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Genetic structure of populations of the threatened eastern massasauga rattlesnake, *Sistrurus c. catenatus*: evidence from microsatellite DNA markers

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Abstract

Throughout its distribution in North America, the threatened eastern massasauga rattlesnake (*Sistrurus c. catenatus*) persists in a series of habitat-isolated disjunct populations of varying size. Here, we use six microsatellite DNA loci to generate information on the degree of genetic differentiation between, and the levels of inbreeding within populations to understand how evolutionary processes operate in these populations and aid the development of conservation plans for this species. Samples were collected from 199 individuals from five populations in Ontario, New York and Ohio. Our results show that all sampled populations: (i) differ significantly in allele frequencies even though some populations are < 50 km apart, and may contain genetically distinct subpopulations < 2 km apart; (ii) have an average of 23% of alleles that are population specific; and (iii) have significant F_{IS} values (mean overall F_{IS} = 0.194) probably due to a combination of Wahlund effects resulting from fine-scale genetic differentiation within populations and the presence of null alleles. Our results imply that massasauga populations may be genetically structured on an extremely fine scale even within continuous populations, possibly due to limited dispersal. Additional information is needed to determine if dispersal and mating behaviour within populations can account for this structure and whether the observed differentiation is due to random processes such as drift or to local adaptation. From a conservation perspective, our results imply that these massasauga populations should be managed as demographically independent units and that each has high conservation value in terms of containing unique genetic variation.

Keywords: conservation genetics, eastern massasauga rattlesnakes, inbreeding, microsatellites, population genetic structure

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Introduction

The secretive habits of most snakes have led to a lack of comprehensive information on key features of the population biology of this taxonomic group (Parker & Plummer 1987). This has meant that the information required to develop appropriate recovery strategies for many threatened species and populations of snakes has been unavailable (e.g. Greene & Campbell 1992). Studies of other vertebrates, mainly mammals and birds, have shown how data from mtDNA and, more recently, nuclear DNA markers can provide otherwise unavailable insights on demographic patterns and how genetic variation is partitioned within and among populations (reviewed in Avise 1994; Moritz 1994; Avise & Hamrick 1996). Despite the clear value of such data, few studies using DNA-based markers have been conducted on snakes which are threatened (Gibbs *et al*. 1994; Villarreal *et al*. 1996; Prior *et al*. in press).

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The eastern massasauga rattlesnake, *Sistrurus c. catenatus* is in decline throughout its range in eastern North America (Greene & Campbell 1992) and is classified as threatened in Canada (Weller & Parsons 1991) and unofficially regarded as such in the United States (R. Refschnider, personal communication). Amongst the largest extant populations are those found in two regions of Ontario, Canada: the eastern shore of Georgian Bay and the Bruce Peninsula (see Fig. 1, Weller & Oldham 1993). Apart from moderately large populations that may persist on the lower peninsula of Michigan, relatively small habitat-isolated remnant populations are all that remain in the states of Illinois, Indiana, Iowa, Minnesota, Missouri, New York, Ohio, Pennsylvania, and Wisconsin (see Johnson & Menzies 1993). The current fragmented distribution of massasaugas appears to be a result of several interacting factors including narrow habitat use (Reinert & Kodrich 1982; Weatherhead & Prior 1992), climatic change and the natural succession of vegetation communities (Weller & Oldham 1993; Johnson 1995), and anthropogenic destruction of habitat (Greene & Campbell 1992).

To develop effective recovery strategies for this species it would be useful to know: (i) how genetically distinct isolated populations are from each other and (ii) the level of inbreeding within populations. Such information would provide an empirical basis for defining local populations and conservation management units (Moritz 1994) and for ranking populations *vis-à-vis* their conservation value as well as insights as to the processes which influence levels of genetic variation in snake populations.

In this study, we address these questions with samples collected from five eastern massasauga rattlesnake populations (two from the United States and three from Ontario) and a set of microsatellite DNA markers isolated from this species (Gibbs *et al.* in press). These hypervariable nuclear DNA markers assay variation in the number of repeats, 1–6 bp in size, at a particular locus (Quellar *et al*. 1993). Because microsatellites are diploid markers they offer two advantages over more commonly used mtDNA-based markers. First, with respect to estimates of population differentiation, microsatellites are less sensitive to the effects of sex-specific differences in migration. Secondly, they enable the calculation of *F*-statistics to estimate levels of nonrandom associations of alleles within populations. While microsatellites are

