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**CONSERVATION GENETICS OF THE EASTERN MASSASAUGA
RATTLESNAKE FROM THE CARLYLE LAKE REGION,
CLINTON COUNTY, ILLINOIS**

Final Report

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I. INTRODUCTION AND OBJECTIVES

The Eastern Massasauga Rattlesnake (*Sistrurus catenatus catenatus*) is listed as endangered in Illinois and is a candidate species for federal protection in the United States (Symanski 1998). State listing in Illinois occurred in 1994 and was motivated by a dramatic decrease in the distribution of the Eastern Massasauga from an historic high of at least 26 counties to just eight counties at the time of listing. Even with state protection, the distribution of the Eastern Massasauga has continued to shrink with populations persisting in just three or four counties in 2009. Only one Illinois population, located around the southern end of Carlyle Lake (Clinton Co.) is thought to be stable. Declines of the Cook Co. population led, in 2009, to rescue efforts in which remaining animals are being captured and integrated into a captive breeding program overseen by the Association of Zoos and Aquariums (AZA) through the Eastern Massasauga Species Survival Plan (SSP) (Earnhardt et al. 2009). Other conservation efforts in Illinois include surveys of historic Eastern Massasauga localities, long-term capture-mark-recapture and telemetry studies at Carlyle Lake, formation of the Illinois Eastern Massasauga Recovery Team, and development of a draft recovery plan.

This project addresses genetic issues associated with the conservation of the Eastern Massasauga. Conservation genetics is a sub-discipline of conservation biology in which genetic principles are applied to biodiversity management and protection (Frankham et al. 2002; Allendorf and Luikart 2007; reviewed for snakes by King 2009). Threats to biodiversity often have impacts on genetic variation that put populations and species at increased risk. For example, small population size promotes loss of genetic diversity through stochastic processes (random genetic drift), resulting in increased homozygosity, expression of deleterious recessive alleles, and inbreeding depression (Crnokrak and Roff 1999; Keller and Waller 2002). As a consequence, individual organisms have reduced survival and reproductive potential, leading to further decreases in population size and promoting further loss of genetic diversity; a process termed the 'extinction vortex' (Gilpin and Soulé 1986). Habitat fragmentation compounds the problem by further reducing population size and slowing the rate of gene flow, thus contributing to the extinction vortex. Small population size also reduces the ability of populations to adapt to local environmental conditions. This can be especially problematic when changing environmental conditions (*e.g.*, invasive species, global climate change) impose new challenges. Genetic concerns also arise in the design of captive breeding programs and the use of head-starting, reintroduction, repatriation, and translocation as management tools (Kingsbury and Attum 2009).

The original objectives of this study were narrowly focused on the Eastern Massasauga population at Carlyle Lake, Clinton County, Illinois and were to:

- 1) Determine the population genetic structure of all populations that have been sampled over the last eight years around the southern periphery of the lake.
- 2) Determine the rates of gene flow, the number of effective migrants per generation, and the genetic similarity of the populations.
- 3) Provide the genetic data necessary to strengthen the population viability modeling.

As the project progressed, the need to expand these objectives to address patterns of genetic variation state-wide and range-wide became evident. Motivated by needs identified in the draft

Illinois Eastern Massasauga Rattlesnake Recovery Plan currently in preparation by the Illinois Eastern Massasauga Recovery Team, this report focuses on the following **Revised Objectives**:

- 1) **Provide range-wide genetic information as needed to guide the design of a captive breeding program.**
- 2) **Optimize molecular techniques as necessary to allow analysis of mitochondrial and both neutral and functional nuclear genetic markers.**
- 3) **Characterize levels of genetic variation within remaining Illinois populations and the captive breeding population as needed to provide base-line data for future genetic monitoring.**

The original objectives of this study are incorporated into objective 3 of these revised objectives.

II. MTDNA POPULATION STRUCTURE IN THE EASTERN MASSASAUGA RATTLESNAKE (*SISTRURUS CATENATUS CATENATUS*): IMPLICATIONS FOR THE GENETIC MANAGEMENT OF THE CAPTIVE BREEDING PROGRAM

A fundamental problem faced by conservation biologists and management agencies is to define “management units,” geographic subdivisions of a species’ range that warrant separate management strategies and tactics (Moritz 1994, 1997). Such units may be defined using demographic criteria (geographic areas or clusters of sub-populations whose population growth characteristics are independent of other areas or clusters), genetic criteria (geographic areas or clusters of sub-populations whose population genetic characteristics are independent of other areas or clusters), or political criteria (geographic areas or clusters of sub-populations defined by jurisdictional boundaries). The use of genetic criteria to identify management units is motivated by recognition that organisms living in different geographic areas may be genetically isolated from each other and adapted to local environmental conditions and thus are not interchangeable with each other (Crandall et al. 2000).

In this study, sequence data for the mitochondrial DNA (mtDNA) gene ND2 were generated from tissue samples collected from throughout the range of the Eastern Massasauga (Fig. 1A). A total of 18 haplotypes (distinct mtDNA sequences) were identified. Analyses using statistical parsimony (Clement et al. 2000) and Geneland (Guillot et al. 2008) identified three subunits corresponding to unique geographic regions (Fig. 1). The western subunit (Iowa, Wisconsin, Illinois) is separated from the central subunit (Indiana, southern and central Michigan, Ohio, far southwestern Ontario) by a minimum of three mutational steps. The central subunit is separated from the eastern subunit (New York, Pennsylvania, northern Michigan, remaining portions of Ontario) by the deletion event.

A manuscript describing this study more fully has been submitted for publication in *Conservation Genetics* and is included as an appendix to this report. Implications for the genetic management of the captive breeding population are excerpted below:

Management of captive breeding programs requires balancing demographic and genetic objectives. For the Eastern Massasauga, rapid population increase might best be achieved by treating the captive population as a single unit in order to immediately maximize the number of breeding individuals. In contrast,

maintaining patterns of genetic variation found in wild populations (e.g., for possible reintroduction or augmentation) requires managing the three subunits identified here separately and may mean that breeding by some individuals is delayed. Furthermore, the number of founders per subgroup is necessarily less than that of the entire population. In opting for this second strategy, developers of the Eastern Massasauga SSP noted that even with a subdivided captive population, 93% of the genetic diversity (expected heterozygosity) of the founders should be retained in the descendant population.

As of January 2010, records of 63 living animals were included in the Eastern Massasauga North American regional studbook (D. Mulkerin, pers.obs.). Of these, 55 can be assigned to geographic subunits based on observed or inferred haplotype or site of origin. These include 12, 32, and 11 animals belonging to the western, central, and eastern subunits, respectively. Three of the nine central subunit haplotypes and two of the four eastern subunit haplotypes are represented. The western subunit is without variation within the living managed population with only one of five haplotypes represented. By increasing number of founders and diversity of haplotypes through cooperation with non-AZA facilities (e.g., Nature Centers), law enforcement agencies, and other sources, genetic diversity of the managed population would increase and improve the long-term prospects for genetic health.

From Ray et al., submitted.

Based on this analysis, the Eastern Massasauga SSP (Earnhardt et al. 2009) specifies a captive breeding plan designed to maintain these three subunits separately within the captive population. Importantly, the SSP is consistent with the draft Illinois Eastern Massasauga Rattlesnake Recovery Plan (in preparation) which calls for having “a stable or growing population with the appropriate genetic representation in an Association of Zoos and Aquariums (AZA) authorized captive breeding program (i.e., Species Survival Plan).”

