Population Genetic Structure in the Black Rat Snake: Implications for Management

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Abstract: *Assessments of population genetic structure and diversity can be of value in formulating management plans for threatened species. Using randomly amplified polymorphic DNA markers, we found evidence of significant genetic structure among black rat snakes (Elaphe obsoleta) sampled at three spatial scales. Highly isolated (1500–1900 km apart) populations were strongly divergent (F_{ST} = 0.242–0.323), whereas populations more proximal (≤465 km apart) although currently isolated, exhibited far less divergence (F_{ST} = 0.019). A considerable proportion (80%) of total genetic diversity was due to differences among individuals within populations, although differences among populations (8%) also were significant. At the scale of sub-populations (local populations 15–50 km apart), differentiation was generally moderate (F_{ST} = 0.058). Our estimates of Nei's genetic distance for sub-populations (0.014) approximated (mean = 0.044) those obtained in other studies that have assessed differentiation between snake populations based on variation in allozymes. The majority (ca. 86%) of total genetic variance across five sub-populations was attributable to differences among individuals, although differences among sub-populations (ca. 13%) also were significant. We found little evidence of genetic structure (F_{ST} = 0.006) between pairs of hibernacula, our finest spatial scale (samples 1–2 km apart), if they were located in natural habitats. In contrast, a pair of hibernacula sampled in an urban area exhibited genetic structure equivalent to some sub-population differences (F_{ST} = 0.039), suggesting interrupted gene flow related to urban development. Our results have direct implications for ranking populations in terms of their conservation value and the genetic management of threatened snakes.*

Estructura Genética Poblacional en la Serpiente Rata Negra: Implicaciones para su Manejo

Resumen: *Evaluaciones de la estructura genética y la diversidad de una población pueden ser de gran valor en la formulación de planes de manejo para especies amenazadas. Utilizando marcadores polimórficos amplificados de ADN, encontramos evidencia de una estructura genética significativa entre serpientes "rata negra" (*Elaphe obsoleta*) muestreada en tres escalas espaciales. Altamente aisladas (1500–1900 km de separación) las poblaciones fueron fuertemente divergentes (F_{ST} = 0.242–0.323), mientras que poblaciones mas cercanas (≤465 km de separación) que, aunque se encontraban aisladas, exhibieron mucho menos divergenica (F_{ST} = 0.019). Una considerable proporción (80%) de la diversidad genética total se debió a las diferencias entre individuos dentro de las poblaciones, sin embargo, diferencias entre poblaciones (8%) también fueron significativas. En la escala de subpoblación (poblaciones locales separadas entre 15–50 km) la diferenciación fue generalmente moderada (F_{ST} = 0.058). Nuestras estimaciones de la distancia Genética de Negi para subpoblaciones (0.014) se aproximó (media = 0.044) a aquellos valores obtenidos en otros estudios que han evaluado la diferenciación entre poblaciones de serpientes en base a la variación de sus alozimas. La mayoría (ca. 86%) de la varianza genética total obtenida entre cinco subpoblaciones fue atribuíble a diferencias entre individuos, aunque las diferencias entre subpoblaciones (13%) fueron también significativas. Encontramos una ligera evidencia de estructura genética (F_{ST} = 0.006) entre pares de hibernáculas, nuestra escala espacial mas fina (muestras separadas de 1–2 km), si estas se encontraban en sus hábitats naturales. En contraste, un par de hibernáculas muestreado en un área urbana exhibió una estructura genética equivalente a algunas differencias*

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subpoblacionales (F_{ST} = *0.039), lo cual sugiere un flujo de genes interrumpido relacionada con el desarrollo urbano. Nuestros resultados tienen implicaciones directas en la categorización de poblaciones en base a su valor de conservación, así como en el manejo genético de serpientes amenazadas.*

