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Genetic Structure of the Copperhead (Viperidae: Agkistrodon Contortrix Mokasen) at its Most Northern Distribution

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GENETIC STRUCTURE OF THE COPPERHEAD (VIPERIDAE: *AGKISTRODON CONTORTRIX MOKASEN*) AT ITS MOST NORTHERN DISTRIBUTION

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By

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ABSTRACT

The focus of molecular studies in North American pitvipers has been on species that warrant conservation concern, such as Timber (*Crotalus horridus*) and Massasauga rattlesnakes (*Sistrurus catenatus*). Scant effort has been directed towards the molecular ecology of the Copperhead (*Agkistrodon contortrix*), other than to evaluate male reproduction in a laboratory setting and phylogenetic history. Thus, employing DNA-based molecular techniques to study the natural history of *A. contortrix* will broaden our knowledge of molecular ecology in North American pitvipers, and particularly in a non-threatened species that can serve as a surrogate for threatened and endangered taxa. Our study will also provide insight into the population genetics of a peripheral population to supplement previous studies regarding the Central-Peripheral Hypothesis and the importance of adaptive genetic variation in peripheral populations as a hedge for environmental change. We employed 23 polymorphic tetra-nucleotide microsatellite loci to explore population parameters in *A. c. mokasen* at its most northern distribution. Six admixed genetic clusters were identified across 9 dens with intra-den relatedness not significantly different from the population average. Mean male and female relatedness did not differ either within hibernacula or in the adult population. Molecular effective population size is 93.6 individuals (CI = 78.7-113.5) whereas adult census size from mark-recapture is 116 individuals. Molecular evidence for a recent genetic bottleneck was noted in the study population. Several litters with known mothers were assigned to more than one male, indicating multiple paternity, with significant paternal skew detected in one litter. Our study detailed population genetic parameters of a non-threatened pitviper that may be useful in comparative studies with threatened and endangered pitvipers, and is the first to document multiple paternity and paternal skew in a free-living population of Copperhead snakes.

This thesis is approved for recommendation to the Graduate Council.

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DEDICATION

This thesis is dedicated to my parents, Eileen Kennedy Levine and Alan Levine, and my sister, Rebecca Levine, who have always supported and encouraged me to pursue my dreams of becoming a biologist.

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I. INTRODUCTION

Species and population-level conservation are of growing concern in the face of anthropogenic climate change and habitat fragmentation (Opdam & Washer 2004, Thomas *et al.* 2004). An estimate of how these stressors impact threatened and endangered species requires population level data. Mark-recapture (Ovaskainen *et al.* 2008) and telemetry studies (Shepard *et al*. 2008) estimate migration and dispersal, yet are limited in their scope and application (Holycross & Douglas 2007). However, molecular methods (Douglas & Douglas 2010; Appendix 1) can broaden and extend more classic methods (Hadfield *et al*. 2006) while also providing unique opportunities to study rare or cryptic forms (Shine 2008) and in fact, may provide more and/or different information than traditional approaches (Gibbs & Weatherhead 2001).

Molecular tools have been used to survey dispersal, migration, inbreeding, effective population size, reproductive success, and genetic diversity across a broad range of biodiversity (Christie 2010, Jones *et al*. 2010). The focus of molecular studies in North American pitvipers has been on species that warrant conservation concern, or those with a broad literature base, such as Timber (*Crotalus horridus*) (Bushar *et al.* 1998, Anderson 2010, Clark *et al.* 2011) and Massasauga rattlesnakes (*Sistrurus catenatus*) (Lougheed *et al.* 2000, Bushar *et al*. 2001, Chiucchi & Gibbs 2010). Scant effort has been directed towards the molecular ecology of non-threatened species, although the latter can often serve as a surrogate for threatened and endangered forms (Rodrigues & Brooks 2007, Caro 2010), particularly when life history aspects are congruent between the two.

Employing genetic techniques to study the natural history of the Copperhead (*Agkistrodon contortrix*) can yield baseline information of a non-threatened pitviper for

comparative studies with threatened and endangered sympatric pitvipers, although to date molecular techniques have been used only to examine male reproduction (Booth & Schuett 2011) and phylogenetic history (Guiher & Burbrink 2008, Douglas *et al.* 2009) in *A. contortrix*. Herein, we aim to broaden our knowledge of molecular ecology in North American pitvipers, and particularly in a non-threatened species that can serve as a surrogate for threatened and endangered forms (Rodrigues & Brooks 2007) that often have lower levels of genetic diversity than similar but non-threatened species (Spielman *et al.* 2004a). We will also provide insight into the population genetics of a peripheral population to supplement previous studies regarding the Central-Peripheral Hypothesis, which predicts reduced population connectivity, size, and genetic diversity at range limits compared to range centers (Garner *et al*. 2004), and the importance of the genetics of peripheral populations for adaptation to environmental change (Eckert *et al.* 2008).

In this study, we employed 23 polymorphic tetra-nucleotide microsatellite (msat) loci to explore population-level parameters in *A. c. mokasen*, a subspecies inhabiting the northeastern range of the species. This non-threatened pitviper species is sympatric throughout much of its range with Timber (*Crotalus horridus*) and Massasauga (*Sistrurus catenatus*) rattlesnakes, two species of conservation concern (Szymanski 1998, Brown 1993). The Copperhead also shares natural and life history characteristics with Timber and Massasauga rattlesnakes (i.e. shared denning preferences with Timber rattlesnakes (Gloyd & Conant 1990)), making it an ideal surrogate species for comparative studies.

It is uncertain as to why *A. c. mokasen* is non-threatened while *C. horridus* and *S. catenatus* are of conservation concern. One reason for declining populations in *C. horridus* compared to *A. contortrix* may be differences in the time required to reach sexual maturity

(although average litter size in overlapping populations of *C. horridus* and *A. contortrix* are similar (Trauth 2004)). The minimum time required to reach sexual maturity has been reported to range from four to six years in *C. horridus* (Trauth *et al*. 2004), whereas sexual maturity has been reached in as few as two to three years in *A. contortrix* (Fitch 1960). However, the average time required to reach sexual maturity, whether or not an individual will reproduce during a given season, and the number of offspring per litter may differ from those estimates reported considering the relationship between reproduction and resource availability and acquisition in squamates (Shine 2003).

The purpose of this study is to use multilocus DNA data to estimate population-level parameters in a northern population of *A. c. mokasen*. We test the following eight hypotheses: (1) The study population has genetic diversity, (2) the study population exhibits genetic structure (i.e. departures from 100% population admixture), (3) each hibernaculum in the study population contains the same genetic cluster(s), (4) multiple paternity occurs in the study population, (5) paternal skew occurs in multiply-sired litters in the study population, (6) average relatedness is greater among individuals with the same den affiliation, (7) the ratio of effective to census population size differs from unity, and (8) the study population has experienced a recent genetic bottleneck.

II. METHODS AND MATERIALS

A. STUDY SPECIES AND SITE

Agkistrodon contortrix evolved west-to-east in North America, with the northeastern 'subspecies' (Northern Copperhead, *A. c. mokasen*) most recently derived (Douglas *et al.* 2009).

The distribution of this form extends from southern Illinois north and east to Connecticut and Massachusetts, in tandem with the Wisconsinian Glacier (Gloyd & Conant 1990). Rapid range expansions may have reduced population heterozygosities in northern populations post-Pleistocene (Douglas *et al.* 2009). The Copperhead snake is active from approximately April to October (Gloyd & Conant 1990, Smith *et al.* 2009) and overwinters in rocky hibernacula often shared with Black Racer (*Coluber constrictor*), Black Rat Snake (*Elaphe obsoleta*), and Timber Rattlesnake, when present (Gloyd & Conant 1990).

The study site is a 1,200 acre plot located within the basalt trap rock ridges of the Central Connecticut River valley, 4.75 kilometers northwest of Meriden (Smith 2007) (Appendix 2) It contains nine known *A. contortrix mokasen* dens but the majority of adults (85%) are found in but five (Smith *et al*. 2009; Appendix 3). These are located on Lamentation Mountain and Chauncey Peak, respectively, and are separated by Bradley Hubbard Reservoir and a straight-line distance of approximately 200 meters (Smith *et al.* 2009; Appendix 3).

The Northern Copperhead has high annual fidelity to hibernacula (Smith 2007) and natal philopatry to maternal hibernacula is hypothesized (Gloyd & Conant 1990). Vitellogenesis occurs early spring with ovulation in late spring (Schuett 1982) followed by a single late summer breeding period (Schuett 1982, Smith 2007). Multiple paternity is common and females store sperm (Schuett & Gillingham 1986) with documented facultative parthenogenesis (Booth & Schuett 2011).

B. MICROSATELLITE PRIMER AND MULTIPLEX OPTIMIZATION

Microsatellites are Mendelian-inherited co-dominant markers consisting of tandem nucleotide repeat motifs that can delineate relationships among individuals and are therefore

useful for the study of population genetics (Jones *et al.* 2010). Their capacity to quantify relationships among individuals increases with number of variable microsatellite loci analyzed (Rew *et al.* 2011) and with the number of family members assayed (Wang 2007). Full siblings, for example, can be distinguished with analysis of 15 to 20 variable microsatellite loci, whereas parent-offspring can be delineated with as few as 10 (Blouin 2003).

Forty-eight uninterrupted tetra-nucleotide potentially amplifiable loci (PALs) were developed from results of a genomics evaluation of *A. contortrix* (Castoe *et al.* 2010). Primers were tested for successful DNA amplification on 15 samples consisting of five *A. contortrix* from throughout the range of the species, three *A. piscivorus* from throughout the range of the species, and 7 species of *Sistrurus* and *Crotalus* (Appendix 1). Polymerase chain reactions (PCRs) were performed on GeneAmp ® PCR System 9700 and Veriti® 96-Well Thermal Cyclers. Reaction volumes were 10 uL and contained 1.8 uL HPLC, 2.0 uL 5x- green PCR buffer, 0.8 uL [25 mM] MgCl, 2.0 uL [1.25 mM] dNTP, 0.2 uL BSA, 1.0 uL [1.0 uM] each of forward and reverse primer, 0.2 uL GoTaq® Flexi DNA polymerase, and 1.0 uL DNA (20 ng/uL). The PCR program included an initial denaturation of 95^{o} C for 3 m followed by 15 cycles of 95^{o} C for 45 s, 55^{o} C for 45 s, and 72^{o} C for 30 s, followed by 25 cycles of 95^{o} C for 30 s, 55^{o} C for 30 s, and 72^{o} C for 15 s, concluding with a final extension of 72^{o} for 5 m. Gel electrophoresis was conducted with 5.0 uL PCR product compared to 2.0 uL PCR standard in 2.0% agarose gel at 100 volts for 37-40 m on an Owl D2 Wide Gel System (Thermo Fisher Scientific). Primers that amplified strong, clear, and variable msat fragments in *A. contortrix* were chosen for multiplex optimization (Appendix 4).