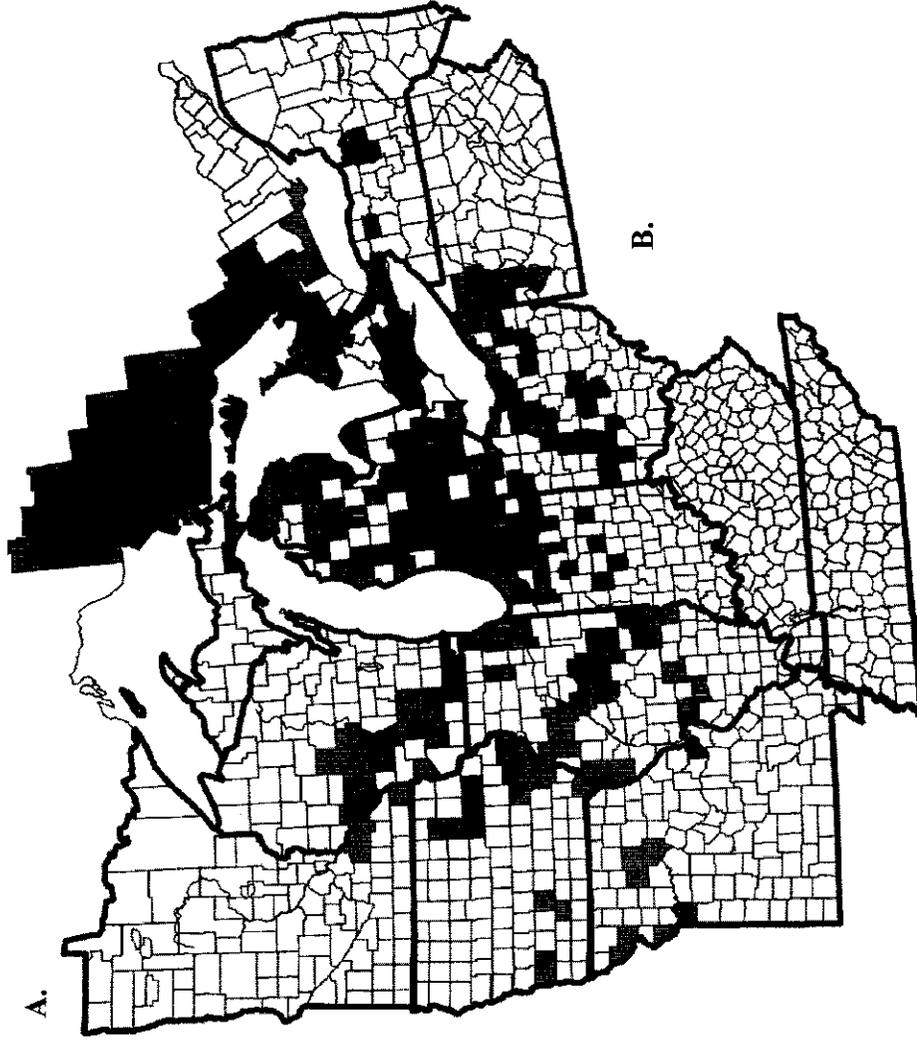


Fig. 1. Genetic structure of the Eastern Massasauga based on mtDNA ND2 sequence data. **A.** Historic range (by county in gray, adapted from Villeneuve 1988; Symanzki 1998) and distribution of genetic subunits (color) of the Eastern Massasauga. Numbers indicate sample sizes from a given county or cluster of counties. **B.** Statistical parsimony network of 18 ND2 haplotypes. Circle sizes are proportional to number of individuals sharing a given haplotype. Black dots represent unrepresented haplotypes; a three-base deletion separates haplotypes 5 and 13, represented by a dashed line.

III. MOLECULAR TECHNIQUES FOR THE ANALYSIS OF NEUTRAL AND FUNCTIONAL NUCLEAR GENETIC MARKERS

Conservation geneticists use a variety of genetic markers to guide biodiversity management and protection. Sequences of the mtDNA gene ND2 described in the previous section represent one type a genetic marker. Other classes of genetic markers include microsatellite DNA and functional genes contained within the nucleus. In animals, mtDNA and nuclear DNA (nuDNA) differ in that mtDNA is haploid (occurring as a single copy) and maternally inherited whereas nuDNA is diploid (occurring in two copies) and biparentally inherited. Furthermore, microsatellite DNA, which consists of short (2-6 nucleotide) units repeated many times in succession, is neither transcribed nor translated into proteins and so is said to be selectively neutral. That is, differences among individuals in genetic constitution at a particular microsatellite DNA locus have no effect on their ability to survive and reproduce. In contrast, differences in functional genes may influence survival and reproduction and thus be the target of natural selection.

Different types of genetic markers can provide different kinds of information relevant to the genetic management of wild and captive populations. For example, the mtDNA sequence data described in the previous section provides a useful characterization of range-wide genetic differentiation in the Eastern Massasauga. In contrast, microsatellite DNA is more useful for revealing genetic differentiation on a finer geographic scale, for assessing whether small population size is contributing to a loss of genetic variation, and for determining relatedness among individuals as might be used in the design of a captive breeding program. Finally, variation in specific functional genes can provide insights into patterns of local adaptation not evident from neutral markers.

In order to have a variety of tools for use in conservation genetic analysis of Eastern Massasaugas, efforts to optimize molecular techniques for use with mtDNA, microsatellite DNA, and a functional nuclear gene were undertaken. Methods for analysis of the mtDNA gene ND2 are summarized in the appendix and will not be discussed further here. Progress with other marker classes is summarized below.

- A. *Microsatellite DNA Loci.* – A large number of microsatellite loci have been developed for use in a wide array of snakes (King 2009). Currently, primer sequences for nine microsatellite loci developed specifically for use in the Eastern Massasaugas are available (Gibbs et al. 1997, 1998; Kropiewnicki 2008). An additional set of primer sequences developed for use in the Desert Massasauga, *Sistrurus catenatus edwardsii* (Anderson et al. 2009) and 20 sets of primer sequences developed for other rattlesnakes, *Crotalus spp.* (reviewed by King 2009) may prove useful in Eastern Massasaugas (e.g., Bushar et al. 2001).

Seven microsatellite loci have been selected to characterize genetic variation in free-ranging Illinois populations and the captive population of Eastern Massasauga. To facilitate comparison with existing data from other populations, this work is being carried out collaboratively with Dr. Bradley Swanson, Central Michigan University. Dr. Swanson's lab recently completed a state-wide microsatellite DNA survey of Eastern

Massasaugas in Michigan (Kropiewnicki 2008). DNA extraction and polymerase chain reaction (PCR) amplification of microsatellite DNA loci is done at NIU and fragment analysis of PCR products is done at CMU. To date, PCR conditions have been optimized to give reliable results for all seven loci and a sample of animals from Piatt County, Illinois have been scored for four loci (next section).

- B. *Functional Nuclear Gene.* – There are a large number of functional nuclear genes and gene complexes that might provide insight into patterns of local adaptation and thus complement available mtDNA and microsatellite DNA markers. One such gene class that has proven useful in conservation genetic analyses is the Major Histocompatibility Complex (MHC). MHC genes are involved in animal immune responses, are highly variable, and are anticipated to evolve rapidly in response to changing disease, pathogen, and parasite assaults. Thus, reduced MHC variation, as might result from small population size, may be a useful indicator of the need for genetic management through translocation of animals among isolated wild populations or augmentation of wild populations with captive breed animals.

MHC genes have been well characterized in birds and mammals but relatively little work has been done in amphibians and reptiles (but see Madsen and Újvári 2006). Based on other studies, *MHC Class IIB exon 2* was selected for this project. *MHC Class IIB exon 2* encodes the antigen-binding site of the β chain protein. Using primers developed for use in birds and crocodylians (Edwards et al. 1995), a sequence of about 160 nucleotide pairs has been successfully amplified in the Eastern Massasauga and its identity confirmed by sequencing and alignment with *MHC Class IIB exon 2* sequences of other vertebrates. Because the MHC consists of a family of highly similar genes, several of which are likely to be amplified using PCR, additional steps are necessary to distinguish among related genes and to characterize patterns of variation at each. Two techniques for separating PCR products are being used; single strand confirmation polymorphism (SSCP) analysis and denaturing gradient gel electrophoresis (DGGE) (Knapp 2005). While techniques for use with Eastern Massasauga are still being perfected, an initial SSCP analysis of 18 Eastern Massasaugas from Piatt County, Illinois reveals 4-6 distinct banding patterns consistent with individual differences in genotype (Fig. 2). Isolation, purification, and re-amplification of PCR products represented by individual SSCP bands, followed by DNA sequencing, is planned to determine the MHC genotype of individual Eastern Massasaugas.



Fig. 2. Genetic variation in *MHC Class IIB exon 2* among nine Eastern Massasaugas from Piatt County, Illinois (numbered 1-9) as revealed by single strand conformation polymorphism. Arrows highlight bands present in some individuals but not others, reflecting genetic differences among individuals for this gene family.