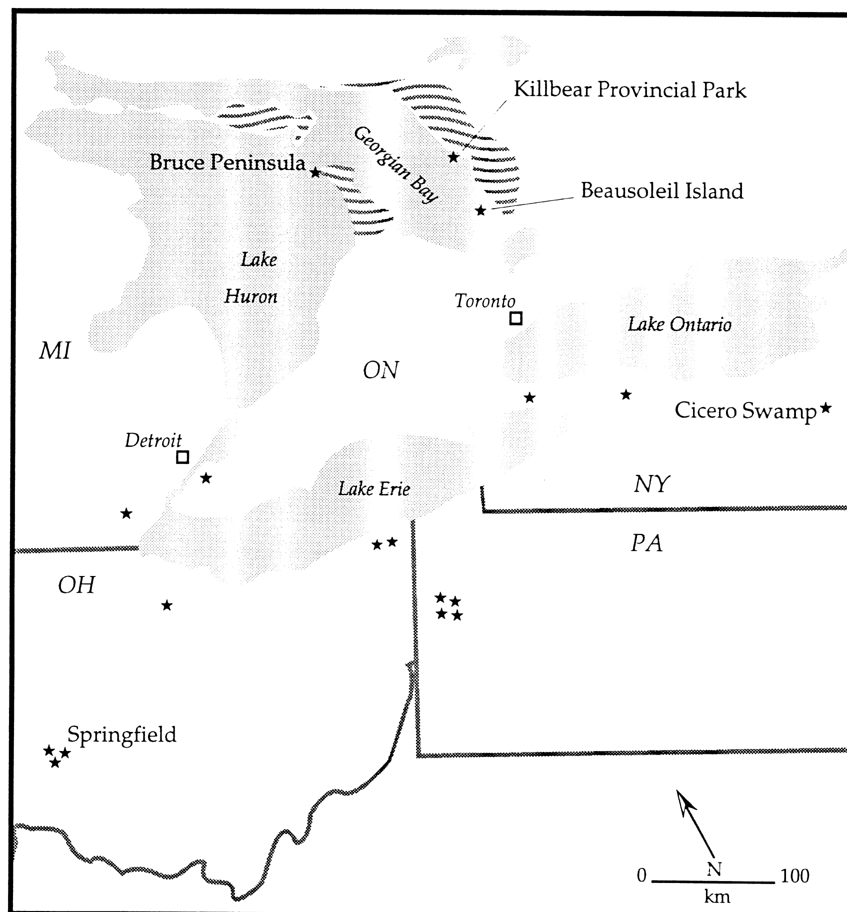

Fig. 1 Distribution of known remnant populations of massasaugas in the Great Lakes region (stars), including those used in the present study: Killbear Provincial Park, Beausoleil Island, Bruce Peninsula National Park, Cicero Swamp, and Springfield. Wavy lines on the eastern shores of Georgian Bay, the Bruce Peninsula, and Manitoulin Island represent the approximate ranges of the largest extant regional populations of this species.

becoming more widely used in conservation genetic analyses for mammals and birds they have rarely been used in studies of threatened reptiles. Indeed, we know of only one study which has isolated microsatellite markers for a snake (i.e. timber rattlesnake, *Crotalus horridus*; Villarreal *et al*. 1996). Thus, our work is amongst the first to illustrate the usefulness of such markers for conservation genetic analyses of snake populations.

Materials and methods

Population samples

Between 1991 and 1994, we and our collaborators collected small samples of blood (50–100 µl) from field-captured adult massasaugas from populations in Ontario and the United States. Upon collection, blood samples were immediately stored in lysis buffer until DNA extraction. At the time of capture, all snakes were permanently marked by caudal scute clipping or passive integrated transponder (PIT) tagging to prevent resampling of individuals at a later date. The samples we obtained were from five geographically distinct populations (see Fig. 1).

1 Bruce Peninsula: 41 individuals from the upper portion (≈ 13 000 ha region) of the Bruce Peninsula, Ontario (81°26′–81°39′ W and 45°09′–45°16′ N, most captured within the Bruce Peninsula National Park) representing a geographically isolated, though moderately large, regional population.

2 Killbear: 80 individuals from within Killbear Provincial Park (*c*. 1760 ha, 80°09′–80°14′ W and 45°19′–45°23′ N), a peninsula on the eastern shores of Georgian Bay, Ontario, and representative of a local population within the isolated and moderately large Georgian Bay regional population (see Fig. 1).

3 Beausoleil: 32 individuals from Beausoleil Island (*c*. 1100 ha, 79°50′–79°54′ W and 44°50′–44°54′ N, part of the Georgian Bay Islands National Park) at the south-eastern end of Georgian Bay and representative of another local population amid the Georgian Bay regional population. Beausoleil and Killbear are only approx. 50 km apart with additional local populations of massasaugas known to occur between the two.

4 Cicero: 25 individuals from the Cicero Swamp Wildlife Management Area (75°59′–76°05′ W and 43°07′–43°10′ N), a 2024 ha peatland complex located near Syracuse, New York. Cicero is one of only two small and highly isolated populations that remain in New York (Johnson 1995).

5 Springfield: 21 individuals from three geographically isolated sites (Prairie Road Fen, Cedar Bog, and Wright–Patterson AFB, each separated by < 35 km) in west-central Ohio (83°43′–84°03′ W and 39°49′–40°03′ N) near Springfield. Populations at each of these locations are thought to have been contiguous historically as they occur within a common wetland / drainage system. Because < 10 samples were obtained from any one locality we pooled these samples and treated them as a single 'population' although, in light of our results (see below) the locations may be more isolated than this grouping implies.

Variation in microsatellites

A detailed description of the procedures used to both process DNA samples and isolate and characterize the microsatellite loci used is given in Gibbs *et al*. (in press). Briefly, massasauga DNA was extracted from blood samples using standard phenol–chloroform procedures or DNAZOL (Gibco), and quantified using a fluorometer. Individuals were then genotyped with each of the six dinucleotide microsatellite loci (Scµ 01, 05, 07, 11, 16 and 26). Based on data from the Killbear population, these loci exhibited a high number of alleles (4–12 alleles per locus for 74 adults assayed), had expected heterozygosities between 0.15 and 0.85, and appeared to segregate in a Mendelian fashion (Gibbs *et al.* in press). To genotype individuals, PCR amplifications were performed in 10 µl reaction volumes using 50 ng of genomic DNA, 0.3 pmole of the forward primer end-labelled with [^{33}P]-ATP (Dupont), 0.4 pmole of unlabelled forward primer, 0.8 pmole of unlabelled reverse primer, 200 µM dNTPs, 0.5 U of AmpliTaq (Perkin Elmer), 0.1 M Tris-HCl, pH 8.3, 0.5 M KCl, and 1.5–2.0 mM $MgCl_2$. Amplifications were run in a Perkin Elmer 480 Thermocycler for 30 cycles with each cycle consisting of 30 s at 94 °C, 30 s at the appropriate annealing temperature (described in Gibbs *et al*. in press) and 30 s at 72 °C. Amplification products (3 µL) were then run on 6% denaturing polyacrylamide gels at 55 W for 2.5 h. Gels were then dried and exposed to Biomax (Kodak) X-ray film overnight. Products were sized by reference to a known sequencing reaction of a control template and 'hot' amplifications of the known-size clones for each locus, both of which were run on the gel at the same time.