Dilution tests were conducted to determine appropriate primer volumes. Groupings were based on the strength of the signal and size of the fragment, with minimal overlap such that

primers with weaker (versus stronger) signals were together (Appendix 4). Six multiplexes were designed, each containing four primers (Appendix 4). The primer in a multiplex with the weakest signal was labeled with blue fluorescent dye (6FAM), the second weakest was labeled with green fluorescent dye (JOE NHS Ester), the second strongest with yellow (TAMRA NHS Ester), and the strongest with red (ROX NHS Ester).

PCRs were conducted on the same samples as before (Appendix 1), with reaction volumes = 2.0 uL 5x- PCR buffer, 0.8 uL [25 mM] MgCl, 2.0 uL [1.25 mM] dNTP, 0.2 uL BSA, 0.5 uL of each forward and reverse primer in the multiplex, 0.1 uL GoTaq® Flexi DNA polymerase, and 1.0 uL DNA. Initial denaturation of 95° C for 3 followed by 15 cycles of 95° C for 45 s, 55° C for 45 s, and 72° for 30 s, followed by 20 cycles of 95° for 30 s, 55° C for 30 s, and 72° C for 15 s, and a final extension of 72° C for 5 m. Fragments were analyzed on an ABI Prism 3730xl, and sized by comparison to an internal standard (LIZ500). Alleles were scored using PeakScanner™ software. Primer volumes, DNA volumes, volumes of the other PCR components, and the PCR program were then adjusted for each multiplex to yield scoreable allele fragments.

C. DATA COLLECTION

One hundred and sixteen blood samples from adults and 137 juvenile skin sheds (N=253 total) were collected by Smith (2007) from 2001 to 2003. DNA was extracted (PureGene® DNA Isolation Kit) and diluted to 20.0 ng/uL. PCR reaction volumes consisted of 0.0-1.2 uL HPLC, 2.0-2.34 uL 5x- PCR buffer, 1.0-1.16 uL [25 mM] MgCl, 2.0 uL [1.25 mM] dNTP, 0.2 uL BSA, 0.2-0.65 uL forward and reverse primer for each primer, 0.1 uL GoTaq® Flexi DNA polymerase, and 1.0-2.0 uL DNA. The temperature profile was an initial denaturation of 95° C for 3 m

followed by 15 cycles of 95° C for 45 s, 55° C for 45 s, and 72° for 30 s, followed by 16-19 cycles of 95° for 30 s, 55° C for 30 s, and 72° C for 15 s, and a final extension of 72° C for 5 m. PCR was conducted on GeneAmp® PCR System 9700 and Veriti® 96-Well Thermal Cyclers. Microsatellite fragments were sized after comparison to LIZ500 size standard on an ABI Prism 3730xl automated sequencer. Alleles were scored using PeakScanner™ (Applied Biosystems) software.

D. GENETIC DIVERSITY AND STRUCTURE

MICRO-CHECKER (Van Oosterhout *et al*. 2004) was used to evaluate genotypic data for null alleles, large allele drop-out, and genotyping errors. Departures from Hardy-Weinberg Equilibrium (HWE) were examined in GENEPOP (Raymond & Rousset 1995) using a Markov Chain Method with 200 batches and 5000 iterations per batch, and with Bonferonni–adjusted P = 0.002. A Markov chain test for linkage disequilibrium (LD) was also conducted in GENEPOP (batches/ iterations as above and with P = 0.05) but only with adults in that juvenile sibships can spuriously suggest LD and departures from HWE. GenAlEx (Peakall & Smouse 2006) was used to calculate observed (H_o) and expected (H_e) heterozygosities at each locus, percent polymorphism, and mean number of alleles per locus (N_a) for all individuals by gender, and for adults by den affiliation. Two-sample t-tests or ANOVAs were used to compare mean H_e by gender, ontogeny, and den affiliation.

A Bayesian assignment method (STRUCTURE 2.1, Pritchard *et al*. 2000) with admixture and correlated allele frequencies was used to test for genetic structure (Falush *et al*. 2003a). Juveniles were excluded because they often consist of sibling and half-sibling groups that can overestimate the actual number of genetic clusters (= K) (Pritchard *et al*. 2000). Simulations

were run from K=1 to K=10 with 12 independent runs for each K, so as to exceed the number of potential partitions (=dens) in the study site (Evanno *et al*. 2005). A burn-in of 10^5 iterations was followed by a run length of 10^6 Markov Chain Monte Carlo (= MCMC) iterations. We employed STRUCTURE HARVESTER (Earl & vonHoldt 2012) to determine the actual K by identifying the greatest value of ΔK (per Evanno *et al*. 2005). We also employed the "Pop ID" option to facilitate grouping individuals by den after analyses have been completed.

E. MULTIPLE PATERNITY, PATERNAL SKEW, AND RELATEDNESS

We employed programs COLONY (Jones & Wang 2010), GERUD 2.0 (Jones 2001, Jones 2005), and CERVUS (Marshall *et al*. 1998) to estimate the prevalence of multiple paternity, as it has been suggested that using multiple methods is better than relying on a single approach (Croshaw *et al*. 2009, Sefc & Koblmuller 2009, Walling *et al*. 2010). All 22 loci (Appendix 5) were included for analyses in program COLONY, which employs a full-pedigree likelihood method to simultaneously estimate full-sib, half-sib, and parent-offspring relationships. Familial relationships were estimated under a model of male and female polygamy and without updating allele frequencies to account for inferred relationships. Four of our most polymorphic loci (AGK11, AGK26, AGK28, and AGK29) were used to estimate the minimum number of males that sired litters with known maternity (n = 21) in the parental reconstruction program GERUD 2.0, as suggested by the manual. Finally, CERVUS, a likelihood-based program, was used to estimate parent-offspring relationships in litters with known maternity (n = 21). Paternal skew was assessed in multiply sired litters by determining if the number of offspring sired by each male in a litter differed significantly from unity (goodness-of-fit x^2 test).

Program COANCESTRY (Wang 2011) was used to estimate relatedness in the study population. We used Queller and Goodnight's (1989) moment estimator of relatedness (*r*) to estimate mean population relatedness, mean adult relatedness, mean adult female relatedness, mean adult male relatedness, mean male and female relatedness within the largest den (Den 141), mean relatedness by den affiliation and mean relatedness among breeding pairs. Mean relatedness was compared among groups with an ANOVA and P = 0.05. To visualize adult relatedness, estimates of relatedness among dyads for all adults in the study population (N = 116) were used to construct a dissimilarity matrix for a Principle Coordinates Analysis (PCoA) in program NTSYSpc (Rohlf 2000).

Family groups were identified in program COLONY by conducting pairwise relatedness tests of adults within the study population (N = 116) to detect first- (parent-offspring or full siblings) and second-order (avuncular or half-siblings) relationships. Individuals with a probability of relatedness of at least 0.85 were retained for family group analyses, as there was a clear drop-off in relatedness probability among individuals beyond this value. Family groups were identified by grouping individuals that were related to all other individuals in a group by first- or second-order relationships. To visualize family group structure, a similarity matrix of relationships among all individuals within a family group was generated by coding relationships between individuals (neither first- nor second-order relationship = 0.00, second-order relationship = 0.25, first-order relationship = 0.50, self-self relationship = 1.00), and this matrix was then used for a PCoA in program NTSYSpc.

F. EFFECTIVE POPULATION SIZE AND GENETIC BOTTLENECK

In an ecological sense, population size (N_c) is measured by enumerating adults at a locality. Effective population size (N_e) is an estimate for an idealized population with the same levels of genetic drift and inbreeding as the study population (Frankham *et al*. 2010), and is most often much smaller than is N_c. We evaluated N_e in our study population by using the linkage disequilibrium method (Hill 1981) corrected for a finite sample size in program LDNE (Waples & Do 2007). Alleles with frequencies <0.02 were excluded and jackknife-adjusted confidence intervals were calculated (Waples & Do 2007, Waples & Do 2010). We then employed the adult census size (n=116; Smith 2007) to derive an N_e:N_c ratio.

Program BOTTLENECK (Piry *et al*. 1999) tests for a recent reduction in N_e by comparing equilibrium expectations in multilocus genetic data against observed heterozygosity. Stepwise Mutation (SMM) and Two-Phase (TPM) models are both employed as standards, with the TPM potentially more appropriate (Cornuet & Luikart 1996). We ran 1000 iterations under the TPM with 95% single-step mutations, 5% multistep mutations, and a multistep mutation variance of 12 (Piry *et al*. 1999). Statistical significance was assessed for both models in BOTTLENECK using the standardized differences test at P = 0.05, as is suggested for the number of loci that we employed (Piry *et al*. 1999). We also used a mode-shift test to assay for the presence of a more proximal bottleneck in our study population (i.e., one that occurred in the most recent few dozen generations, Luikart *et al*. 1998). Proximal bottlenecks can be rejected when a frequency distribution of alleles is significantly L-shaped.

III. RESULTS

A. GENETIC DIVERSITY AND STRUCTURE

A single msat locus (i.e., AGK36) was removed from subsequent analyses because it displayed HWE disequilibrium and evidence of a null allele (p = 0.12). GENEPOP detected LD between 3 pairs of loci but these comparisons were less than expected by chance alone at P= 0.05.