IV. LEVELS OF GENETIC VARIATION WITHIN FREE-RANGING ILLINOIS POPULATIONS AND THE CAPTIVE POPULATION

Low genetic variation within a population can be a warning sign of isolation and small population size and can lead to further population declines as a result of inbreeding depression (e.g., Madsen et al. 1995). This process can be reversed through management strategies aimed at promoting naturally occurring gene flow among populations (e.g., via habitat corridors) or by human-transport of individuals among populations (= 'genetic restoration'). Notable successes in reversing losses of genetic variability and reproductive failure due to inbreeding depression have been achieved in European adders, Florida panthers, and Illinois prairie chickens (Madsen et al. 1999, 2004; Land and Lacy, 2000; Westemeier et al. 1998). Base-line data on levels of genetic variation together with follow-up genetic monitoring provides managers with the information needed to counter further losses of genetic variation (e.g., by promoting *in situ* population growth), thus potentially avoiding more extreme management actions such as translocation or reintroduction. Furthermore, other kinds of information can be extracted from data collected

through genetic monitoring efforts, including estimates of population size, population trends, and patterns of gene flow. Collection of base-line data on genetic variation in Illinois populations of Eastern Massasaugas is ongoing. Results obtained to date are used to compare levels of genetic variation within Illinois populations

- A. *mtDNA*. – The three geographic subunits described previously (Section II) are each characterized by a single common mtDNA haplotype and three to four relatively rare haplotypes (Table 1). Frequently, a single haplotype is shared by all individuals from a given location although at a number of sites, two or three haplotypes are represented. Larger sample sizes would likely reveal greater haplotype richness within sites. It is worth noting that the Carlyle Lake population (Clinton Co., Illinois) is unusual in that three haplotypes are represented in nearly equal numbers.
- B. *Microsatellite DNA*. – To date, microsatellite DNA loci have been used to provide information on patterns of genetic variation within populations of Eastern Massasauga in Ontario, Ohio, and New York (six loci, Gibbs et al 1997, 1998); Illinois, Indiana, and Ohio (three to four loci, Andre 2003, this study); and Michigan (seven loci, Kropiewnicki 2008) representing 21 distinct sampling locations (Table 2). These data can be used to characterize genetic variation within populations in two ways; allelic richness (the average number of alleles per locus), and observed heterozygosity (the average proportion of individuals that are heterozygous at a given locus).

Among the 21 locations, allelic richness ranges from 2.5 (Kalkaska Co., MI) to 9.3 (Parry Sound District, ONT). Illinois sampling locations fall between these extremes, ranging from 3.3 to 6.0 (Table 2). However, because allelic richness increases with increasing sample size, assessing whether a given site shows greater or less allelic richness than expected requires controlling for sample size (Fig. 2). Based on the relationship between allelic richness and sample size, allelic richness appears to be unexpectedly low (Kalkaska Co., MI) or high (Wyandott Co., OH) at some locations. However, allelic richness at Illinois locations does not appear to be out of the ordinary (Fig. 2).

Observed heterozygosity also shows marked variation among locations, ranging from 0.37 (Kalkaska Co., MI) to 0.65 (Wyandott Co., OH; Kalamazoo Co., MI) (Fig. 3). Two Illinois locations, one in Piatt Co. and one in Clinton Co., fall at the lower end of this distribution with observed heterozygosity of 0.41 and 0.42 (Fig. 3). Should this pattern persist following analysis of additional individuals and loci, possible strategies for genetic management might be considered.

Table 1. Geographic distribution of Eastern Massasauga ND2 haplotypes. Listed are sampling locations (by county and state or province), total number of individuals (n), number of individuals exhibiting a given haplotype, and the number of different haplotypes observed at each location (haplotype richness). Colors correspond to the haplotype groups and geographic subunits shown in Figure 1. Haplotypes 7, 11, 12, and 18 (Figure 1B) were found only among captive animals. These haplotypes are all associated with the central subgroup identified in Figure 1. Three individuals were excluded from this table because only partial sequences were obtained and they could not be assigned unambiguously to one of the haplotypes shown here (Bremer Co., IA – haplotype 1 or 2; Kalkaska Co., MI – haplotype 13 or 14; Venango Co., PA – haplotype 13 or 16).

County, State/Province	n	Haplotype			Haplotypes Richness
	1				1
	4				2
	1				1
	1				1
	4				1
	10				3
	7				1
	4				2
	4				1
	4				1
	2				1
	5				1
	1				1
	5				2
	4				1
	4				3
	2				1
	3				1
	2				1
	4				2
	3				1
	3				1
	9				1
	6				1
	3				1
	6				1
	4				2
	7				1
	1				1
	1				1
Total	115				14

Table 2. Summary of genetic variation in eastern massasauga rattlesnakes based on analysis of three to six microsatellite DNA loci. Number of alleles per locus and observed heterozygosity were averaged across loci.

State/ Province	County (locality)	Sample Size	Number of loci	Number of alleles per locus	Observed heterozygosity
Illinois	Piatt ¹	8	4	3.5	0.41
Illinois	Clinton (SSW) ²	32	3	6.0	0.42
Illinois	Clinton (Hazlet) ²	25	3	5.3	0.47
Illinois	Clinton (Dam) ²	13	3	3.3	0.61
Indiana	LaGrange ²	10	3	5.3	0.60
Ohio	Wyandot ²	10	3	8.3	0.65
Ohio	Clark (Springfield) ³	21	6	7.5	0.47
New York	Onondaga (Cicero Swamp) ³	25	6	4.5	0.45
Ontario	Parry Sound(Killbear NP) ³	80	6	9.3	0.54
Ontario	Simcoe (Beausoleil Island) ³	32	6	6.5	0.51
Ontario	Grey (Bruce Peninsula) ³	41	6	9.0	0.61
Michigan	Allegan ⁴	17	5	4.4	0.55
Michigan	Barry ⁴	15	5	5.0	0.58
Michigan	Kalamazoo ⁴	17	5	4.8	0.65
Michigan	Kalkaska ⁴	12	5	2.5	0.37
Michigan	Lenawee ⁴	20	5	4.8	0.55
Michigan	Livingston ⁴	32	5	5.6	0.60
Michigan	Mackinac ⁴	43	5	3.8	0.52
Michigan	Montmorency ⁴	26	5	4.8	0.58
Michigan	Oakland ⁴	30	5	5.8	0.55
Michigan	Van Buren ⁴	12	5	4.4	0.54

