact specimens are not available, trapping individuals is not feasible, or projects span a large geographic range (Woods et al. 1999). In such cases, only small amounts of DNA may be obtained (e.g. from hair), and the ability to determine sex from such samples is an important attribute of these markers. The beaver-specific SRY protocol was found to be more reliable in sex identification than the zinc-finger protocol. This protocol was more consistent and produced distinct bands representing the SRY and cytochrome *b* genes. The short length of the SRY marker may make it ideal for use on samples in which DNA is degraded.

The presence of additional bands down the lane of the gel using the zinc-finger markers sometimes made sex determination more ambiguous. These bands may be the result of incomplete elongation of the Zfy and Zfx regions, or they may indicate that primers bind non-specifically throughout the genome. Furthermore, the absence of distinct bands at 1350-bp and 1200-bp occurred in nearly 28% of our samples. This may be due to a lack of high molecular weight DNA, especially for samples that were subjected to suboptimal storage conditions. However, incomplete amplification also occurred for samples in which high quality DNA was present. Because the fragments produced by this method are long, additional fragments may indicate that PCR failed to

elongate the zinc-finger fragments entirely. In contrast, the ease and efficiency to which sex was determined using the SRY protocol suggests that this method is preferred, especially when the quality of DNA is not ideal. However, the SRY method did fail to amplify the SRY fragment from 4 known males, leading to their misidentification. In cases in which results from field and SRY methods conflict, an additional test using the zinc-finger marker may aid in correct sex identification of beavers. I was able to correctly identify sex for all beavers using all 3 methods. This may be important when accurate sex identification is necessary in studies of sex-biased behaviors or during reintroductions when specific sex ratios are required.

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