Average adult H_o and H_e were 0.64 ($\pm$ 0.04) and 0.64 ($\pm$ 0.04), while juvenile values were 0.62 ($\pm$ 0.04) and 0.57 ($\pm$ 0.04), respectively, with mean H_e values statistically different ($p < 0.05$). Average male H_e (0.61 $\pm$ 0.04) and female H_e (0.65 $\pm$ 0.004) also differed significantly ($p < 0.05$), although this difference may not be biologically significant considering that both heterozygosities represent medium values of genetic diversity. There were no significant H_e differences ($p > 0.05$) among dens within the study site (141's Den, Chauncey's Slide Den, Middle Chauncey Den, Middle Lamentation Den, and Point of Lamentation Den) (Table 1)

A total of 144 alleles were recorded across 22 loci in the adult population (averaging 6.54 $\pm$ 0.56 per locus). The minimum number of alleles at a locus was two while the maximum was 12 (Appendix 7). The average number of alleles per locus in each of 5 main dens ranged from 3.96 ($\pm$ 0.3) to 5.82 ($\pm$ 0.44) (Table 1).

Six genetic clusters were identified ($\Delta K_{MAX} = 38.450$) (Figure 1), the same number identified by employing the traditional method (Pritchard *et al*. 2000) of estimating the greatest mean log probability of the data [mean Ln $P(K)_{MAX} = -6511.108$] (not shown). Therefore, the null hypothesis of no genetic structure in this population of Northern Copperheads is rejected.

We also rejected the null hypothesis of each hibernaculum as a distinct genetic cluster, as all instead represent an admixture of clusters (Figure 2), albeit in different proportions. Middle

Lamentation Den (n = 7) and C29 Chauncey Slide Den (n = 7) were assigned membership to cluster 5 but their genotypic proportions differed by 33.6%. In general, differences among local hibernacula are small, but elevate considerably when compared with those approximately 3 kilometers distant. For example, 2.6% (± 0.02) of individuals from local den 141s (n = 62) were assigned to cluster 6, as compared to 90.7% (± 0.04) from Black Pond den (n = 4) (Figure 4). Despite the disparity in sample size between these locations, only 12.2% of individuals in den 141 fell into cluster 6, again supporting regional differentiation.

B. MULTIPLE PATERNITY, PATERNAL SKEW, AND RELATEDNESS

Fourteen of 21 litters with known maternity were assigned paternity with a probability greater than 0.9967 by program COLONY. Of these, 10 litters were singly sired (71%) and four (29%) were sired by two males. The number of sires assigned paternity to litters with known maternity in program COLONY was equal to the minimum number of sires assigned to each litter by program GERUD 2.0. The mean numbers of offspring per singly-sired litter (7.0 ± 2.75) and multiply sired litter (8.5 ± 3.12) did not differ significantly ($p > 0.05$).

Of the 10 singly-sired litters identified, six were assigned paternity to males that were collected by Smith (2007) while the remaining four were assigned to four different unknown males. Interestingly, one male (NCOA 99) sired three single paternity litters. Additionally, when the identity of the male who singly sired a litter was known, it and the female consistently had fidelity to the same hibernaculum. Of the four doubly-sired litters, three males were identified as those previously captured by Smith (2007). The remaining five males were identified as unknown males. CERVUS and COLONY both assigned litter paternities to the same males, when male identities were known.

In multiply-sired litters, one male sired an average of 70.1% (± 0.13) offspring while the second male sired an average of 29.9% (± 0.13) (Figure 5). Of these multiply sired litters, significant paternal skew ($p < 0.05$) was present in only one litter (number of offspring in litter two = 8) while the number of offspring sired by each male in the remaining litters (number of offspring in litters one, three, and four = seven, six, and 13, respectively) did not differ from unity (Figure 5). It must be noted, however, that our power to detect paternal skew is low considering the mean litter size of 8.5 (± 3.12) offspring. It is therefore possible, that paternal skew exists in the remaining multiple paternity clutches, despite our inability to detect it.

Mean relatedness (*r*) among adults in the population equals -0.01 (± 0.15). There is no significant difference ($P > 0.05$) among mean male (-0.015 ± 0.16) and female (-0.018 ± 0.15) *r* in the adult population. There were also no significant differences ($p > 0.05$) in mean *r* among dens (Table 2), nor among males (-0.04 ± 0.15) and females (-0.03 ± 0.16) within Den 141's. Mean *r* among breeding pairs (0.09 ± 0.18, Table 3) also did not differ significantly ($p > 0.05$) from mean adult *r* in the study population.

The PCoA of the dissimilarity matrix of relatedness among adults in the study population grouped the majority of adults together (Figure 6). Four adults (NCOA 35, NCOA 62, NCOA 75, and NCOA 103) appear as outliers to the rest of the individuals. The first three eigenvalues generated by this analysis accounted for 34.81%, 27.31%, and 24.18% of the variance in the distance matrix, respectively.

Four separate family groups were identified with program COLONY. The majority of adults in the study population fell into one family group (group two = 99 individuals), while the other three family groups have fewer members (group one = eight individuals, group three = three individuals, group four = four individuals). A plot of the results of a PCoA of the similarity

matrix of relationships among individuals within family group 2 allowed for the visualization of family group structure (Figure 7). The first three eigenvalues generated by this analysis accounted for 2.43%, 2.39%, and 2.28% of the variance in the distance matrix, respectively.

Family groups consisted almost entirely of individuals from the same general area, supporting the results of the genetic structure analyses. Individuals assigned to family group one were collected at Black Pond Den, with the exception of a single individual from Middle Chauncey Den. Every individual assigned to family group two had fidelity to a den within the immediate study site (Den 141, Point of Lamentation Den, Middle Lamentation Den, C29 Den, or Middle Chauncey Den). Interestingly, the three individuals assigned to family group three also had fidelity to dens within the immediate study site, and may therefore represent recent immigration events. Finally, family group four consisted of only individuals from outside the study site (Wassel Reservoir Den or Old Lyme Den).

Eight first-order relationships were detected among adults within the study population. Of these, four relationships occurred between individuals with affiliation to Den 141, two relationships occurred between individuals from Den 141 and Middle Lamentation Den, one relationship occurred between individuals from Black Pond Den, and one relationship occurred between individuals from Old Lyme Den. One hundred and seventy eight second-order relationships were also detected among adults within the study population. The majority of second-order relationships (N = 175) occurred between adults from the main study area. It is of interest, however, that a single second-order relationship was detected between an individual from Middle Chauncey Den and an individual from Black Pond Den, as these dens are separated by approximately three kilometers.

C. EFFECTIVE POPULATION SIZE AND GENETIC BOTTLENECK

Effective population size (N_e) was equal to 93.6 (jackknifed lower and upper CI = 78.7 and 113.5, respectively), and the population census size (N_c) falls above the upper bound of the N_e CI. The N_e:N_c ratio (93.6:116) = 0.801. Little consensus exists as to the value of an average N_e:N_c ratio in wild populations (Waples 2002) with estimates ranging from 10^{-6} to 0.8 (Frankham 1995). However, it important to note that our census estimate is likely conservative, considering that several offspring were assigned paternity to males that we failed to capture and would not have detected had we not conducted paternity analyses (See multiple paternity, paternal skew, and relatedness results). Considering that sperm storage occurs in *A. c. mokasen* (Schuett & Gillingham 1986), it is also possible that offspring assigned to unknown males were the result of the female mating with a male that died prior to the study of Smith (2007).

The null hypothesis of no significant heterozygosity excess was rejected under both SMM and TPM (P = 0.00001 and 0.009, respectively). The allele frequency distribution in the mode-shift test was distinctly L-shaped (Figure 5), rejecting evidence for a proximal bottleneck and instead indicating a more historic occurrence than the last few dozen generations, where a generation interval (= average age of reproduction; Hill 1979) is three years in Northern Copperhead (Fitch 1960).

The standardized differences test in program BOTTLENECK identifies bottlenecks that occurred within the past $2N_e$ to $4N_e$ generations. Using N_e = 93.6 (CI = 78.7-113.5) (Waples & Do 2007) and multiplying it by two and four (as above), the genetic bottleneck in this population must have occurred 187.2 to 374.4 generations ago. Assuming a three-year average generation interval for Northern Copperhead, the genetic bottleneck would have occurred during the span from 561.6 to 1123.2 years prior to the start of the study (year 2001).

IV. DISCUSSION

A. GENETIC DIVERSITY

Average population and intra-den H_o, H_e, and N_a per locus clearly demonstrate genetic variability in the study population and are similar to those reported for other North American pitvipers, including Timber Rattlesnake (Clark *et al*. 2008, Clark *et al*. 2010), Eastern Massasauga Rattlesnake (Gibbs *et* al. 1997, Chiucchi & Gibbs 2010), Desert Massasauga Rattlesnake (Anderson *et al*. 2009), New Mexico Ridge-Nosed Rattlesnake (Holycross & Douglas 2007), and Midget Faded Rattlesnake (Oyler-McCance & Parker 2010). Heterozygosity estimates do not differ significantly from those found in other North American squamates. Our observed within-hibernaculum heterozygosities were not significantly different to those found by Lougheed *et al*. (1999) in Black Rat Snake (*Elaphe obsoleta obsoleta*), a non-venomous colubrid that is often sympatric to *A. contortrix mokasen* (Table 2). Average H_o and H_e in this population are not significantly different from those reported for other North American colubrids including Eastern Foxsnake (Row *et al*. 2010) and Northern Water Snake (Prosser *et al*. 1999). Genetic diversity in the study population is therefore congruent with estimates from other snakes.

The estimated N_a per locus in our study population is similar to those reported for other species of conservation concern, including Timber Rattlesnake (Anderson 2010) and Eastern Massasauga Rattlesnake (Gibbs *et al*. 1997). Somewhat surprisingly, N_a is lower than that reported for threatened Desert Massasauga Rattlesnake, although allelic diversity in the latter is also greater than for other threatened species (Anderson *et al*. 2009). However, average N_a per locus in this population is greater than reported for a Wyoming population of Midget Faded Rattlesnake (Oyler-McCance & Parker 2010) but similar to those reported for Eastern Foxsnake (Prosser *et al*. 1999) and Northern Water Snake (Row *et al*. 2010). Differently, N_a per locus in

Eastern Rat Snake (N_a = 9-40) generally exceeds that found in this population (Lougheed *et al*. 1999).