¹this study

²Andre, 2003

³Gibbs et al., 1997

⁴B. Swanson, 2007, unpublished report to Michigan Natural Features Inventory

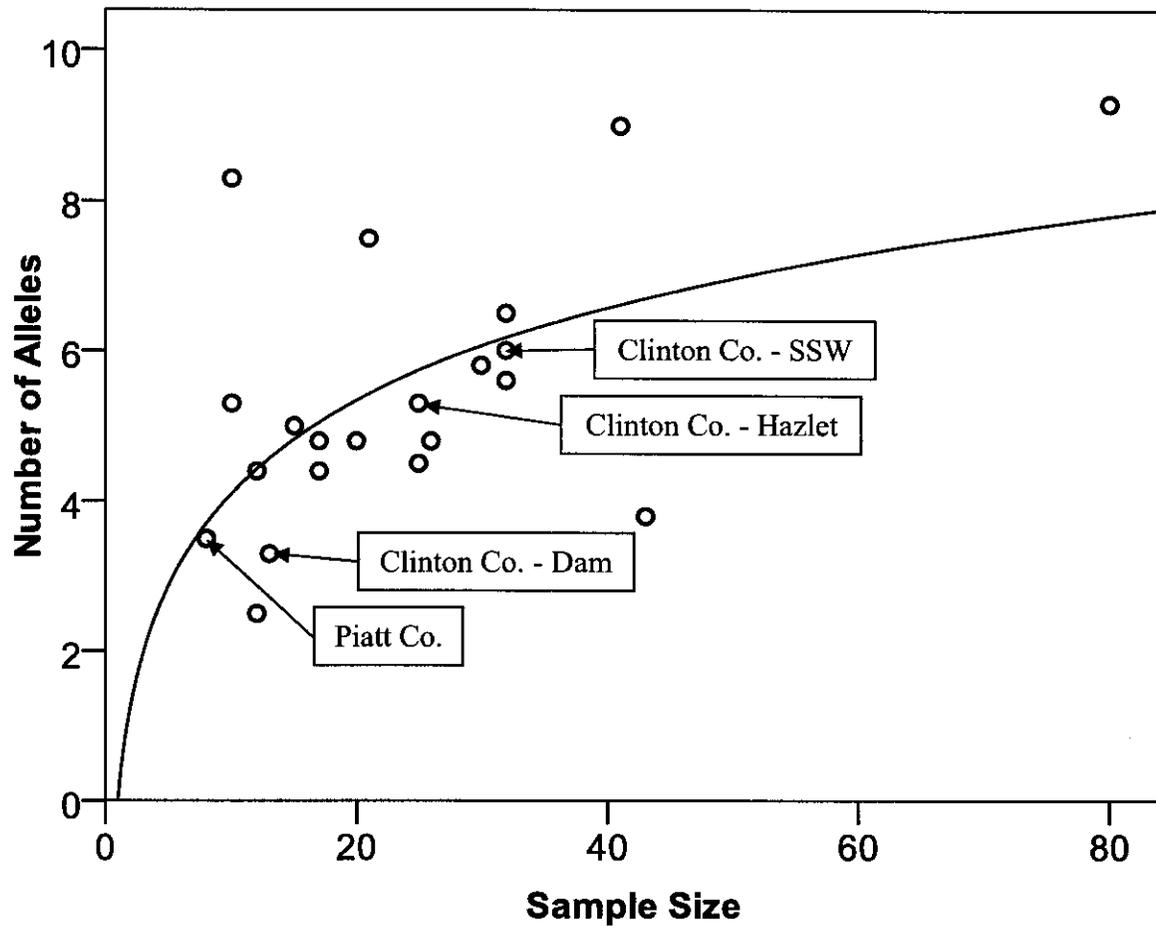


Figure 2. Relationship between sample size and allelic richness (mean number of alleles per locus) based on microsatellite DNA analyses of Eastern Massasaugas from 21 locations (Table 2). Line represents the best-fit logarithmic regression through the origin ($r^2 = 0.93$, $P < 0.001$). Four Illinois sampling locations, three in Clinton Co. and one in Piatt Co., are labeled.

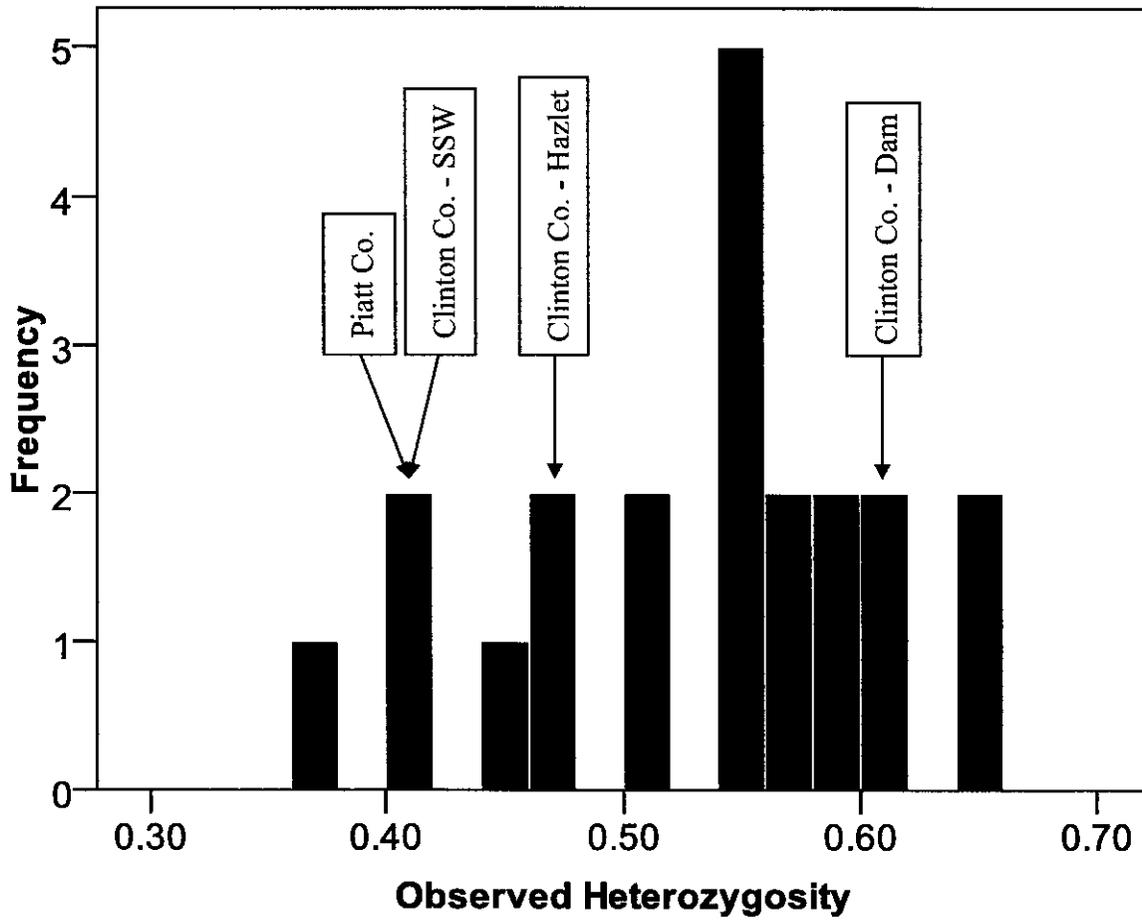


Figure 3. Frequency distribution of observed heterozygosity based on microsatellite DNA analyses of Eastern Massasaugas from 21 locations (Table 2). Four Illinois sampling locations, three in Clinton Co. and one in Piatt Co., are labeled.

V. ONGOING WORK

Genetic analysis of Illinois Eastern Massasaugas is ongoing. One immediate goal is to complete the characterization of genetic variation within the Cook Co. population, Piatt Co. population, and three Clinton Co. populations (Hazlet, Dam, SSW) using seven microsatellite DNA loci and target sample sizes of 30 individuals per population or subpopulation. [Given the current status of the Cook Co. population, it is likely that fewer animals will be sampled from there.] Should tissue samples become available from Eastern Massasaugas elsewhere in Illinois, they will be included in this analysis. Information obtained will provide base-line data for future genetic monitoring, insight into whether loss of genetic variation represents a current threat to any of the Illinois populations, and information on rates of gene flow, especially among Clinton Co. subpopulations. A second immediate goal is to characterize genetic variation within the captive population using these same seven microsatellite DNA loci. This analysis will provide information on relatedness among captives that will aid in the design of captive breeding (some captive individuals are suspected to be siblings). This analysis will also provide information on how well the captive population represents genetic variation seen in the wild, thus guiding acquisition of further individuals for inclusion in the captive breeding program. This work is being conducted by NIU graduate student C. Jaeger in collaboration with field researchers (M. Dreslik and colleagues) and the Applied Technology in Conservation Genetics Laboratory at Central Michigan University (B. Swanson). It is anticipated that the analyses outlined above (seven microsatellite loci for 30 individuals per population or subpopulation and captive animals) will be completed during 2010.

A second, longer-term goal is to characterize variation in the *MHC Class IIB exon 2* gene family in wild populations in Illinois and elsewhere and in the captive population using methods developed during this project (section III). Information gained in this analysis will provide insight into the degree of local adaptation within populations and can be used in making decisions about possible augmentation, translocation, and reintroduction. This analysis will also provide additional information on how well the captive population represents genetic variation seen in the wild. This work is being conducted by NIU graduate student C. Jaeger. It is anticipated that information on MHC variability within Illinois populations, based on analysis of ca. 5 individuals from Cook Co., Piatt Co., and Clinton Co. will be available later in 2010. Information on MHC variability from locations outside of Illinois and for the captive population will be generated in subsequent years.

A related project being conducted by NIU graduate student C. Jaeger in collaboration with B. Jellen (now a graduate student at St. Louis University) involves using microsatellite DNA markers to assess the frequency with which multiple paternity occurs within litters of newborn Eastern Massasaugas. Currently, tissue samples from females and their offspring from a study site in Pennsylvania are being used in this analysis (collected at by B. Jellen). However, results are expected to apply to populations throughout the snakes' range. Information obtained from this analysis will provide insight into the breeding system of the Eastern Massasauga which can be incorporated into population projections. If warranted, tissue samples from females and their offspring from the Carlyle Lake population will also be analyzed as they become available.

Finally, long-term collection of tissue samples from the Carlyle Lake population, now spanning a decade, provides an opportunity to assess genetic changes that have occurred over time and to build a marker-based pedigree for this population. With this in mind, the initial characterization of genetic variation at Carlyle Lake is focusing on samples collected in 2005 and 2006, thus allowing for future comparisons with samples collected earlier and later in time.

Together with the information included in this report, results from ongoing work will provide a strong empirical basis for the genetic management of wild Illinois and captive populations of the Eastern Massasauga and a baseline to which the results for future monitoring efforts can be compared. These results will be provided to the IDNR as they become available.