Analysis of variation

We quantified differentiation amongst populations by testing for differences in allele frequencies and by calculating fixation and distance indices. Some loci used in this study have disjunct distributions of alleles within populations (discussed by Gibbs *et al*. in press), raising the possibility that mutations at these loci may not follow the step-wise mutation model assumed by recent measures of population differentiation developed for microsatellites (e.g. R_{ST} (Slatkin 1995) and dmµ (Goldstein *et al*. 1995)) although we have not explicitly tested this possibility (e.g. Estoup *et al*. 1995). Therefore, we present fixation and distance measures based both on infinite alleles (F_{ST} and Nei's D) and stepwise (R_{ST} and dmµ) mutation models. We used

several analytical software packages to calculate these measures including: GENEPOP (Raymond & Rousett 1995) to test for differences in allele frequencies and heterozygote deficiencies; FSTAT (Goudet 1995) to calculate and test F_{ST} values; and MICROSAT (Minch *et al*. 1995) to generate Nei's D and R_{ST} values.

We estimated levels of nonrandom association of alleles within massasauga populations by: (i) testing for both locus-specific and overall heterozygote deficiencies within populations using the exact tests in GENEPOP and, (ii) calculating F_{IS} values for each locus and then an overall F_{IS} value using data pooled across all loci using FSTAT. The significance of the overall F_{IS} value for each population was tested using the permutation procedure in FSTAT.

Results

Population differentiation

Table 1 shows the genetic characteristics of all six loci in each of the five populations surveyed. In general, based on levels of heterozygosity, numbers of alleles and mean allele frequencies, Scμ 01, 05, 07 and 11 are more variable than the other two loci (Scμ 16 and 26) although there is substantial variation in these characteristics amongst different populations. Overall, the most important result is that these loci appear to detect high levels of intraspecific variation in most populations surveyed, making them suitable for studies of population differentiation.

Tests which simultaneously compared the frequency of alleles in each of the five populations for each locus ($n = 6$ tests) were all highly significant ($P = 0$ for each). Next, we compared allele frequency distributions for all pair-wise combinations of populations at each locus ($n = 60$ tests). Because of the large number of tests performed we used the sequential Bonferoni procedure described by Rice (1989) to adjust the P value used to judge significance of each test. The majority (49 out of 60 = 82%) of comparisons yielded highly significant differences ($P < 0.00001$). Only two such tests were nonsignificant (Beausoleil vs. Killbear for Scμ 05 and Cicero vs. Killbear for Scμ 26). Thus, geographically separate massasauga populations differ significantly in allele frequencies. This pattern appears to be consistent amongst populations regardless of the geographical distance between them.

In addition to allele frequency differences, each massasauga population we sampled also contained a substantial proportion of unique alleles. Between 14.8% and 32.7% (mean = 22.7%) of all alleles detected within population samples were found to be population specific (Table 2). Furthermore, between 2.0% and 7.2% (mean = 5.5%) of these unique alleles occurred at frequencies of ≥ 5%. The number of individuals sampled per population does not appear to have had a significant effect on the proportion of population-specific alleles detected because the proportion of alleles classified as unique is approximately the same in populations from which the largest (Killbear) and smallest (Springfield) number of snakes were sampled. These results indicate that each of our study populations contain a distinct subset of the overall genetic variation resolved by the microsatellite markers we used.

Distance and fixation indices are measures of differentiation which incorporate information on both the frequency and identity of alleles. We calculated fixation ($F_{ST)}$ and distance (Nei's D) measures to describe the overall differences between pairs of populations (Table 3). F_{ST} values ranged from a low of 0.085 calculated for geographically isolated populations occupying opposite sides of Georgian Bay (Bruce and Beausoleil) to a high of 0.261 for the comparison of Killbear and Cicero populations. The overall F_{ST} value of 0.164 was significant ($P < 0.001$, FSTAT permutation procedure), indicating that within-population variation averaged 16% of the overall variation detected. Nei's distances were also all significantly different from zero (95% confidence intervals did not overlap zero; data not shown). The largest Nei's D value was calculated for Cicero vs. Springfield (0.779), whereas our contrast of Bruce vs. Beausoleil resulted in the smallest genetic distance (0.173).

R_{ST} values, which are the step-wise equivalent of F_{ST}, tend to be larger (Table 3); the mean R_{ST} averaged across all pair-wise population comparisons is 0.308. All individual R_{ST} values are significantly greater than zero as none have 95% confidence intervals that overlap zero. dmμ values, which measure the squared difference in mean allele sizes between populations, are also all large and positive like Nei's D; however, the variance in this measure is larger as 9 out of 10 values have confidence intervals which overlap zero (data not shown).

Overall, our results suggest that geographically separate populations of massasaugas are both demographically and genetically distinct and that such populations harbour a unique and substantial portion of the total genetic variation found in this subspecies.