Parallels in genetic diversity estimates among Northern Copperhead snakes and other North American pitvipers, particularly Timber rattlesnake and Eastern Massasauga rattlesnake, may be the result of similar natural histories. Northern Copperhead, Timber and Eastern Massasauga rattlesnake are sympatric, although not necessarily syntopic, throughout much of their respective ranges, with Northern Copperhead and Timber Rattlesnake often sharing overwintering dens (Gloyd & Conant 1990). Both also share habitats in that they are common to rocky woodlands (Trauth *et al*. 2004) yet differ from Eastern Massasauga rattlesnakes which often hibernate in crayfish burrows in open fields (Hulse *et al*. 2001). Additionally, these three species are viviparous and are under thermal constraints due to their ectothermy (Angilletta 2009). Comparable habitat preferences and life histories among these species may produce similar selective agents and induce similar levels of genetic diversity, as has been shown in freshwater invertebrates (Marten *et al*. 2006).

The Central-Peripheral Hypothesis predicts a decline in abundance and genetic diversity across taxa when moving from the geographical center of a range to its periphery due to discontinuities in suitable habitat available, and therefore reduced gene flow among populations (Mayr 1963, Lewontin 1974, Brussard 1984). While genetic diversity at the periphery of a distribution has been extensively examined, similarities across studies are difficult to discern (Eckert *et al*. 2008). Estimates of average H_o and H_e in Northern Copperhead, for example, are similar to those reported for peripheral populations of Eastern White Cedar (Pandey and Rajora 2012), Blue-Tailed Damselfly (Wellenreuther *et al*. 2011), and Westslope Cutthroat Trout (Taylor *et al*. 2003). Average H_e in the study population is significantly greater ($p<0.05$) than in

a Timber Rattlesnake population located in the center of the species' range (Anderson 2010), a Timber Rattlesnake population located near the range periphery (Bushar *et al*. 1999), and a peripheral population of Collared Lizard (Blevins *et al*. 2011). However, genetic diversity may reflect more than mere geographic location, in that landscape features have also been implicated in patterns of allelic diversity and heterozygosity (Manel *et al.* 2003, Blevins *et al*. 2011). For example, mean H_o and H_e in Northern Copperhead are greater than in a similarly sized population of Wyoming Midget Faded Rattlesnake (H_o = 0.41 to 0.55) also found at the northern extreme of its range and similarly subdivided by a reservoir (Oyler-McCance & Parker 2010). Additionally, barriers to dispersal and gene flow between populations due to habitat discontinuities at range peripheries differ depending on the species in question, leading to specious conclusions with regards to cross-taxa comparisons of genetic diversity at range limits.

B. GENETIC STRUCTURE

No genetic structure was found among local dens in this study, congruent with results for other pitvipers [Timber Rattlesnake in Missouri (Anderson 2010), Desert Massasauga Rattlesnake in Arizona and New Mexico (Anderson *et al*. 2009), Eastern Massasauga in Ohio, Pennsylvania, and Illinois (Chiucchi & Gibbs 2010)].

Differences in genetic structure among local populations versus those approximately three kilometers distant suggest limited gene flow between these areas, and is (again) consistent with other North American pitvipers. Chiucchi and Gibbs (2010) found high genetic differentiation among local populations of Eastern Massasauga Rattlesnake separated by less than 50 kilometers. Additionally, Clark *et al*. (2010) found that a minor road separating Timber Rattlesnake hibernacula contributed significantly to their genetic structure. The low level of gene

flow inferred through analyses of genetic structure is further supported by the results of the sib-ship analyses, from which only a single half-sibling relationship was identified between the main study site and Black Pond Den.

Copperheads have strong fidelity and return annually to the same overwintering site (Smith *et al*. 2009), much like other North American pitvipers (Clark *et al*. 2008, Chiucchi & Gibbs 2010). Copperheads are thermally constrained and may spend more than six months underground during winter at Northern latitudes (Gloyd & Conant 1990). Long-term den fidelity and its resulting genetic structure (Bohonak 1999) may result from few thermally acceptable hibernation sites at the Northern extreme of the range. Greater genetic structure has also been found in Timber Rattlesnake populations at their more northern range (Bushar *et al*. 1998, Clark *et al*. 2010) compared to the center of their range (Anderson 2010)

Genetic structure in the study population, and in Northern Timber Rattlesnake populations, may also result from the Central-Peripheral Hypothesis. Populations existing on the periphery of species' geographic range are often both isolated by distance from central populations and more isolated from each other than in the range center due to a paucity of suitable environmental patches (Brussard 1984). Immigration and emigration among peripheral populations may therefore be infrequent, contributing to increased likelihood of genetic bottleneck, decreased effective population size, and extinction (Garner *et al*. 2004). Additionally, Northern peripheral populations may have lower genetic diversity and greater genetic differentiation due to population expansion from refugia following glacial events (Hewitt 1999). Douglas *et al*. (2009) found that *A. contortrix* expanded rapidly north and east following the last glacial maximum. It is important to note, however, that we have not yet sampled individuals from the center of the range of the Copperhead. Further research on genetic diversity and

structure in Copperheads from the range center will therefore be required to test these conjectures.

Natal philopatry may be another explanation for genetic structuring in this population. It has been shown to contribute to genetic structuring in Timber Rattlesnake populations (Clark *et al*. 2008, Anderson 2010), as the return of offspring to maternal hibernacula results in aggregated family groups. Support for natal philopatry and den fidelity can also be gained through parentage analyses (below).

The effect of site fidelity and natal philopatry on population genetic structure is not restricted to Serpentes. Site fidelity and/or natal philopatry have been implicated in the genetic structuring of a variety of organisms including Australian Sea Lions (Lowther *et al*. 2012), Vespertilionid bats (Arnold 2007), freshwater midges (Krosch *et al*. 2011), Diamondback Terrapins (Sheridan *et al*. 2010), Walleye (Stepien *et al*. 2009), and Spotted Salamanders (Zamudio & Wieczorek 2007).

Hibernacula within this population do not represent discrete genetic demes, as can be seen by considerable genetic admixture (Figure 3). Although this population exhibits high den fidelity, genetic admixture is indicative of gene flow among hibernacula (Clark *et al*. 2008). Mating between Copperheads with fidelity to different dens can also be supported through parentage analyses (below).

Additionally, our study supports the notion that genetic differentiation among hibernacula can occur over relatively short distances. We found statistically significant ($p<0.05$) differentiation between hibernacula separated by three kilometers. These results support the modest to high levels of genetic differentiation previously found among Timber and Eastern Massasauga rattlesnake.

C. MULTIPLE PATERNITY AND PATERNAL SKEW

Multiple paternity has been documented across vertebrate taxa (Karl 2008, Avise *et al*. 2011), and is thought to occur in greater than 50% of wild squamate clutches (Uller & Olsson 2008). However, few studies have documented multiple paternity in wild *Crotalinae* populations and with the use of genetic data (Siminov & Wink 2011). Multiple paternity in *A. contortrix* has previously been documented through laboratory crosses and analyses of phenotypic data (Schuett & Gillingham 1986). To our knowledge, this is the first study to document multiple paternity in a free-living population of *A. contortrix* and to employ microsatellite data.

The maximum number of males detected as sires in litters in the study population was two, and multiple paternity occurred in 29% of clutches in which paternity could be assigned with a high probability. Our paternity estimates are congruent with that found for another species within *Crotalinae*, *Gloydius halys*, in which a study estimated the presence of two fathers in a single female's litter (Siminov & Wink 2011). The number of sires identified in our study and that of Siminov and Wink (2011), however, is smaller than the number of sires identified in multiple paternity litters in other reptiles. For example, up to four males have been estimated to sire litters in *Vipera berus* (Ursenbacher *et al*. 2009b), up to three in *Elaphe obsoleta* (Blouin-Demers *et al*. 2005), and a minimum of five sires in loggerhead sea turtle clutches (Zbinden *et al*. 2007). The frequency of multiple paternity in the study population (29%) is lower than that found in another study of Viperidae (69%) (Ursenbacher *et al*. 2009b), although it should be noted that the latter relied solely on program CERVUS for output, and this program has relatively weak confidence in individual paternity assignments (Walling *et al*. 2010).

Multiple paternity in *A. c. mokasen* may be due to females mating multiply within a season, female storage of sperm from different males across different breeding seasons, or a

combination of these two (Uller & Olsson 2008). Sperm storage has been documented in *A. contortrix* through laboratory crosses (Schuett & Gillingham 1986). There are no estimates of the number of males that a female can be inseminated by per season, although multiple mating within a season by males in the study population has been documented (Smith 2007). Additionally, sperm competition may obscure the actual number of males that mate with a female, particularly if sperm from some males have not fertilized eggs (Uller & Olsson 2008). Therefore, it will require further study to determine whether multiple paternity in *A. c. mokasen* is due to multiple mating within a season or cross-season sperm storage. Although multiple paternity is common in squamates, estimates of paternal skew are less well understood. We detected significant paternal skew in only a single clutch. Therefore, males sired an equivalent number of offspring in 75% of clutches that we could assign multiple paternity to, with the previously detailed caveat that paternal skew may have been detected in these litters if they were of greater size. Equivalent male contributions to multiple paternity litters may decrease variance in male reproductive success and increase effective population size (Lotterhos 2011), although some argue that equal male contributions simply prevent multiple paternity from having a negative impact on effective population size (Karl 2008). Although we detected only a single litter with paternal skew, multiple paternity studies in other taxa have found different results. For example, Zbinden *et al*. (2007) found significant paternal skew in 45% of loggerhead sea turtle clutches, although clutch size in sea turtles is undoubtedly much larger than in Copperheads. Significant paternal skew, however, was also found in the majority of clutches studied in a population of Ocoee salamanders (with average clutch sizes = 15 eggs) (Adams *et al*. 2005), and the extent of paternal skew was found to depend on the number of sires in red-backed

salamanders (with clutch sizes of 5-11 eggs) (Leibgold *et al*. 2006). Among invertebrates, significant paternal skew was found in the majority of crayfish broods (Walker *et al*. 2002)