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APPENDIX

**MTDNA POPULATION STRUCTURE OF THE EASTERN MASSASAUGA RATTLESNAKE
(*SISTRURUS CATENATUS CATENATUS*): IMPLICATIONS FOR THE GENETIC
MANAGEMENT OF CAPTIVE BREEDING PROGRAM**

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Title mtDNA population structure of the Eastern Massasauga Rattlesnake (*Sistrurus catenatus catenatus*): implications for genetic management of the captive breeding program

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Abstract The Eastern Massasauga Rattlesnake is a declining species with a recently established captive breeding program. In order to effectively manage wild and captive populations, an understanding of the genetic diversity within the species is necessary. We analyzed mtDNA sequences of 147 individuals representing the geographic distribution of wild snakes and including most members of the captive population. Eighteen different haplotypes were found, comprising three geographically and genetically distinct subunits and reflecting post-Pleistocene range expansion from unglaciated to glaciated regions. In order to maintain natural genetic variation, preserve diversity in captive lineages, and allow future augmentation or reintroduction, these subunits are being maintained separately within the captive breeding program.

Keywords Biogeography, Conservation genetics, Landscape genetics, Management units, ND2

Introduction

Genetic analyses of species of conservation concern are accumulating rapidly but their utility in guiding management is sometimes unclear, making the associated cost difficult to justify when funding is limited (Howes et al. 2009). One place where conservation genetic analyses have clear management implications is in the design and implementation of captive breeding and reintroduction programs (Ralls and Ballou 2004; Kozfkay et al. 2008; Sharma et al. 2009; Tzika et al. 2009). The Eastern Massasauga (*Sistrurus catenatus catenatus*), a subspecies of Massasauga rattlesnake that ranges across the North American Midwest, is threatened by habitat destruction, fragmentation, and persecution (Szymanski, 1998). Except in Michigan, where it is a species of special concern, *S. c. catenatus* is listed as endangered within each state or province where it occurs (Levell, 1998) and is a candidate for federal listing under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2008). Recently, the Association of Zoos and Aquariums (AZA) established a Species Survival Plan (SSP®) for the Eastern Massasauga aimed at increasing the size and preserving genetic diversity of the captive population for possible use in future reintroduction and augmentation (Earnhardt et al. 2009).

Previous conservation genetic analyses of *S. c. catenatus* using microsatellite DNA loci have demonstrated significant population subdivision over relatively short distances (Gibbs et al. 1997; Andre 2003; Kropiewnicki 2008). However, range-wide analyses designed to identify management units or guide captive breeding and reintroduction have not been conducted. We utilized mitochondrial DNA (mtDNA) sequence data to identify genetically defined geographic subunits, characterize representation of those subunits among captives, and provide recommendations regarding captive breeding. We anticipate that our results will also provide a genetic basis for reintroduction, augmentation and other population-level management decisions.

Materials and methods

Tissue and DNA samples from across the range of *S. c. catenatus* were provided by museums, colleagues, and SSP participating institutions (Ray 2009, Supplementary Table 1). DNA was extracted using DNeasy Blood and Tissue Kits and Puregene Core Kits (Qiagen, Inc., Valencia, CA) following manufacturer protocols. DNA extracts were used as templates for polymerase chain reaction (PCR) amplification of the mitochondrial gene NADH dehydrogenase subunit II (ND2). PCR reactions were performed using Fail-safe PCR systems premix H (Epicentre Technologies Corp., Madison, WI) with primers CE2330 and CE2331 (Janzen et al. 2002). Two related taxa were included for comparison, *S. c. tergitinus* (Western Massasauga) and *S. c. edwardsii* (Desert Massasauga).

Amplification reactions began with a 2-min denaturing step at 94° C followed by 40 cycles of amplification using a 30 sec denaturation step at 94° C, a 30 sec primer annealing step at 50° C, and a 1.5-min elongation step at 72° C. After a final 7-min elongation period at 72° C, PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, OH) and sequenced using a commercial service (Macrogen, Inc., Seoul, South Korea). Sequencing reactions used amplification primer CE2330 and an internal primer, L5238s (ACT TGA CAG AAA ATT GCC CCC) modified from the L5238 primer (de Quieroz et al. 2002) and resulted in sequences of 922 bases.

Sequences were inspected, trimmed and aligned using Geneious (Drummond et al. 2006). TCS 1.21 (Clement et al. 2000) was used to identify haplotypes and create a haplotype network connected at a minimum 95% significance level. Geneland 1.0.7 (Guillot et al. 2008) was used to analyze the spatial pattern of genetic variation and infer number of populations (K). Estimates of K were generated using five replicates of 100,000 iterations with a thinning value of 100 and post-sampling burn-in of 100 iterations. Possible values of K were allowed to vary from 1 to 32 (the total number of separate geographic locations sampled) with geographic uncertainty of approximately 5 km.

Results

One hundred fifty-three individuals were sequenced, 147 Eastern Massasaugas, one Western Massasauga, one Desert Massasauga, and four captive individuals of uncertain identity. Eastern Massasaugas differed from Western and Desert Massasaugas by more than 100 nucleotides whereas Western and Desert Massasaugas differed from each other by nine nucleotides. During sequence alignment, four captives lacking locality data (two law enforcement confiscations and two that originated from a private collection) showed genetic profiles similar to Western or Desert Massasaugas. Although these four had tentatively been identified as Eastern Massasaugas, they have since been excluded from the Eastern Massasauga SSP.

Among the 147 Eastern Massasauga sequences, 19 synonymously variable bases and one deletion event were identified, resulting in 18 haplotypes (GenBank Accession No. GQ359794 – GQ359809). Four haplotypes contained the same inferred 3-base deletion, the location and length of which did not alter the reading frame. Whereas other haplotypes contained the tandem repeat CCTCCT, these haplotypes contained only one copy, CCT, suggestive of a deletion due to slipped-strand mispairing.

Statistical parsimony resulted in identification of three subunits (Fig. 1) corresponding to unique geographic regions (Fig. 2). The western subunit (Iowa, Wisconsin, Illinois) is separated from the central subunit (Indiana, southern and central Michigan, Ohio, far southwestern Ontario) by a minimum of three mutational steps. The central subunit is separated from the eastern subunit (New York, Pennsylvania, northern Michigan, remaining portions of Ontario) by the deletion event.

Analyses of spatial patterning used 100 Eastern Massasauga sequences for which geographic data were available. Geneland indicated that $K=3$ had the highest probability (mean posterior probability = 0.58, SE = 0.015; Fig. 3). Populations inferred using Geneland corresponded completely with subunits identified using statistical parsimony (Fig. 2).

Discussion

Our analysis reveals the existence of three geographic subunits within the Eastern Massasauga (Fig. 2). A lack of samples from northwestern Indiana, where the Eastern Massasauga is now rare or absent, makes it difficult to determine where the boundary between the western and central subunits lies. However, boundaries between the central and eastern subunit fall between collection sites separated by about 32 km in Crawford and Kalkaska Co. in northern Michigan and about 64 km in Ashtabula Co., Ohio and Butler and Venango Co., Pennsylvania.

Based on glacial history and inferred directionality of the deletion event, we interpret this geographic pattern as resulting from range expansion from unglaciated (Iowa, Wisconsin, central and southern Illinois) to glaciated regions. Assuming that the deletion occurred just once, which is likely given the scarcity of indels in ND2 (no other instances observed), three alternative range expansion scenarios are possible. Range expansion may have proceeded clockwise around Lake Huron, Georgian Bay, Lake Erie, and Lake Ontario from northern Michigan into Ontario, New York, and Pennsylvania. Alternatively, range expansion may have proceeded counterclockwise around these lakes from Ohio into Pennsylvania, New York, Ontario, and northern Michigan. Finally, range expansion may have followed a bifurcating route, proceeding from southern Michigan into southwestern Ontario (between Lake Erie and Lake Huron) and then counterclockwise around Lake Huron and Georgian Bay into northern Michigan and clockwise around Lake Ontario into New York and Pennsylvania. Clear barriers to range expansion along these routes are lacking except for an elevation increase of ca. 100 m going from sites in Ashtabula Co., Ohio to those in Butler and Venango Co., Pennsylvania. At present, genetic data do not allow us to distinguish among these alternatives.

Management of captive breeding programs requires balancing demographic and genetic objectives. For the Eastern Massasauga, rapid population increase might best be achieved by treating the captive population as a single unit in order to immediately maximize the number of breeding individuals. In contrast, maintaining patterns of genetic variation found in wild populations (e.g., for possible reintroduction or augmentation) requires managing the three subunits and may mean that breeding by some individuals is delayed. Furthermore, the number of founders per subgroup is necessarily less than that of the entire population. In opting for this second strategy, developers of the Eastern Massasauga SSP noted that even with a subdivided captive population, 93% of the genetic diversity (expected heterozygosity) of the founders should be retained in the descendant population.