Nonrandom associations of alleles

Comparisons of observed vs. expected numbers of heterozygotes predicted at each locus under Hardy–Weinberg expectations indicate that heterozygote deficits commonly occur in the massasauga populations we sampled. In 25 of 30 (83%) locus–population comparisons, the observed number of heterozygotes was less than expected (Table 1). Pooling across all six loci, we found a significant heterozygote deficit in each of the five populations ($P < 0.00001$ for Bruce, Killbear, Beausoleil, and Springfield and $P = 0.001$ for Cicero as tested by GENEPOP).

Table 1 Genetic characteristics of microsatellite loci in five rattlesnake populations. N = number of individuals genotyped; No. alleles = numbers of different sized alleles present in the population; H_{exp} and H_{obs} = expected and observed heterozygosities as calculated using GENEPOP. r_c and r_b are estimates of the frequency of null alleles as described by Chakraborty *et al*. (1992) and Brookfield (1996), respectively. $r_c = (H_{exp} - H_{obs})/(H_{exp} + H_{obs})$ and $r_b = (H_{exp} - H_{obs})/(1 + H_{exp})$

	Population				
Locus	Bruce Peninsula	Springfield	Cicero	Beausoleil	Killbear
Scμ 01					
N	41	21	25	32	80
No. alleles	14	9	8	15	16
Size range (in bp)	147–213	137–191	151–191	149–197	135–233
Mean frequency (± SD)	0.071 ± 0.067	0.111 ± 0.11	0.125 ± 0.14	0.067 ± 0.098	0.063 ± 0.067
H_{exp}	0.879	0.819	0.795	0.811	0.872
H_{obs}	0.805	0.429	0.560	0.686	0.663
r_c	0.044	0.31	0.17	0.084	0.14
r_b	0.039	0.21	0.13	0.069	0.11
Scμ 05					
N	40	21	25	32	79
No. alleles	5	5	5	4	12
Size range (in bp)	186–238	186–240	184–198	186–240	186–244
Mean frequency (± SD)	0.20 ± 0.24	0.20 ± 0.27	0.20 ± 0.27	0.25 ± 0.19	0.083 ± 0.13
H_{exp}	0.587	0.621	0.521	0.654	0.726
H_{obs}	0.300	0.429	0.520	0.719	0.595
r_c	0.32	0.18	0.001	–0.047	0.010
r_b	0.18	0.12	0.001	–0.039	0.076
Scμ 07					
N	42	20	25	31	79
No. alleles	10	9	4	9	9
Size range (in bp)	158–186	168–186	168–186	158–186	156–186
Mean frequency (± SD)	0.10 ± 0.090	0.11 ± 0.11	0.25 ± 0.21	0.11 ± 0.13	0.11 ± 0.10
H_{exp}	0.836	0.813	0.629	0.768	0.812
H_{obs}	0.714	0.650	0.360	0.613	0.671
r_c	0.079	0.11	0.27	0.11	0.095
r_b	0.066	0.090	0.17	0.088	0.078
Scμ 11					
N	42	20	25	31	79
No. alleles	15	9	5	2	9
Size range (in bp)	100–258	100–234	126–238	126–250	120–254
Mean frequency (± SD)	0.067 ± 0.13	0.11 ± 0.081	0.20 ± 0.18	0.50 ± 0.68	0.11 ± 0.16
H_{exp}	0.709	0.857	0.683	0.032	0.677
H_{obs}	0.619	0.750	0.840	0.032	0.633
r_c	0.068	0.067	–0.10	0.0	0.034
r_b	0.053	0.058	–0.093	0.0	0.026
Scμ 16					
N	42	21	25	31	81
No. alleles	4	4	4	2	5
Size range (in bp)	167–179	155–173	167–177	167–173	155–175
Mean frequency (± SD)	0.25 ± 0.29	0.25 ± 0.19	0.25 ± 0.36	0.50 ± 0.30	0.20 ± 0.28
H_{exp}	0.512	0.336	0.656	0.419	0.487
H_{obs}	0.548	0.048	0.400	0.258	0.469
r_c	–0.034	0.75	0.24	0.24	0.019
r_b	–0.024	0.22	0.15	0.11	0.012
Scμ 26					
N	42	20	25	32	80
No. alleles	6	9	1	7	5
Size range (in bp)	167–179	155–179	167–171	165–198	171–179
Mean frequency (± SD)	0.17 ± 0.17	0.11 ± 0.10		0.14 ± 0.14	0.20 ± 0.37
H_{exp}	0.703	0.828		0.748	0.257
H_{obs}	0.667	0.500		0.719	0.188
r_c	0.026	0.25		0.020	0.16
r_b	0.021	0.18		0.017	0.055

Table 2 Distribution of alleles found only in single populations of rattlesnakes. T is the total number of alleles of different sizes found in a given population, U is the number of alleles which are unique to that population with the value in parentheses equal to the number of these alleles that occur at a frequency of ≥ 5%, and the percentage is $(U/T) \times 100$

	Populations														
	Bruce Peninsula			Springfield			Cicero			Beausoleil			Killbear		
Locus	T	U	%	T	U	%	T	U	%	T	U	%	T	U	%
Scμ 01	14	2 (0)	14.3	9	4 (2)	44.4	8	0	0.0	15	5 (1)	26.7	16	5 (1)	31.3
Scμ 05	5	1 (1)	20.0	5	1 (1)	20.0	5	2 (1)	40.0	4	0 (0)	0.0	11	6 (1)	54.6
Scμ 07	10	0	0.0	9	0	0.0	4	0	0.0	9	1 (0)	11.1	9	1 (0)	11.1
Scμ 11	15	5 (2)	33.3	9	4 (3)	44.4	5	2 (1)	40.0	2	0	0.0	9	4 (2)	44.4
Scμ 16	4	1	25.0	4	1 (0)	25.0	4	0	0.0	5	0	0.0	5	1 (0)	20.0
Scμ 26	6	0	0.0	9	2 (3)	33.3	1	0	0.0	7	3 (1)	42.9	5	1 (0)	20.0
Total	54	9 (3)	16.7	45	13 (9)	28.9	27	4 (2)	14.8	39	8 (1)	20.5	55	18 (1)	32.7