D. RELATEDNESS

Home range and den sharing by related individuals has been shown to increase in some animals when availability of dens decline (Kitchen *et al*. 2005, Banks *et al*. 2011), increasing inclusive fitness as predicted by kin selection theory (Hamilton 1964). For an ectothermic population that occurs at the northern extreme of a geographic range, as is the case with the study population, higher relatedness within dens compared to the population average would be expected due to relative scarcity of suitable hibernation sites. In a Pennsylvania population of Timber Rattlesnake, individuals from the same hibernaculum were more closely related to one another than to individuals from more distant hibernacula (Bushar *et al*. 1998), supporting this theory. In the study population, however, genetic relatedness within dens is not different from overall relatedness. The lack of genetic relatedness among *A. c. mokasen* individuals within dens could be due to several reasons. First, hibernacula within the study site are near to each other (< 200 m) (Smith 2007), differing from the study site of Bushar *et al*. (1998). Males and females within the study population move annual mean distances of 3993.5 m and 1714.1 m, respectively (Smith 2007). Therefore, lack of relatedness within dens may be because dens are relatively close to one another, making movement between them easier and thermally feasible. Secondly, lack of genetic relatedness among individuals sharing the same hibernacula may simply be due to random mating. In the study population, individuals move to a summer breeding ground where breeding occurs in the late summer, differing from more southern populations in which breeding occurs both in the spring and fall (Smith *et al*. 2009). Because breeding does not occur upon

emergence from hibernation as it does in the more southern range of the species, individuals from different hibernacula may mate at the same breeding ground. Mating between individuals from different hibernacula has been documented in Timber Rattlesnake (Anderson 2010). In Timber Rattlesnake, however, natal philopatry to the maternal hibernaculum does occur, increasing genetic relatedness within hibernacula (Bushar *et al*. 1998, Clark *et al*. 2008, Anderson 2010). Lack of genetic relatedness within hibernacula in the study population may therefore be due to a lack of natal philopatry as well, as has been observed in Black Rat Snake (Blouin-Demers & Weatherhead 2002). Parturition occurs close to the maternal hibernaculum in the study population (Smith 2007), however, leading us to believe that the lack of genetic relatedness within hibernaculum is not due to a lack of natal philopatry.

Evidence of sex-biased dispersal from hibernacula would include individuals of one sex within the hibernaculum that are more closely related to one another (higher *r*) than found in the other sex (Pernetta *et al*. 2011). In the study population, however, there was no significant difference between average male and female relatedness in the total population and within the largest den (Den 141s). Male-biased dispersal, however, has been observed to occur in Timber Rattlesnake populations (Clark *et al*. 2008).

It is not surprising that mean genetic relatedness among individuals in breeding pairs did not differ significantly from population mean relatedness. Mating between unrelated individuals reduces fixation of deleterious alleles and genetic drift (Allendorf & Luikart 2007). We do not know, however, if active kin recognition is occurring to prevent breeding among related individuals, although kin recognition in rattlesnakes has been documented through laboratory trials (Clark 2004).

E. EFFECTIVE POPULATION SIZE

N_e falls within the range reported for other Viperid snakes [Eastern Massasauga Rattlesnake (Chiucchi & Gibbs 2010), Timber Rattlesnake (Clark *et al*. 2008), Adder (Ursenbacher *et al*. 2009a)], but is lower than that reported for the sympatric Black Rat Snake (Lougheed *et al*. 1999). An estimated N_e of ~500-1000 individuals is a baseline value deemed necessary to offset long-term environmental change (Franklin & Frankham 1998), and these values are likely underestimates due to the fact that they assume the population in question is idealized (i.e. equal sex ratios, all adult individuals reproduce, etc.). The N_e of the study population (93.6) is but 19% of the lower estimate (N_e=500) of Franklin & Frankham (1998) thus suggesting a potential vulnerability in the study population to stochastic environmental events (Palstra & Ruzzante 2008).

N_e can be influenced by many factors that can disrupt an "ideal" population. These include overlapping generations, unequal sex ratio, non-random mating, unequal reproductive success among individuals, and fluctuating population size (Frankham 1995, Waples 2002). Northern Copperheads experience many of these, to include overlapping generations and (potentially) biased reproductive success. Multiple paternity (see above), for example, would impinge upon reproductive success, but disagreement exists as to how this would impact N_e (Lotterhos 2011). Some (Karl 2008) suggest it decreases N_e by increasing male reproductive variance, while others (Sugg & Chesser 1994, Pearse & Anderson 2009) argue the contrary. The impact of multiple paternity on N_e is further muddled in the study population by the presence of singly-sired clutches, variance in number of offspring (Smith 2007), and sperm storage (Schuett & Gillingham 1986).

F. GENETIC BOTTLENECK

Environmental disturbances and reductions in N_e can often occur simultaneously (England *et al*. 2011), and we suggest they may have occurred in our study population. We detected an historical bottleneck that occurred somewhere within the time span of years 877 to 1439. Interestingly, we failed to detect a bottleneck that would have coincided with the construction of the Bradley Hubbard Reservoir at the center of the study site. Construction of this reservoir occurred in the late 1800's and was continued in 1927 (http://www.ctparks.net/meriden/giuffrida/history.htm). The expansion of Storrs (CT) surprisingly did not appear to have promoted a bottleneck either, which would have been expected had it caused a decline in census size or a disruption in gene flow (Broquet *et al*. 2010). Both have had similar effects on other North American pitvipers, including Timber Rattlesnake (Clark *et al*. 2011) and Midget Faded Rattlesnake (Oyler-McCance & Parker 2011).

Genetic effects of bottlenecks may require a more extensive time span to manifest themselves, depending upon the size of the population and the number of generations that have lapsed (Frankham *et al*. 2010), and this may explain why a bottleneck associated with the construction of Bradley Hubbard Reservoir was not detected. For example, fewer lapsed generations may be the reason for higher genetic diversity in a population of Northern Map turtles that was fragmented by the construction of dams in the past 100 years (Bennett *et al*. 2010). Additionally, the effect of a bottleneck on genetic diversity in our Connecticut study population may be countered by gene flow that reduces inbreeding and introduces genetic diversity into the population (Wright 1969).

Genetic bottlenecks can also promote other deleterious population-level effects, such as a loss of genetic diversity, increased inbreeding, and vulnerability to environmental, demographic,

and genetic stochasticity (Frankham *et al*. 2010). While we do not have historical genetic or demographic data for this population, assumptions can be made based upon apparent reverberations within the present data. For example, average H_e has not declined in this population, suggesting a single rather than a multigenerational bottleneck due to the fact that loss of heterozygosity following a genetic bottleneck declines exponentially with the number of generations in which effective population size is truncated (Allendorf 1986). Despite having little impact on H_e, single generation bottlenecks reduce the average number of alleles per locus due to loss of rare alleles and multiple equally frequent alleles (Allendorf 1986).

G. CONCLUSION

Population genetic parameters are often not investigated in threatened and endangered species until these species have become a focus of conservation concern, leaving us with little baseline information regarding natural genetic variation in these species (Storfer 1996, Hendrickson *et al.* 2003). Although imperfect, studies of non-threatened species can provide valuable information for the management of those that are threatened and endangered (Rodrigues & Brooks 2011, Caro 2010) and have proven successful in this regard (Hendrickson *et al*. 2003). Additionally, there is continual disagreement over the importance of genetic diversity in peripheral vs. central populations in the maintenance of threatened and endangered species (Garner *et al.* 2004).

Our study provides valuable baseline information of a non-threatened pitviper that may be used to inform management decisions for those threatened and endangered. Of note is that our study population has maintained genetic diversity and gene flow among hibernacula in spite of a relatively recent genetic bottleneck. Genetic diversity and structure in this bottlenecked

population can be compared to that in recently (~100-200 years ago) fragmented populations of Timber and Massasauga rattlesnakes to inform us as to whether threatened sympatric pitvipers experience more deleterious effects of genetic bottlenecks. Additionally, estimates of multiple paternity and paternal skew in the study population, and their effects on genetic effective population size, can be used for comparison in potential future studies of multiple paternity and paternal skew in Timber and Massasauga rattlesnakes.

Our study also supplements other studies of genetic diversity in peripheral populations. To the best of our knowledge, however, there have been no studies of genetic diversity and structure in *A. contortrix* populations from the center of the species' range. Future studies of *A. contortrix* population genetics should therefore be conducted in the center of the range of the species in order to test our conjectures regarding the Central-Peripheral hypothesis.

Finally, our study yielded a detailed genetic description of a population of free-living *A. c. mokasen*, a species that has previously only been the focus of laboratory and phylogenetic studies. To the best of our knowledge, our study is the first to document multiple paternity with genetic data in a free-living population of *A. contortrix mokasen*, and one of few genetic studies in sub-family *Crotalinae* to document paternal skew in multiply sired litters. Through our study, we have broadened our knowledge of the genetics of wild North American pitviper populations, contributing to a more thorough understanding of a taxon which is often under the threat of habitat fragmentation and the target of public fear.

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VI. TABLES

TABLE 1. Average expected (H_e) and observed (H_o) heterozygosities, and average numbers of alleles per locus (N_a) at 22 tetra-nucleotide microsatellite loci for N=116 adult *Agkistrodon contortrix mokasen* from 9 dens (=Den) in central Connecticut, although dens with less than 7 individuals may be temporary.

Den	N	H_o	H_e	N_a
141's	62	0.61 ± 0.04	0.63 ± 0.04	5.82 ± 0.44
Chauncey's Slide	7	0.66 ± 0.05	0.60 ± 0.04	3.96 ± 0.30
Middle Chauncey	10	0.63 ± 0.04	0.60 ± 0.04	4.64 ± 0.33
Middle Lamentation	7	0.66 ± 0.05	0.62 ± 0.05	4.55 ± 0.32
Point of Lamentation	14	0.69 ± 0.05	0.63 ± 0.04	4.96 ± 0.38
Black Pond	4	0.64 ± 0.07	0.58 ± 0.04	3.14 ± 0.21
Rock Wall	1	0.73 ± 0.09	0.36 ± 0.05	1.73 ± 0.10
Old Lyme	3	0.61 ± 0.08	0.43 ± 0.05	2.55 ± 0.24
Wassel Reservoir	1	0.77 ± 0.10	0.39 ± 0.05	1.77 ± 0.09

TABLE 2. Mean relatedness (*r*) with standard deviation (± SD) among adult individuals within each of five main *Agkistrodon contortrix mokasen* dens in Central Connecticut.

Den	*r*	± SD
141's	-0.02	0.16
Chauncey's Slide	-0.17	0.15
Middle Chauncey	-0.12	0.15
Middle Lamentation	-0.17	0.12
Point of Lamentation	-0.08	0.15

TABLE 3. Relatedness (*r*) among male and female *Agkistrodon contortrix mokasen* individuals that produced offspring (as inferred through parentage analyses) in a Connecticut population.