As of January 2010, records of 63 living animals were included in the Eastern Massasauga North American regional studbook (D. Mulkerin, pers.obs.). Of these, 55 can be assigned to geographic subunits based on observed or inferred haplotype or site of origin. These include 12, 32, and 11 animals belonging to the western, central, and eastern subunits, respectively. Three of nine central subunit haplotypes and two of four eastern subunit haplotypes are represented. The western subunit is without variation within the captive population with only one of five haplotypes represented. Increasing the number of founders and diversity of haplotypes through cooperation with non-AZA facilities (e.g., Nature Centers), law enforcement agencies, and other sources, would improve long-term prospects for genetic health.

Use of mtDNA as a marker in phylogeographic and conservation genetic analyses has supporters (e.g., Zink and Barrowclough 2008; Barrowclough and Zink 2009) and critics (e.g., Edwards and Bensch 2009; Galtier et al. 2009). With respect to our analysis, the most relevant concern is whether mtDNA data alone are sufficient to guide captive breeding, reintroduction and augmentation. The strong geographic signal seen in our analyses suggests that the answer is 'yes.' However, we recognize that other markers could yield contradictory results. For example, significant Eastern Massasauga population subdivision seen in microsatellite DNA studies (Gibbs et al. 1997; Andre 2003; Kropiewnicki 2008) might argue for maintaining greater site specificity in captive breeding programs (i.e., more subdivisions within the captive population). Such a strategy might also more successfully maintain locally adapted gene complexes. But these benefits need to be weighed against practical considerations of founder numbers, the desire for rapid captive population growth to minimize loss of genetic variation and adaptation to captivity (Frankham 2008; Robert 2009; Williams and Hoffman 2009), and the need to provide sufficient numbers of animals for release. In fact, use of microsatellite DNA loci for assessing levels of and temporal trends in genetic variation, relatedness, and paternity in the captive population is planned.

Given the recency of glacial retreat and Eastern Massasauga range expansion, other classes of genetic markers such as nuclear gene sequences (nuDNA) may reveal a lack of geographic structure and suggest that subdivision of the captive population is not necessary. Indeed it is just this possibility that favors the use of mtDNA over nuDNA sequence data in analyses such as ours (Zink and Barrowclough 2008; Barrowclough and Zink 2009). Alternatively, nuDNA markers might reveal a geographic signal that differs from that seen in mtDNA, suggesting that different subunits be recognized. Of particular interest would be geographic patterns of differentiation in functional genes such as those contributing to venom composition (e.g., Gibbs et al. 2009) or immune function (e.g., Madsen and Ujvari 2006). Such investigations are underway (venom: Gibbs et al. 2009; major histocompatibility loci: C. P. Jaeger and R. B. King, unpublished) and will allow modification of captive and wild population management as warranted. Such markers will also prove useful as monitoring tools should augmentation and reintroduction become part of future Eastern Massasauga management (e.g., Swanson et al. 2006; Smith and Hughes 2008).

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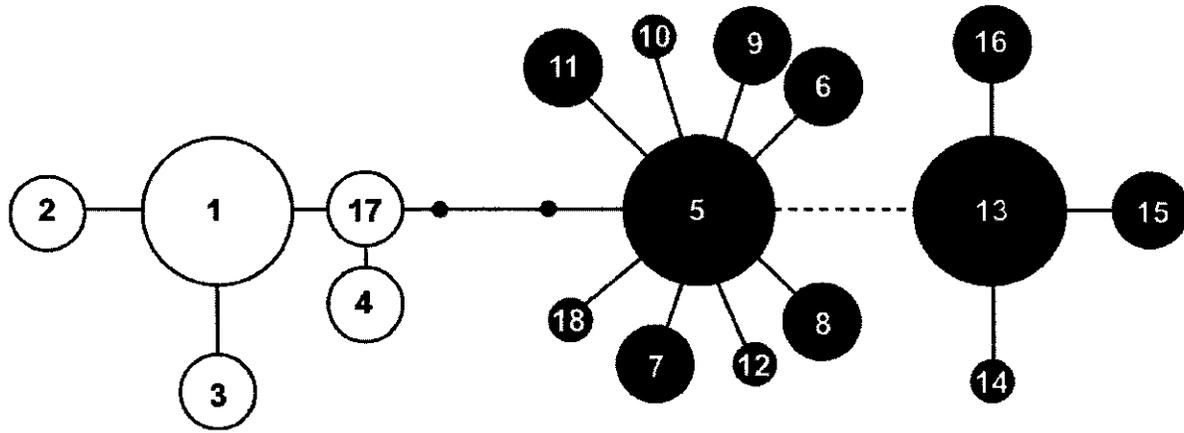
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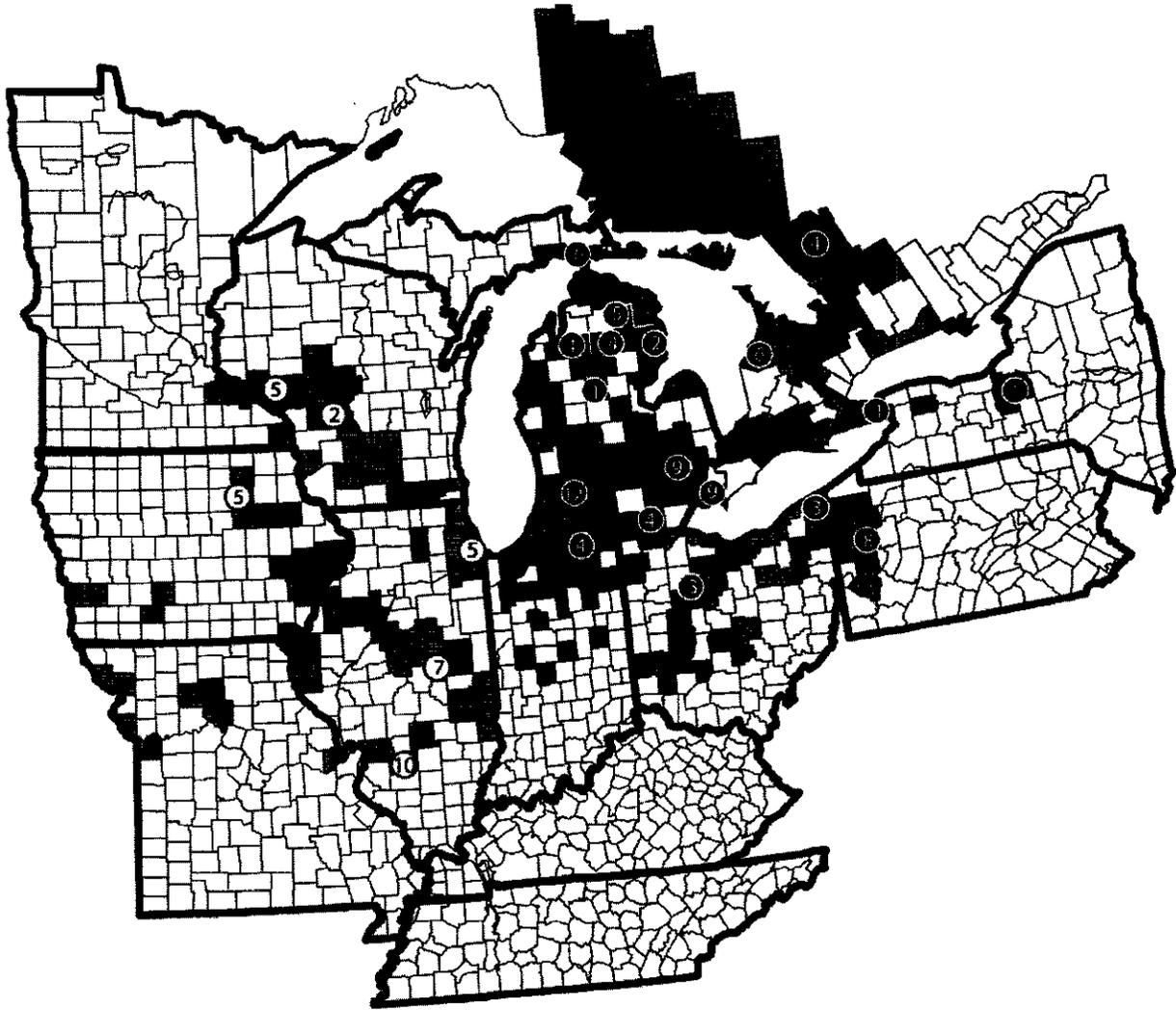
Figure Captions