We also calculated the inbreeding coefficient (F_{IS}) for each locus, which measures the extent of nonrandom mating within populations (Hartl & Clark 1987). The majority (25 out of 28 = 90%) of F_{IS} values for all populations across all loci were greater than zero (Table 4), indicating possible inbreeding within each population. Overall F_{IS} values (pooling across loci) for each population ranged from 0.135 to 0.353 (mean = 0.194) and all were significantly different from zero ($P < 0.006$, tested by FSTAT permutation procedure). These results demonstrate that nonrandom associations of alleles frequently occur in the populations sampled.

Other than inbreeding, two possible explanations for positive F_{IS} values are: (i) the existence of nonamplifying or null alleles (cf. Brookfield 1996), or (ii) unrecognized genetic structure within populations which result in heterozygote deficiencies within samples taken from such populations (the Wahlund effect; Hartl & Clark 1987). Assuming that the entire heterozygote deficiency is due to null alleles, we used the formulae presented by Chakraborty *et al.* (1992) and Brookfield (1996) to estimate the frequency of null alleles (r_c and r_b, respectively) in each population at each locus. The estimator r_c assumes that null homozygotes are not present in the sample whereas r_b allows such genotypes to occur. Values for r_c and r_b for each population by locus combination are shown in Table 1 and range from negative or zero values to a high of 0.31 for r_c for Scμ 01 in the Springfield population. Values averaged across loci for each population range from 0.076 (Georgian Bay) to 0.278 (Springfield) with an overall mean value (± SD) of 0.134 ± 0.128 for r_c and 0.048 (Georgian Bay) to 0.146 (Springfield) with an overall mean of 0.081 ± 0.060 for r_b. In summary, these estimates suggest that if heterozygote deficiencies are entirely due to nonamplifying alleles then such alleles are present, on average, at relatively high frequencies (approximately 10%) in these snake populations. However, as emphasized by Brookfield (1996) this assumes that all of the heterozygote deficiency is due to null alleles; this seems to be unlikely for these snakes (see below).

Other evidence for null alleles comes from the observation that a small number of individual snakes (population (n not amplifying): Killbear (5); Bruce Peninsula (3); Georgian Bay (3); Springfield (1); Cicero (1)) failed to amplify at 1–3 loci despite repeated attempts using new sample dilutions, whereas they were successfully

Table 3 Measures of population differentiation based on a) infinite alleles (F_{ST} and Nei's D) and b) stepwise (R_{ST} and dmμ) mutation models for all pair-wise combinations of snake populations. Population fixation measures (F_{ST} and R_{ST} are shown above the diagonals whereas distance (D and dmμ) values are shown below the diagonal

a) F_{ST} and Nei's D

	Bruce Peninsula	Springfield	Cicero	Beausoleil	Killbear
Bruce Peninsula	–	0.144	0.176	0.085	0.106
Springfield	0.539	–	0.237	0.236	0.254
Cicero	0.462	0.799	–	0.261	0.176
Beausoleil	0.173	0.783	0.628	–	0.130
Killbear	0.266	1.133	0.464	0.266	–

b) R_{ST} and dmμ

	Bruce Peninsula	Springfield	Cicero	Beausoleil	Killbear
Bruce Peninsula	–	0.298	0.216	0.185	0.175
Springfield	29.61	–	0.370	0.321	0.415
Cicero	8.44	42.35	–	0.427	0.410
Beausoleil	85.38	44.51	84.08	–	0.266
Killbear	25.87	76.87	43.06	112.65	–

Table 4 F_{IS} values for each microsatellite locus–population combination. Overall, F_{IS} values were tested using the permutation procedures in FSTAT; values for Bruce Peninsula, Springfield, Cicero and Killbear had $P < 0.001$ whereas the value for Beausoleil had $P = 0.006$. NA: the F_{IS} value could not be calculated because only a single allele was present in the population (Cicero) or a second allele was present in only a single individual (Beausoleil)

	Population				
Locus	Bruce Peninsula	Springfield	Cicero	Beausoleil	Killbear
Scμ 01	0.085	0.483	0.300	0.154	0.241
Scμ 05	0.492	0.316	0.002	–0.100	0.182
Scμ 07	0.148	0.205	0.433	0.204	0.175
Scμ 11	0.128	0.128	–0.235	NA	0.066
Scμ 16	–0.072	0.861	0.395	0.388	0.037
Scμ 26	0.053	0.403	NA	0.039	0.273
Combined	0.137	0.353	0.187	0.118	0.161

genotyped at the other loci. However, of the 16 individual–locus amplifications that failed, six (38%) involved just two individuals (three failed amplifications each) which suggests that the failure to amplify for a significant proportion of these individual–locus combinations is probably due to experimental difficulties such as template contamination.

Direct evidence against the presence of null alleles in the Killbear population was reported by Gibbs *et al*. (in press) who used the original sequence from clones to design sets of new primers that were located 0–10 bp upstream or downstream from the original primer sequences for the three loci (Scμ 01, 07 and 26) which showed the highest levels of heterozygote deficiency in this population. When all individuals which had originally been classified as homozygotes were reamplified using the new primer sets, all individuals originally scored as homozygotes at a particular locus remained classified as such using the new primers. This suggests that null alleles due to point mutations in the template sequence matched by the original primer sets are not the cause of the heterozygote deficiencies.