Female ID	Male ID	*r*
NCOA020	NCOA033	-0.04
NCOA017	NCOA026	-0.28
NCOA004	NCOA076	0.20
NCOA029	NCOA099	0.15
NCOA106	NCOA099	0.28
NCOA062	NCOA099	0.05
NCOA028	NCOA090	-0.02
NCOA010	NCOA065	0.24
NCOA104	NCOA069	0.20
-	Mean	0.09
-	± SD	0.18

VII. FIGURES

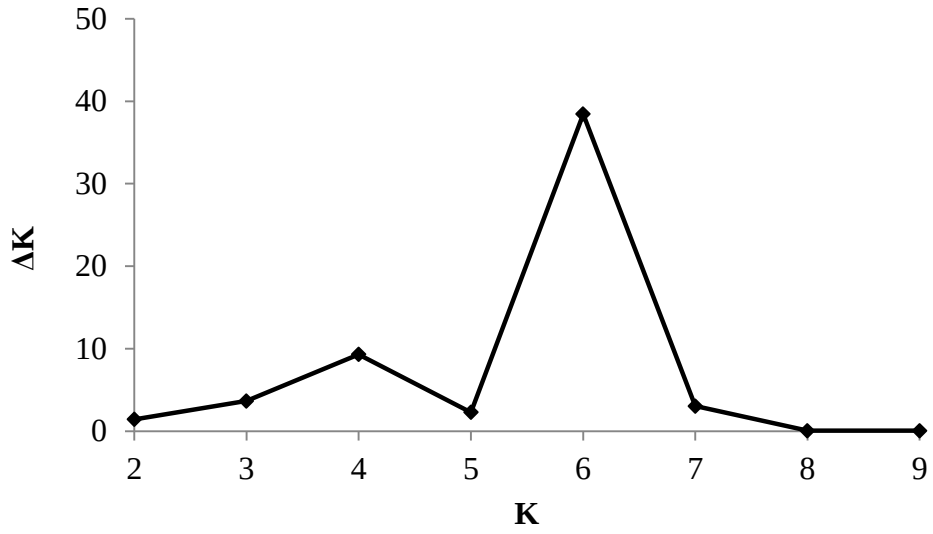

FIGURE 1. Six genetic clusters (K = 6) of adult *Agkistrodon contortrix mokasen* (n = 116) from nine dens in Central Connecticut identified by STRUCTURE HARVESTER (with Evanno Method) after Bayesian clustering (STRUCTURE 2.1). $\Delta K = \text{mean}(|L(K)|)/\sigma(L(K))$, where L(K) is the likelihood of K and σ(L(K)) is the standard deviation of the likelihood of K.

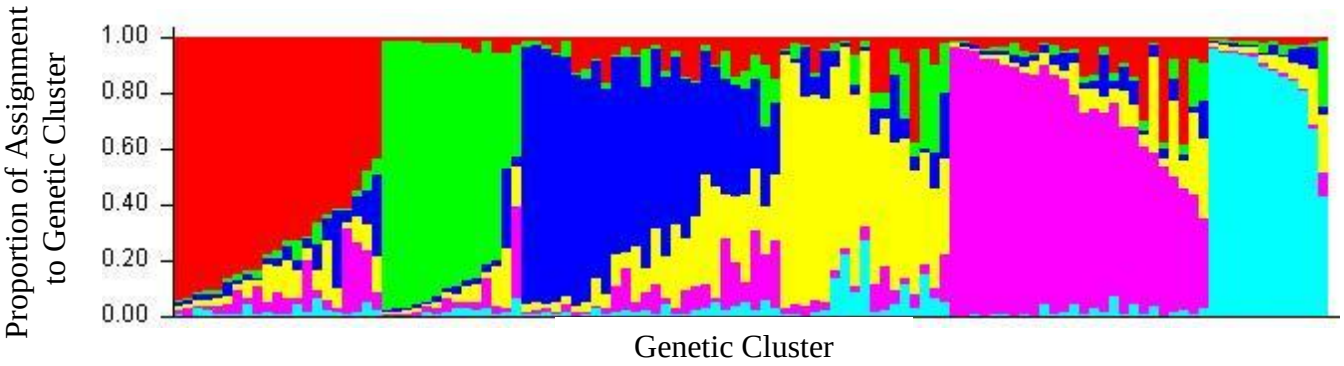

FIGURE 2. Clusters of adult *Agkistrodon contortrix mokasen* (n=116) in Central Connecticut identified by STRUCTURE 2.1. Considerable admixture is present in most individuals. Red = Cluster 1, Green = Cluster 2, Dark Blue = Cluster 3, Yellow = Cluster 4, Magenta = Cluster 5, Light Blue = Cluster 6.

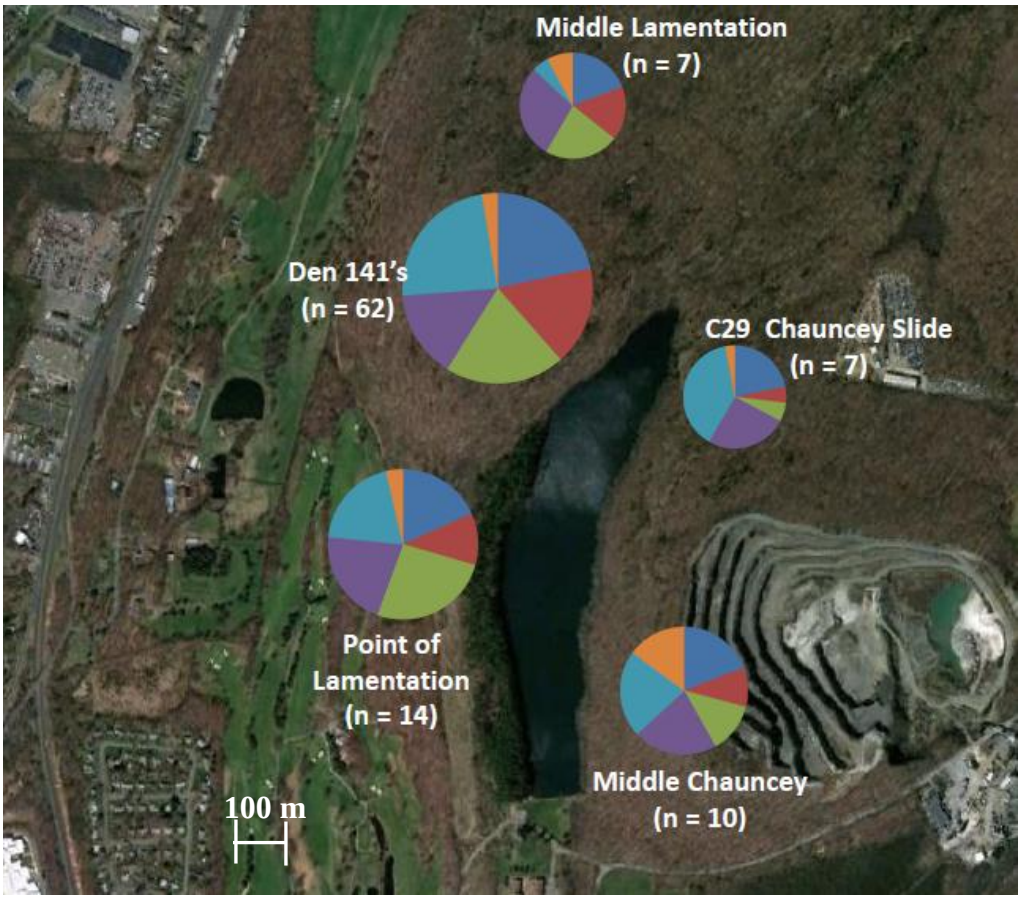

FIGURE 3. Proportion of adult *Agkistrodon contortrix mokasen* from each of 5 main dens within the Central Connecticut study site assigned to each of 6 genetic clusters (K) identified by the Bayesian clustering algorithm employed in STRUCTURE 2.1. N is equal to the number of individuals sampled from each den. Dark Blue = Cluster 1, Maroon = Cluster 2, Green = Cluster 3, Purple = Cluster 4, Light Blue = Cluster 5, Orange = Cluster 6.

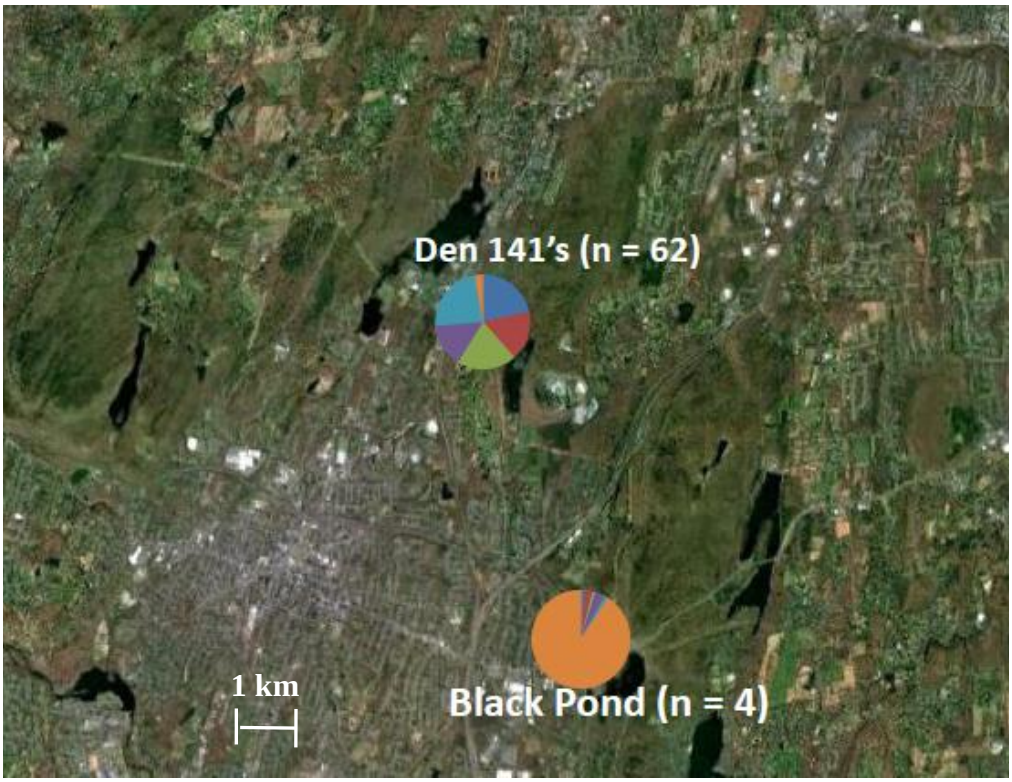

FIGURE 4. Genetic structure of *Agkistrodon contortrix mokasen* at the main study site (represented by Den 141s) differs from that of Black Pond Den, 3 kilometers distant. N is equal to the number of individuals sampled from each den. Dark Blue = Cluster 1, Maroon = Cluster 2, Green = Cluster 3, Purple = Cluster 4, Light Blue = Cluster 5, Orange = Cluster 6.