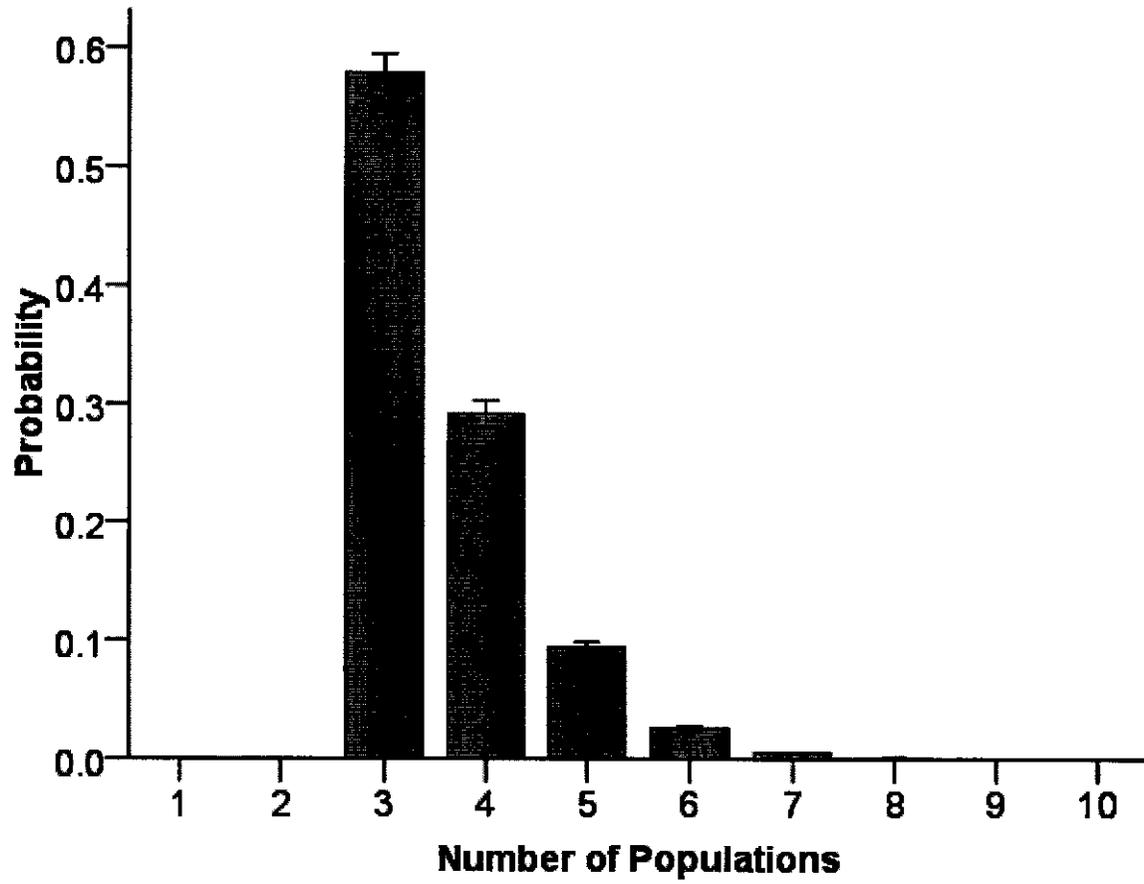
Fig. 1 Statistical parsimony network of Eastern Massasauga ND2 haplotypes. Small, medium, and large symbols represent 1, 2-5, and 19-61 individuals, respectively. Dots denote unrepresented haplotypes; a three-base deletion separates haplotypes 5 and 13 (dashed line)

Fig. 2 Historic Eastern Massasauga range (by county, adapted from Villeneuve 1988; Symanzki 1998) and distribution of mtDNA subunits. Numbers indicate sample sizes from a given county or cluster of counties

Fig. 3 Posterior probabilities (standard errors) of values of K (number of populations) obtained using Geneland







Supplemental Table 1. Source locality, haplotype, and geographic subgroup membership of wild and captive Eastern Massasauga Rattlesnakes. Entries are divided into six data partitions corresponding to wild-caught and captive snakes sequenced for ND2 and for which locality data (State/Province, County) are available (Partition 1), captive snakes sequenced for ND2 for which locality data are lacking (Partition 2), captive snakes for which ND2 haplotype was inferred based on the haplotype of a matrilineal relative (Partition 3), captive snakes for which geographic subgroup was inferred based on collection location (Partition 4), captive snakes for which haplotype and subgroup membership is currently unknown (Partition 5), and Desert and Western Massasauga Rattlesnakes sequenced for ND2 for comparison with Eastern Massaugas (partition 6). Captive snakes are identified by studbook number. Those individuals that were alive and part of captive breeding program as of January 2010 are denoted by a + in the SSP column (a - in this column indicates a captive snake that died). W, C, and E refer to the Western, Central, and Eastern subgroups (Fig. 2). Haplotype numbers correspond to symbols in Fig. 1. Snakes for which incomplete sequences were obtained are identified in the comments column. In all cases, these snakes could be assigned unambiguously to geographic subgroup and most could be assigned unambiguously to haplotype.

Data Partition	ID	State/Province	County	Studbook #	SSP	Subgroup	Haplotype	Comments	DNA/Tissue Provider
1	144	Illinois	Clinton			W	2		M. Dreslik
1	145	Illinois	Clinton			W	2		M. Dreslik
1	842	Illinois	Clinton			W	2		M. Dreslik
1	751	Illinois	Clinton			W	3		M. Dreslik
1	833	Illinois	Clinton			W	3		M. Dreslik
1	834	Illinois	Clinton			W	3		M. Dreslik
1	744	Illinois	Clinton			W	3		M. Dreslik
1	183	Illinois	Clinton			W	4		M. Dreslik
1	733	Illinois	Clinton			W	4		M. Dreslik
1	745	Illinois	Clinton			W	4		M. Dreslik
1	22299	Illinois	Cook	405	+	W	1		AZA
1	Sica Cook 11	Illinois	Cook	406	+	W	1		M. Redmer
1	Sica Cook	Illinois	Cook			W	1		M. Redmer
1	WS 12	Illinois	Cook			W	1	incomplete sequence	M. Redmer
1	Sica Cook 1	Illinois	Cook			W	1		M. Redmer
1	001	Illinois	Platt			W	1		C. Phillips
1	002	Illinois	Platt			W	1		C. Phillips
1	028	Illinois	Platt			W	1		C. Phillips
1	031	Illinois	Platt			W	1		C. Phillips
1	032	Illinois	Platt			W	1		C. Phillips
1	035	Illinois	Platt			W	1		C. Phillips
1	036	Illinois	Platt			W	1		C. Phillips
1	HB086	Iowa	Bremer			W	1		T. VanDeWall
1	HB088	Iowa	Bremer			W	17		T. VanDeWall
1	HB087	Iowa	Bremer			W	17		T. VanDeWall
1	HB085	Iowa	Bremer			W	17		T. VanDeWall
1	Iowa 5	Iowa	Bremer			W	1 or 2	incomplete sequence	T. VanDeWall
1	H1283	Wisconsin	Buffalo	308	+	W	1		AZA
1	H1019	Wisconsin	Juneau	250	-	W	1		AZA