In contrast, there is evidence for unrecognized genetic structure within populations which can also result in reduced heterozygosity within samples. We examined this possibility in three of our populations (Springfield, Bruce Peninsula and Killbear) by testing for differentiation between clusters of samples taken at specific sites within the geographical range over which all samples were collected from each population. In all cases, despite small sample sizes for each cluster, there is evidence for even finer genetic structure within each larger 'population'. In Springfield, samples from each of the three subpopulations (Prairie Road Fen (n = 4), Cedar Bog (n = 10), and Wright–Patterson AFB (n = 7), each separated by < 35 km, showed significant differences (all $P < 0.034$; exact test in GENEPOP) in allele frequencies at five out of six loci with a combined P value of < 0.0001 and an overall F_{ST} value of 0.183 ($P < 0.001$). In the Bruce Peninsula population, sets of samples from Cyprus Lake (n = 11) and Emmett Lake (n = 7) sites, located approximately 5 km apart, were significantly different in allele frequencies at only two out of six loci ($P < 0.034$) but showed a highly significant overall p value (0.0148) and a marginally significant ($P = 0.059$) overall F_{ST} value of 0.033. Finally, as described by Gibbs *et al*. (in press), although samples from within the Killbear population were collected from an extremely limited area (9 km^2), most samples could be clustered into two groups (Twin Points (n = 27) and Blind Bay (n = 41)) which had been collected at sites whose geographical centres were approximately 1.5 km apart. Comparisons of these samples showed highly significant ($P < 0.0033$) differences in allele frequencies at five out of six loci (overall p value < 0.0001) and a highly significant overall F_{ST} value of 0.040 ($P < 0.001$). Interestingly, the F_{IS} values for four of the six loci (all $P < 0.031$) as well as the overall value (0.161; $P < 0.001$) remained significant, suggesting that significant nonrandom associations of alleles still persist even in these closely related subpopulations in Killbear. Overall, these results from three different sites support the interpretation that microgeographic genetic differentiation on a scale of < 5 km exists within at least some of the sampled populations and may provide at least a partial explanation for the positive F_{IS} values observed for each population.

Discussion

Our analysis of microsatellite variation in eastern massasauga rattlesnakes has shown that the geographically separate populations that we sampled are strongly genetically differentiated and that nonrandom associations of alleles within populations are probably due to a combination of additional fine-scale differentiation within sampled populations and the presence of null alleles. These results raise several interesting biological issues and have important implications for the conservation of eastern massasauga rattlesnakes. We consider the biological issues first.

Biological issues

Our first major finding was that all sampled populations are genetically distinct and that each contains population-

specific alleles. For some populations (e.g. Cicero and Springfield) this result was not surprising, because these populations are isolated from other populations by large landscape barriers composed of unsuitable urban and agricultural habitats. However, we found similar levels of genetic distinctiveness for populations around Georgian Bay. These populations are geographically quite close to each other (Beausoleil and Killbear sites are ≈ 50 km apart; Fig. 1), between which there exists high-quality habitat occupied by other massasauga populations (Weller & Oldham 1993). Nonetheless, these populations differ significantly in allele frequencies at most loci, have positive values for each distance and fixation measure, and contain substantial numbers of unique alleles.

These population-level results suggest that gene flow between populations is very restricted and that the populations we sampled have been genetically isolated from each other for some time. Measures of gene flow between populations based on estimates of *Nm* from F_{ST} values (Hartl & Clark 1987) are relatively small, ranging in value from 0.7 to 2.7 (mean = 1.3). The mean value for the three Ontario populations is larger (2.2) but still implies restricted gene flow between these nearby populations. The substantial number of unique alleles in each population suggests that this isolation has occurred on an evolutionary timescale, allowing mutation to generate new alleles within each population, some of which have existed for long enough to increase to significant levels (> 5%).

We can estimate the time since common ancestry for any two populations using the method developed by Goldstein *et al*. (1995) that integrates information on genetic distance, mutation rates, and generation time. They propose that the time since separation *t* (in generations) equals $d\mu/2\mu$, where $d\mu$ is as defined in the Materials and methods and μ is the mutation rate for the locus or loci used to estimate $d\mu$. To determine values of *t* for the three Georgian Bay populations we used values of $d\mu$ given in Table 3 and a generation time of 3 years (Keenlyne 1978; C. Parent, unpublished data), and a mutation rate of 5.4×10^{-4} which is the average value based on data from 30 human dinucleotide microsatellite loci as given in Goldstein *et al*. (1995). All times since common ancestry were estimated to exceed 71 000 years. These estimates are obviously biologically unrealistic given that the sample sites were glaciated no more than 10 000 years ago (Pielou 1991). Goldstein *et al*. (1995) assume in their calculation that loci follow a strict step-wise mutation model, an assumption that may not be met by at least some of the loci we used (Gibbs *et al.* in press) Thus, when the $d\mu$ values reported here are interpreted under such a model, they may greatly overestimate the number of mutational events separating populations, and hence the time since they shared a common ancestor. Whatever the actual time that these populations have been isolated, it certainly exceeds the several hundred years that encompasses the period of European settlement, with its accompanying large-scale alteration of habitat (e.g. logging). Thus, low levels of gene flow and genetic isolation appear to be the natural state for eastern massasauga populations, rather than being recently (anthropogenically) induced.