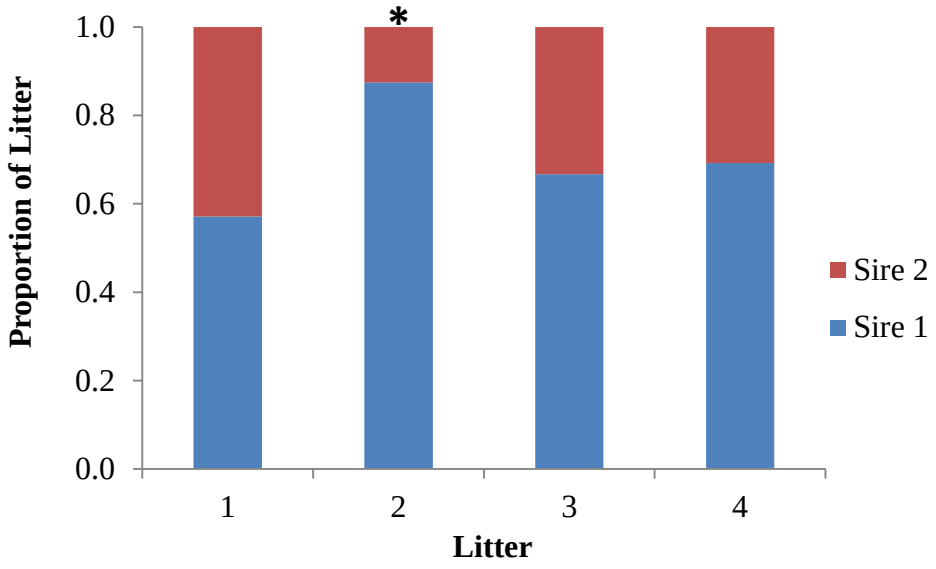

FIGURE 5. The proportion of offspring sired by each male in four Connecticut *Agkistrodon contortrix mokasen* multiple paternity litters as identified by paternity analyses of 22 tetra-nucleotide microsatellite loci. Litters with significant paternal skew are indicated with an asterix. The number of offspring in litters one through four are seven, eight, six, and 13, respectively.

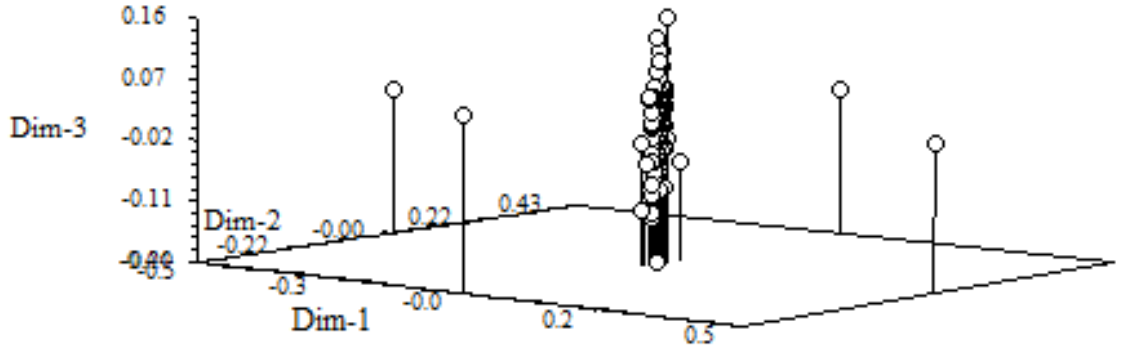

FIGURE 6. A plot of the results of a Principle Coordinates Analysis conducted on a dissimilarity matrix of pairwise relatedness values of 116 adult Northern Copperheads (*Agkistrodon contortrix mokasen*) from a central Connecticut population. Dim-1 = Principle Coordinates Axis 1, Dim-2 = Principle Coordinates Axis 2, and Dim-3 = Principle Coordinates Axis 3. Individuals are represented as empty circles.

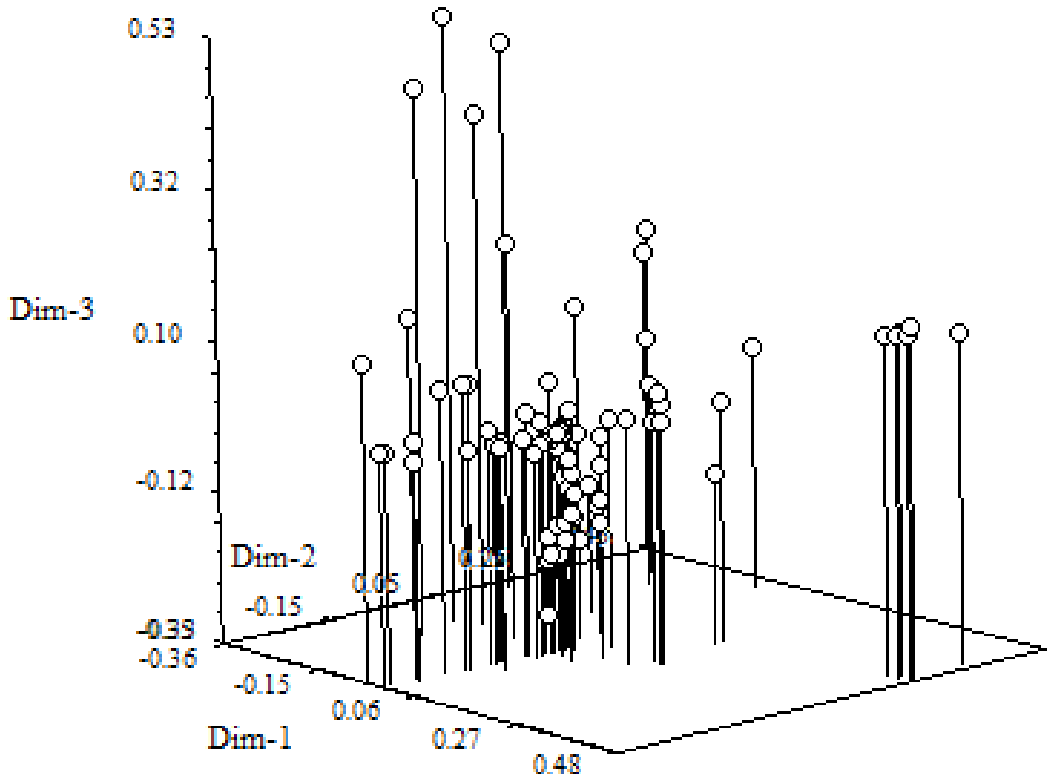

FIGURE 7. A plot of the results of a Principle Coordinates Analysis conducted on a similarity matrix of relatedness values (coded from relationship categories) among 99 adult Northern Copperheads (*Agkistrodon contortrix mokasen*) belonging to a family group from a central Connecticut population. Dim-1 = Principle Coordinates Axis 1, Dim-2 = Principle Coordinates Axis 2, and Dim-3 = Principle Coordinates Axis 3. Individuals are represented by the empty circles in the plot.

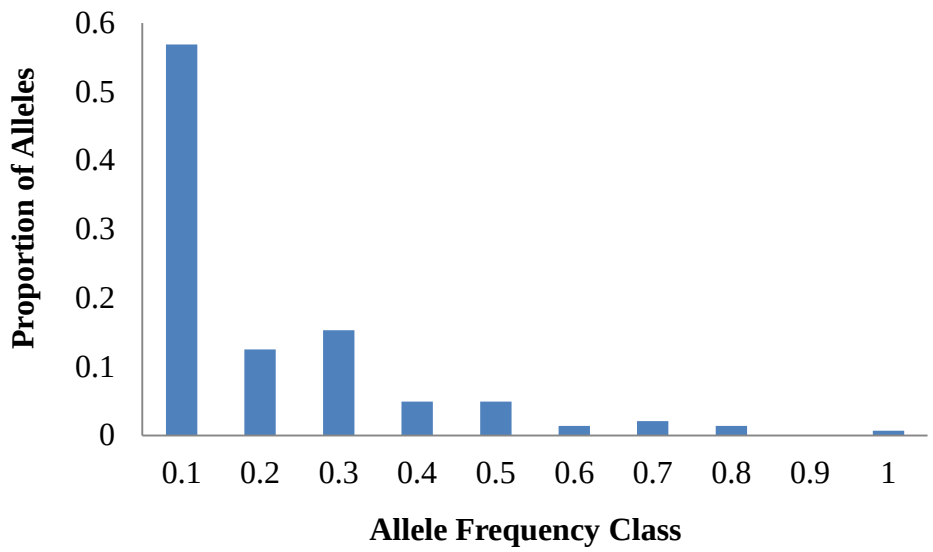

FIGURE 8. The proportion of alleles in 22 microsatellite DNA loci apportioned by frequency class for 116 adult *Agkistrodon contortrix mokasen* from the central Connecticut study population. The L-shaped distribution indicates a bottleneck history extending beyond the previous few dozen generations.

VIII. APPENDICES

APPENDIX 1. Species used for optimization of 23 tetra-nucleotide microsatellite primers for a population genetics study of a Connecticut population of *Agkistrodon contortrix mokasen*. *Negative* signifies a blank lane used for quality control.