1	MCZ 1073	Wisconsin	Monroe				W	1		C. Berg
1	MO436	Wisconsin	Trempealeau	292	+		W	1		AZA
1	MCZ 1075	Wisconsin	Trempealeau				W	1		C. Berg
1	MCZ 1076	Wisconsin	Trempealeau				W	1		C. Berg
1	MCZ 1079	Wisconsin	Trempealeau				W	3		C. Berg
1	3	Indiana	La Grange				C	5		J. Marshall
1	11	Indiana	La Grange				C	5		J. Marshall
1	7	Indiana	La Grange				C	8		J. Marshall
1	9	Indiana	La Grange				C	8		J. Marshall
1	NM 8504	Michigan	Alcona				C	5	incomplete sequence	B. Swanson
1	NM 4504	Michigan	Alcona				C	5		B. Swanson
1	SALL 04	Michigan	Allegan				C	5		B. Swanson
1	SALL 32	Michigan	Allegan				C	5	incomplete sequence	B. Swanson
1	SALL 0329	Michigan	Allegan				C	5	incomplete sequence	B. Swanson
1	SBARR 04	Michigan	Barry				C	5		B. Swanson
1	SBARR 2842	Michigan	Barry				C	5		B. Swanson
1	SBAFM 01	Michigan	Cass				C	5		B. Swanson
1	SBAFM 03	Michigan	Cass				C	5		B. Swanson
1	NM 81600	Michigan	Clare				C	5		B. Swanson
1	Gray 5	Michigan	Crawford				C	5		C. Smith/B. Kingsbury
1	Gray 6	Michigan	Crawford				C	5		C. Smith/B. Kingsbury
1	Gray 7	Michigan	Crawford				C	5		C. Smith/B. Kingsbury
1	Gray 8	Michigan	Crawford				C	5		C. Smith/B. Kingsbury
1	SGN 6	Michigan	Kalamazoo				C	5		B. Swanson
1	SGN 8	Michigan	Kalamazoo				C	5		B. Swanson
1	Saucro 4	Michigan	Kalamazoo				C	9		B. Swanson
1	SGN 2	Michigan	Kalamazoo				C	10	incomplete sequence	B. Swanson
1	SGN 1	Michigan	Kalamazoo				C	5 or 11	incomplete sequence	B. Swanson
1	IR Crunchy	Michigan	Lenawee				C	5		B. Swanson
1	IR D5F33	Michigan	Lenawee				C	5		B. Swanson
1	IR 36C38	Michigan	Lenawee				C	5		B. Swanson

1	IR 91164	Michigan	Lenawee				C	5	incomplete sequence	B. Swanson
1	SBL 276	Michigan	Livingston				C	5		B. Swanson
1	SBL 41	Michigan	Livingston				C	5		B. Swanson
1	SBL 39	Michigan	Livingston				C	5		B. Swanson
1	SBL 308	Michigan	Livingston				C	5		B. Swanson
1	NRH 001	Michigan	Montmorency				C	5		B. Swanson
1	NRH 082	Michigan	Montmorency				C	5		B. Swanson
1	NRH 094	Michigan	Montmorency				C	5		B. Swanson
1	NRH 373	Michigan	Montmorency				C	5		B. Swanson
1	NRH 97	Michigan	Montmorency				C	5		B. Swanson
1	S822	Michigan	Oakland				C	5		B. Swanson
1	S259-37	Michigan	Oakland				C	5		B. Swanson
1	SHOL 1	Michigan	Oakland				C	5		B. Swanson
1	SSL 867	Michigan	Oakland				C	6		B. Swanson
1	SSL 84	Michigan	Oakland				C	6		B. Swanson
1	Sica 712	Ohio	Ashtabula				C	11		H.L. Gibbs
1	Sica 718	Ohio	Ashtabula				C	11		H.L. Gibbs
1	Sica 729	Ohio	Ashtabula				C	11		H.L. Gibbs
1	Sica 734	Ohio	Wyandot				C	5		H.L. Gibbs
1	Sica 736	Ohio	Wyandot				C	5		H.L. Gibbs
1	Sica 737	Ohio	Wyandot				C	5		H.L. Gibbs
1	38775	Ontario	Essex	328	-		C	5		AZA
1	38784	Ontario	Essex	345	+		C	5		AZA
1	R06028	Ontario	Essex	366	+		C	5	incomplete sequence	AZA
1	778901	Ontario	Essex	367	+		C	5	incomplete sequence	AZA
1	R06029	Ontario	Essex	370	+		C	5	incomplete sequence	AZA
1	SCL758	Ontario	Essex				C	5		S. Loughheed
1	SCL761	Ontario	Essex				C	5		S. Loughheed
1	SCL762	Ontario	Essex				C	5		S. Loughheed
1	SCL771	Ontario	Essex				C	5		S. Loughheed
1	NSM 061	Michigan	Kalkaska				E	13		B. Swanson

1	NSM 292	Michigan	Kalkaska			E	13		B. Swanson
1	NSM 870	Michigan	Kalkaska			E	13		B. Swanson
1	NSM 331	Michigan	Kalkaska			E	13 or 14	incomplete sequence	B. Swanson
1	BOBL 590	Michigan	Mackinac			E	13		B. Swanson
1	BOBL 602	Michigan	Mackinac			E	13		B. Swanson
1	BOBL 829	Michigan	Mackinac			E	13		B. Swanson
1	BOBL 52	Michigan	Mackinac			E	13		B. Swanson
1	BOBL 03-4	Michigan	Mackinac			E	13		B. Swanson
1	BOBL 12-4	Michigan	Mackinac			E	13		B. Swanson
1	Sica 42	New York	Onondaga			E	13		H.L. Gibbs
1	Sica 23	Ontario	Grey			E	13		H.L. Gibbs
1	BPM25	Ontario	Grey			E	13		S. Loughheed
1	BPM26	Ontario	Grey			E	13		S. Loughheed
1	BPM27	Ontario	Grey			E	13		S. Loughheed
1	BPM28	Ontario	Grey			E	13		S. Loughheed
1	BPM32	Ontario	Grey			E	13		S. Loughheed
1	97743	Ontario	Parry Sound	263	-	E	16	incomplete sequence	AZA
1	99R16	Ontario	Parry Sound	271	+	E	13		AZA
1	101433	Ontario	Parry Sound	294	+	E	15	incomplete sequence	AZA
1	Sica 143	Ontario	Parry Sound			E	13		H.L. Gibbs
1	Yagi	Ontario	Welland			E	14		S. Loughheed
1	Sica 187	Pennsylvania	Butler			E	13		B. Jellen
1	Sica 208	Pennsylvania	Butler			E	13		B. Jellen
1	Sica 245	Pennsylvania	Butler			E	13		B. Jellen
1	Sica 339	Pennsylvania	Butler			E	13		B. Jellen
1	Sica 340	Pennsylvania	Butler			E	13		B. Jellen
1	Sica 38	Pennsylvania	Butler			E	13		H.L. Gibbs
1	Sica 41	Pennsylvania	Butler			E	13		H.L. Gibbs
1	Ven 250	Pennsylvania	Venango			E	13	incomplete sequence	B. Jellen
2	6541			242	+	C	5		AZA
2	7022			243	+	C	5		AZA

3					365	+	C	5	haplotype inferred from matrilineal relative	AZA
3					368	+	C	5	haplotype inferred from matrilineal relative	AZA
3					369	+	C	5	haplotype inferred from matrilineal relative	AZA
3	22087				386	+	E	13	haplotype inferred from matrilineal relative	AZA
3					387	+	E	13	haplotype inferred from matrilineal relative	AZA
3	22088				388	+	E	13	haplotype inferred from matrilineal relative	AZA
3	22091				391	+	E	13	haplotype inferred from matrilineal relative	AZA
3	22092				392	+	E	13	haplotype inferred from matrilineal relative	AZA
3					393	+	E	13	haplotype inferred from matrilineal relative	AZA
3					407	+	C	5	haplotype inferred from matrilineal relative	AZA
3					408	+	C	5	haplotype inferred from matrilineal relative	AZA
3					409	+	C	5	haplotype inferred from matrilineal relative	AZA
3					411	+	C	5	haplotype inferred from matrilineal relative	AZA
3					412	+	C	5	haplotype inferred from matrilineal relative	AZA
3					413	+	C	5	haplotype inferred from matrilineal relative	AZA
3					414	+	C	5	haplotype inferred from matrilineal relative	AZA
3					415	+	C	5	haplotype inferred from matrilineal relative	AZA
3	22377				417	+	W	1	haplotype inferred from	AZA

3	22378														matrilineal relative		
					418		+	W		1					haplotype inferred from matrilineal relative		AZA
3	22379				419		+	W		1					haplotype inferred from matrilineal relative		AZA
3	22380				420		+	W		1					haplotype inferred from matrilineal relative		AZA
4					422		+	W							subgroup inferred from collection location		AZA
4			Illinois	Clinton	423		+	W							subgroup inferred from collection location		AZA
4			Illinois	Clinton	424		+	W							subgroup inferred from collection location		AZA
4			Illinois	Clinton	425		+	W							subgroup inferred from collection location		AZA
5					245		+										AZA
5					304		+										AZA
5					394		+										AZA
5					395		+										AZA
5					396		+										AZA
5					401		+										AZA
5					402		+										AZA
5					403		+										AZA
6	R1488														Desert Massasauga		AZA
6	203051														Western Massasauga		AZA
6	203052														Western Massasauga		AZA
6	R1532														Western Massasauga		AZA
6	020985														Western or Desert Massauga		AZA
6	F2														Western or Desert Massauga		R. Carmichael