The basis for the heterozygote deficiencies in all populations is probably due to a combination of the presence of null alleles combined with a Wahlund effect due to previously unrecognized structure within each population sample. Although we have direct evidence for both effects in terms of individuals which fail to amplify at one or a few loci (null alleles) and observed genetic differentiation between subpopulations (the Wahlund effect), it is not possible with our data to quantifiably evaluate the importance of each factor as causes of the observed heterozygote deficiences. Confirming the presence of null alleles will require large-scale analyses of patterns of allele segregation in families of massasaugas as described by Gibbs *et al*. (in press) as well as direct molecular analysis of the sequence in the region of the genome where null alleles are hypothesized to be present in homozygote or nonamplifying individuals to see whether mutations which prevent primer binding have in fact occurred. Additional fine-scale sampling within populations and genetic analyses of these samples with other highly variable DNA-based markers (e.g. mtDNA control region and minisatellite loci) would confirm the microgeographic differentiation reported here.

Low heterozygosity and high F_{IS} values are also consistent with populations having some degree of inbreeding. The fact that we detected genetic differences between snakes sampled only a few km apart suggests that natal dispersal is very limited, which in turn should increase the likelihood of inbreeding. A related possibility is that the fine-scale structure we detected may be a consequence of sampling sets of closely related individuals. Such a phenomenon could result if very few females produce most of the offspring in a particular area. To assess the merit of these various biological explanations for the observed heterozygote deficiencies will require studies that document the reproductive success of individual males and females, as well as dispersal patterns and survival rates of juveniles and adults in an area where microgeographic differentiation has been detected. We are currently conducting such a study on individuals in the Killbear population (Parent *et al.* unpublished data).

These results contrast with those reported by Gibbs *et al*. (1994) who used RAPDs to genotype small numbers (n = 10) of individuals from each of two of these populations (Bruce Peninsula and Georgian Bay) and found no evidence for differentiation between populations. In comparison, microsatellite data show highly significant differences in allele frequencies between these

populations. Although comparisons between these studies are complicated due to large differences in sample sizes they suggest that microsatellites may be more sensitive at detecting differentiation between recently established populations, possibly due to their higher levels of variability. A larger-scale analysis with RAPDs of the same samples used in this study and then a direct comparison of the results based on the same estimators of differentiation (e.g. F_{ST} values) could be used to examine this possibility.

All of these results raise several general questions. First, are eastern massasauga rattlesnake populations really as finely structured genetically as our results suggest and, if so, what is the biological basis for such a pattern? To answer this question will require an analysis of genetic patterns among populations conducted at a much finer scale than the present study and the detailed study of an individual population as described above. Second, if massasauga populations are highly structured genetically, what is the biological basis for this structure and how typical is this of snakes in general? A literature review of studies reporting population differentiation in other snakes, at a scale similar to that used in the present study (Prior *et al*. in press), has shown that genetic distances calculated using allozyme data are roughly an order of magnitude smaller than those we derived using microsatellite data. This disparity could be a consequence either of real differences in the population structure of massasaugas and other species and/or of differences in the sensitivity of the genetic markers used.

DNA-based analyses of population structure in other snake species will resolve whether the difference between our results for massasaugas and the allozyme studies of other snakes reviewed by Prior *et al*. (in press) are methodological or biological. However, a recent study on the geographical variation in venom of the Malayan pit viper (*Calloselasma rhodostoma*) suggests an intriguing mechanism that could favour restricted dispersal in vipers and other venomous snakes and thus generate fine-scaled genetic structure. Over the distribution of the species they studied, Daltry *et al*. (1996) found significant geographical variation in the biochemical composition of the vipers' venom. They attributed this biochemical structuring to local adaptation, where venom biochemistry evolved to match local prey specialization. Daltry *et al*. (1996) were able to rule out the possibility that the snakes were able to phenotypically match their venom to their prey, indicating that venom variation is probably genetically controlled. Thus, geographical variation in prey specialization that favours matching variation in venom specialization could produce genetic structure in continuous populations of venomous snakes through local adaptation. If this hypothesis is correct, then we would expect variation in venom composition to map on to the genetic structure of the population. Contrary to this prediction, Wilkinson *et al*. (1991) found no evidence that variation in the venom of Mojave rattlesnakes (*Crotalus s. scutulatus*) coincided with allozyme variation. However, this may again be a case of allozymes having inadequate resolution.

Conservation implications

Our data help define the spatial and ecological scale at which massasauga populations should be monitored and managed for conservation. At the very least, we now should consider the regional populations of the Bruce Peninsula and Georgian Bay to be composed of a series of partially independent local populations or management units (Moritz 1994). However, the preliminary results on the differentiation of subpopulations emphasizes that a more precise delimitation of what constitutes a local population of these snakes awaits additional fine-scale genetic data (e.g. locality-based samples < 10 km apart). The subpopulation results suggest that the genetic neighbourhood (cf. Wright 1946), or set of individuals with which an organism mates at random, may have an extremely limited spatial distribution even within continuously distributed massasauga populations.

One practical implication of our results is that if local populations in regions such as Georgian Bay suffer catastrophic population reductions or extinctions (e.g. via fire or disease), natural repopulation through immigration is likely to be very slow. If so, active managed translocations might be necessary to re-establish populations. Furthermore, given the genetic distinctiveness of individual populations, the source of donor individuals for such translocations would have to be carefully evaluated if maintaining the current genetic structure is deemed an important conservation goal.

Finally, the fact that individual massasauga populations appear to contain unique portions of the total genetic variation suggests that each population is likely to be of particularly high conservation value. This argument relies on a long-standing assumption in conservation genetics that presumably neutral variation such as that detected using microsatellites is correlated with variation in traits which would preserve the evolutionary potential of this species (e.g. Avise 1994). We have no information on such a relationship in these animals, but such a correlation has been documented in other species (e.g. Vrijenhoek *et al*. 1985). Another important conservation genetic issue that needs to be resolved is whether any significant phylogenetic relationships, and hence distinct evolutionary lineages, occur amongst populations of this subspecies (cf. Moritz 1994). Analyses of mtDNA sequence variation among populations would be useful for exploring this possibility and we are currently conducting such work (Gibbs *et al*. unpublished data).