DNA Samples (20 ng/uL)		
Lane	**Species**	**Common Name**
1	*A. contortrix* (Texas)	Southern Copperhead
2	*A. contortrix* (Nebraska)	Osage Copperhead
3	*A. contortrix* (Pennsylvania)	Northern Copperhead
4	*A. contortrix* (Connecticut)	Northern Copperhead
5	*A. contortrix* (Connecticut)	Northern Copperhead
6	*A. piscivorus* (Texas)	Western Cottonmouth
7	*A. piscivorus* (Florida)	Florida Cottonmouth
8	*A. piscivorus* (Kentucky)	Western Cottonmouth
9	*Sistrurus catenatus*	Massasauga Rattlesnake
10	*Crotalus lepidus klauberi*	Banded Rock Rattlesnake
11	*C. willardi willardi*	Arizona Ridge-Nose Rattlesnake
12	*C. horridus*	Timber Rattlesnake
13	*C. viridis viridis*	Prairie Rattlesnake
14	*C. oreganus oreganus*	Northern Pacific Rattlesnake
15	*C. atrox*	Western Diamond-Backed Rattlesnake
16	*Negative*	Negative

APPENDIX 2. *Agkistrodon contortrix mokasen* DNA samples (N = 253) were collected from dens (N = 9) located in Central Connecticut (= yellow star) for genotyping at 23 tetra-nucleotide microsatellite loci.

APPENDIX 3. Eighty-five percent of adult DNA samples genotyped in a population genetic study of *Agkistrodon contortrix mokasen* were collected from five dens (= yellow star) (Middle Lamentation (n = 7), Den 141's (n = 62), Point of Lamentation (n = 14), Middle Chauncey (n = 10), and C29 Chauncey Slide (n = 7)) in Central Connecticut.

APPENDIX 4. Twenty-four *Agkistrodon contortrix mokasen* tetra-nucleotide microsatellite primers (= Locus) were grouped into six multiplexes for microsatellite amplification for a genetic study of a Connecticut population. Dye = fluorescent dye that binds to microsatellite primer for identification of loci after genotyping.

Multiplex	Locus	Dye
MP1	AGK-01	6FAM
	AGK-04	JOE NHS Ester
	AGK-03	TAMRA NHS Ester
	AGK-12	ROX NHS Ester
MP2	AGK-11	6FAM
	AGK-09	JOE NHS Ester
	AGK-22	TAMRA NHS Ester
	AGK-10	ROX NHS Ester
MP3	AGK-26	6FAM
	AGK-13	JOE NHS Ester
	AGK-21	TAMRA NHS Ester
	AGK-19	ROX NHS Ester
MP4	AGK-33	6FAM

	AGK-46	JOE NHS Ester
	AGK-38	TAMRA NHS Ester
	AGK-16	ROX NHS Ester
	AGK-29	6FAM
MP5	AGK-31	JOE NHS Ester
	AGK-39	TAMRA NHS Ester
	AGK-44	ROX NHS Ester
	AGK-28	6FAM
MP6	AGK-24	JOE NHS Ester
	AGK-36	TAMRA NHS Ester
	AGK-20	ROX NHS Ester

APPENDIX 5. Expected (H_e) and observed (H_o) heterozygosities for each of 23 microsatellite loci in a Connecticut population of *Agkistrodon contortrix mokasen*. Loci with significant departures from Hardy-Weinberg Equilibrium are in bold and denoted with an asterisk, and were not included in further population genetic analyses.

Locus	**H_e**	**H_o**
AGK-01	0.421	0.362
AGK-03	0.681	0.655
AGK-04	0.503	0.552
AGK-09	0.679	0.655
AGK-10	0.702	0.647
AGK-11	0.731	0.733
AGK-12	0.723	0.716
AGK-13	0.082	0.086
AGK-16	0.752	0.724
AGK-19	0.763	0.828
AGK-20	0.554	0.543
AGK-21	0.722	0.750
AGK-22	0.412	0.448
AGK-24	0.771	0.707
AGK-26	0.712	0.759
AGK-28	0.852	0.845
AGK-29	0.784	0.784
AGK-31	0.705	0.767

AGK-33	0.630	0.600
AGK-36	**0.656***	**0.491***
AGK-38	0.691	0.681
AGK-39	0.698	0.647
AGK-44	0.397	0.422

APPENDIX 6. Diversity and frequency of alleles (= Allele) at each of 23 tetra-nucleotide microsatellite loci (= Locus) in adult (N = 116) and juvenile (N = 137) *Agkistrodon contortrix mokasen* from a Connecticut population. Loci with deviations from Hardy-Weinberg Equilibrium are denoted with an asterix.

Locus	Allele	Adults	Juveniles
AGK01	134	0.698	0.819
	138	0.302	0.181
AGK03	208	0.017	0.000
	212	0.078	0.063
	214	0.220	0.261
	224	0.487	0.496
	244	0.159	0.132
	248	0.039	0.048
AGK04	159	0.181	0.199
	171	0.103	0.165
	179	0.026	0.023
	183	0.672	0.613
	187	0.017	0.000
AGK09	157	0.401	0.436

	161	0.013	0.034
	165	0.371	0.327
	169	0.022	0.011
	173	0.147	0.192
	177	0.004	0.000
	193	0.004	0.000
	205	0.017	0.000
	209	0.004	0.000
	213	0.013	0.000
	217	0.004	0.000
AGK10	251	0.000	0.004
	255	0.013	0.026
	259	0.004	0.000
	263	0.384	0.489
	267	0.026	0.034
	271	0.211	0.239
	275	0.022	0.015
	279	0.323	0.149
	283	0.017	0.045
AGK11	193	0.095	0.059
	197	0.013	0.004

	201	0.009	0.000
	205	0.444	0.444
	209	0.069	0.052
	213	0.220	0.230
	217	0.091	0.156
	221	0.017	0.000
	225	0.034	0.048
	229	0.009	0.007
AGK12	197	0.280	0.239
	201	0.030	0.034
	205	0.341	0.250
	209	0.276	0.409
	213	0.073	0.068
AGK13	176	0.957	0.896
	180	0.043	0.104
AGK16	222	0.013	0.000
	226	0.224	0.202
	230	0.302	0.320
	234	0.181	0.173

	238	0.272	0.305
	242	0.009	0.000
AGK19	239	0.056	0.073
	243	0.289	0.401
	247	0.267	0.252
	251	0.138	0.077
	255	0.246	0.193
	259	0.004	0.004
AGK20	278	0.030	0.038
	282	0.616	0.655
	286	0.142	0.159
	290	0.211	0.148
AGK21	158	0.004	0.000
	174	0.052	0.129
	178	0.280	0.305
	182	0.427	0.305
	186	0.078	0.040
	190	0.060	0.103
	194	0.056	0.040
	198	0.043	0.077

AGK22	129	0.009	0.004
	131	0.004	0.000
	137	0.039	0.052
	141	0.750	0.722
	145	0.052	0.030
	149	0.147	0.193
AGK24	205	0.082	0.094
	209	0.043	0.004
	213	0.306	0.391
	217	0.004	0.004
	221	0.289	0.241
	225	0.034	0.034
	229	0.039	0.023
	233	0.203	0.211
AGK26	103	0.444	0.485
	107	0.013	0.007
	115	0.004	0.000
	119	0.000	0.004
	123	0.241	0.201
	127	0.129	0.146

	131	0.039	0.099
	135	0.121	0.058
	139	0.009	0.000
AGK28	150	0.121	0.109
	154	0.069	0.109
	158	0.022	0.000
	162	0.198	0.120
	166	0.250	0.339
	170	0.043	0.055
	174	0.013	0.004
	178	0.116	0.131
	182	0.086	0.066
	186	0.052	0.037
	190	0.017	0.007
	194	0.013	0.011
	198	0.000	0.011
AGK29	164	0.004	0.000
	168	0.056	0.007
	172	0.284	0.412
	176	0.142	0.151
	180	0.147	0.173

	184	0.293	0.217
	192	0.069	0.040
	196	0.004	0.000
AGK31	113	0.216	0.195
	117	0.039	0.059
	121	0.091	0.099
	125	0.470	0.460
	129	0.073	0.088
	133	0.112	0.099
AGK33	148	0.022	0.019
	150	0.074	0.045
	154	0.278	0.257
	158	0.070	0.049
	162	0.530	0.604
	166	0.026	0.026
AGK36*	244	0.088	0.109
	248	0.046	0.073
	252	0.162	0.128
	256	0.532	0.529
	260	0.157	0.146

	264	0.009	0.015
	268	0.005	0.000
AGK38	119	0.034	0.047
	123	0.069	0.051
	127	0.060	0.015
	131	0.517	0.668
	135	0.095	0.026
	139	0.039	0.077
	143	0.043	0.007
	147	0.138	0.106
	151	0.004	0.004
AGK39	204	0.444	0.346
	208	0.013	0.015
	212	0.013	0.004
	216	0.086	0.033
	220	0.203	0.279
	223	0.004	0.000
	224	0.237	0.324
AGK44	167	0.004	0.000
	171	0.754	0.722

175	0.168	0.207
179	0.073	0.070

APPENDIX 7. Number of alleles per locus (N_a), observed heterozygosity (H_o), and expected heterozygosity (H_e) for 22 tetra-nucleotide microsatellite loci (=Locus) in adult *Agkistrodon contortrix mokasen* (N = 116) from central Connecticut.

Locus	N	N_a	H_o	H_e
AGK01	116	2	0.36	0.42
AGK03	116	6	0.66	0.68
AGK04	116	5	0.55	0.50
AGK09	116	11	0.66	0.68
AGK10	116	8	0.65	0.70
AGK11	116	10	0.73	0.73
AGK12	116	5	0.72	0.72
AGK13	116	2	0.09	0.08
AGK16	116	6	0.72	0.75
AGK19	116	6	0.83	0.76
AGK20	116	4	0.54	0.55
AGK21	116	8	0.75	0.72
AGK22	116	5	0.45	0.41
AGK24	116	8	0.71	0.77
AGK26	116	8	0.76	0.71
AGK28	116	12	0.85	0.85
AGK29	116	8	0.78	0.78
AGK31	116	6	0.77	0.71
AGK33	115	5	0.60	0.63

AGK38	116	9	0.68	0.69
AGK39	116	6	0.65	0.70
AGK44	116	4	0.422	0.397
Mean	-	6.54	0.64	0.64
±SD	-	0.56	0.04	0.04