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References

Avise JC (1994) *Molecular Markers, Natural History, And Evolution*. Chapman and Hall, New York.

Avise JC, Hamrick JL (1996) *Conservation Genetics: Case Studies From Nature.* Chapman and Hall, New York.

Brookfield JFY (1996) A simple new method for estimating null allele frequency from heterozygote frequency. *Molecular Ecology*, **5**, 453–455.

Chakraborty R, De Andrade M, Daiger SP, Budowle B (1992) Apparent heterozygote deficiencies observed in DNA typing data and their implications in forensic applications. *Annals of Human Genetics*, **56**, 45–57.

Daltry JC, Wuster W, Thorpe RS (1996) Diet and snake venom evolution. *Nature*, **379**, 537–540.

Estoup A, Garney L, Solignac M, Cornuet J-M (1995) Microsatellite variation in honey bee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. *Genetics*, **140**, 679–695.

Gibbs HL, Prior KA, Parent C (1997) Characterization of DNA microsatellite loci from a threatened snake, the eastern massasauga rattlesnake (*Sistrurus c. catenatus*) and their use in population studies. *Journal of Heredity*, in press.

Gibbs HL, Prior KA, Weatherhead PJ (1994) Genetic analysis of populations of threatened snake species using RAPD markers. *Molecular Ecology*, **3**, 329–337.

Goldstein DB, Linares AR, Cavalli-Sforza LL, Feldman M (1995) Genetic absolute dating based on microsatellites and the origin of modern humans. *Proceedings of the National Academy of Sciences USA*, **92**, 6723–6727.

Goudet J (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. *Journal of Heredity*, **86**, 485–486.

Greene HW, Campbell JA (1992) The future of the pitvipers. In *Biology of the Pitvipers* (eds Campbell JA, Brodie ED), pp. 421–427. Selva, Tyler, Texas.

Hartl D, Clark AG (1987) *Principles of Population Genetics*. 2nd edn. Sinauer Associates, Sunderland, Massachusetts.

Johnson G (1995) *Spatial ecology, habitat preference, and habitat management of the eastern massasauga, Sistrurus c. catenatus in a New York weakly-minerotrophic peatland*. PhD dissertation, SUNY, Syracuse, New York.

Johnson B, Menzies V (1993) *International Symposium And Workshop On The Conservation Of The Eastern Massasauga Rattlesnake*. Metro Toronto Zoo, Toronto.

Keenlyne KD (1978) Reproductive cycles of two species of rattlesnakes. *American Midland Naturalist*, **100**, 368–375.

Minch E, Ruiz-Linares A, Goldstein D, Feldman M, Cavalli-Sforza LL (1995) MICROSAT (version 1.4c): a computer program for calculating various statistics on microsatellite allele data. WWW: http//lotka.stanford.edu/research/distance.html.

Moritz C (1994) Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology*, **3**, 401–411.

Parker WS, Plummer MV (1987) Population ecology. In: *Snakes: Ecology and Evolutionary Biology* (eds Seigel RA, Collins JT, Novak SS), pp. 253–301. Macmillan, Toronto.

Pielou EC (1991) *After The Ice Age: The Return Of Life To Glaciated North America*. The University of Chicago Press, Chicago.

Prior KA, Gibbs HL, Weatherhead PJ (1997) Population genetic structure in the black rat snake: implications for management. *Conservation Biology*, in press.

Quellar DC, Strassman JE, Hughes CR (1993) Microsatellites and kinship. *Trends in Ecology and Evolution*, **8**, 285–288.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): A population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Reinert HK, Kodrich WR (1982) Movements and habitat utilization by the massasauga, *Sistrurus catenatus catenatus*. *Journal of Herpetology*, **16**, 162–171.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

Slatkin M (1995) A measure of population subdivison based on microsatellite allele frequencies. *Genetics*, **139**, 457–462.

Villarreal X, Bricker J, Reinert HK, Gelbert L, Bushar LM (1996) Isolation and characterization of microsatellite loci for use in population genetic analysis in the timber rattlesnake, *Crotalus horridus*. *Journal of Heredity*, **87**, 152–155.

Vrijenhoek RC, Douglas ME, Meffe GK (1985) Conservation genetics of endangered fish populations in Arizona. *Science* **229**, 400–402.

Weatherhead PJ, Prior KA (1992) Preliminary observations of habitat use and movements of the eastern massasauga rattlesnake (*Sistrurus c. catenatus*). *Journal of Herpetology*, **26**, 447–452.

Weller WF, Oldham MJ (1993) Historic and current distribution and status of the eastern massasauga rattlesnake, *Sistrurus catenatus*, in Ontario, Canada. In: *International Symposium And Workshop On The Conservation Of The Eastern Massasauga Rattlesnake*. (eds Johnson B, Menzies, V), pp. 35–39. Metro Toronto Zoo, Toronto.

Weller WF, Parsons HJ (1991) *Status Of The Eastern Massasauga Rattlesnake,* Sistrurus catenatus, *in Canada.* COSEWIC, Ottawa.

Wilkinson JA, Glenn JL, Straight RC, Sites JW Jr (1991) Distribution and genetic variation in venom A and B populations of the Mojave rattlesnake (*Crotalus scutulatus scutulatus*) in Arizona. *Hepetologica*, **47**, 54–68.

Wright S (1946) Isolation by distance under diverse systems of mating. *Genetics*, **31**, 39–59.

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