Introduction

Genetic data now serve an important role in guiding the management of endangered species (e.g., Hedrick & Miller 1992; Loeschcke et al. 1994). Conservation biologists commonly use both data that define the genetic structure of populations and data that provide comparative measures of within-population diversity. Data on the genetic structure of populations are of particular interest because they may reveal evidence of restricted gene flow or genetic isolation that is undetectable through traditional demographic studies (e.g., capture-recapture data). Such knowledge can be used to identify genetically-based "management units" (Moritz 1994), enabling conservation practices to be focused appropriately. Estimates of within-population diversity also are of value because they can reveal recent, or on-going, changes in population structure and dynamics. For instance, habitat destruction could fragment previously contiguous populations, thereby disrupting gene flow and resulting in a loss of within-population variation via genetic drift (Franklin 1980). Conservation of genetic diversity is prudent given evidence of a positive correlation between genetic variability and both individual fitness traits and population viability (e.g., McAlpine 1993; Jimenez et al. 1994; but see Milligan et al. 1994). Thus, estimates of current genetic variability within populations may allow one to rank populations with respect to their genetic conservation status (e.g., relative vulnerability).

The availability of both types of genetic data can contribute to the management of endangered species by providing an informed basis for allocating limited funds for conservation and for assessing the efficacy of conservation strategies (e.g., has genetic variability changed as a result of management?). Whereas the benefits of genetic studies seem apparent, relatively few taxonomic groups (mostly mammals, birds, and fish) have been examined (reviewed by Avise et al. 1995). In particular, snakes are poorly represented in studies of conservation genetics (Dodd 1993). Our general goal is to provide one of the first studies of the population genetic structure of a threatened snake from a conservation perspective.

Five distinct sub-species of *Elaphe obsoleta* are currently recognized (Conant & Collins 1991). The black rat snake (*Elaphe o. obsoleta*) is the most widely distributed of the five, ranging across much of the eastern half of the U.S. (Fig. 1a). Although abundant in some regions (Durner & Gates 1993), in Ontario this sub-species occurs in as few as five isolated populations in two separate parts of the province. The populations of "Skunk's Misery," "Big Creek," "Oriskany," and "Niagara" are all found in southwestern Ontario (north of Lake Erie), whereas the "Frontenac Axis" population is located in eastern Ontario (Fig. 1a). The small size and high degree of isolation of these remnant populations have raised concerns regarding their conservation status, resulting in black rat snakes being provisionally designated as "threatened" in Canada (Prior & Weatherhead, unpublished data).

Our primary objective was to quantify genetic population structure and estimate levels of gene flow in black rat snakes at three distinct spatial scales: (1) geographically separate regional populations (>100 km apart); (2) local sub-populations (15–50 km) within a regional population; and (3) communal hibernacula (1–5 km apart) within local populations (Fig. 2). Our reasons for choosing these scales for comparison were twofold. First, previous work by Paik and Yang (1987) on a congeneric species (*Elaphe dione*) found some evidence of genetic structure at the regional scale. Second, sampling at the scale of hibernacula reflects a non-arbitrary social/ecological assortment of individuals within local populations. That is, across much of the northern half of their range, black rat snakes hibernate communally at traditional sites to which individuals exhibit strong interannual fidelity (i.e., >95%, Prior & Weatherhead, unpublished data). Gene flow among neighboring hibernacula is expected to be high for three reasons: (1) mating takes place during the late spring and early summer after dispersal from the hibernacula (Weatherhead & Hoysak 1989); (2) the activity ranges of adults from adjacent hibernacula regularly overlap; (3) females may lay their eggs remote from the maternal hibernacula, so many juveniles may hibernate at sites different from those used by their mothers.

Our secondary objective was to characterize the genetic diversity of the small, isolated rat snake populations found in Ontario relative to larger, non-isolated populations sampled in the central core of the species' range. We predicted that, in the absence of gene flow, small, isolated populations would exhibit reduced levels of genetic diversity (heterozygosity) as a function of genetic drift (e.g., Lesica & Allendorf 1995).

Collectively, these data will contribute to an understanding of the ecology and biogeography of black rat snakes. More specifically, our results are expected to help clarify the genetic status and composition of Ontario's threatened populations of black rat snakes, thus aiding those charged with preparing plans for the management of the species in Canada.

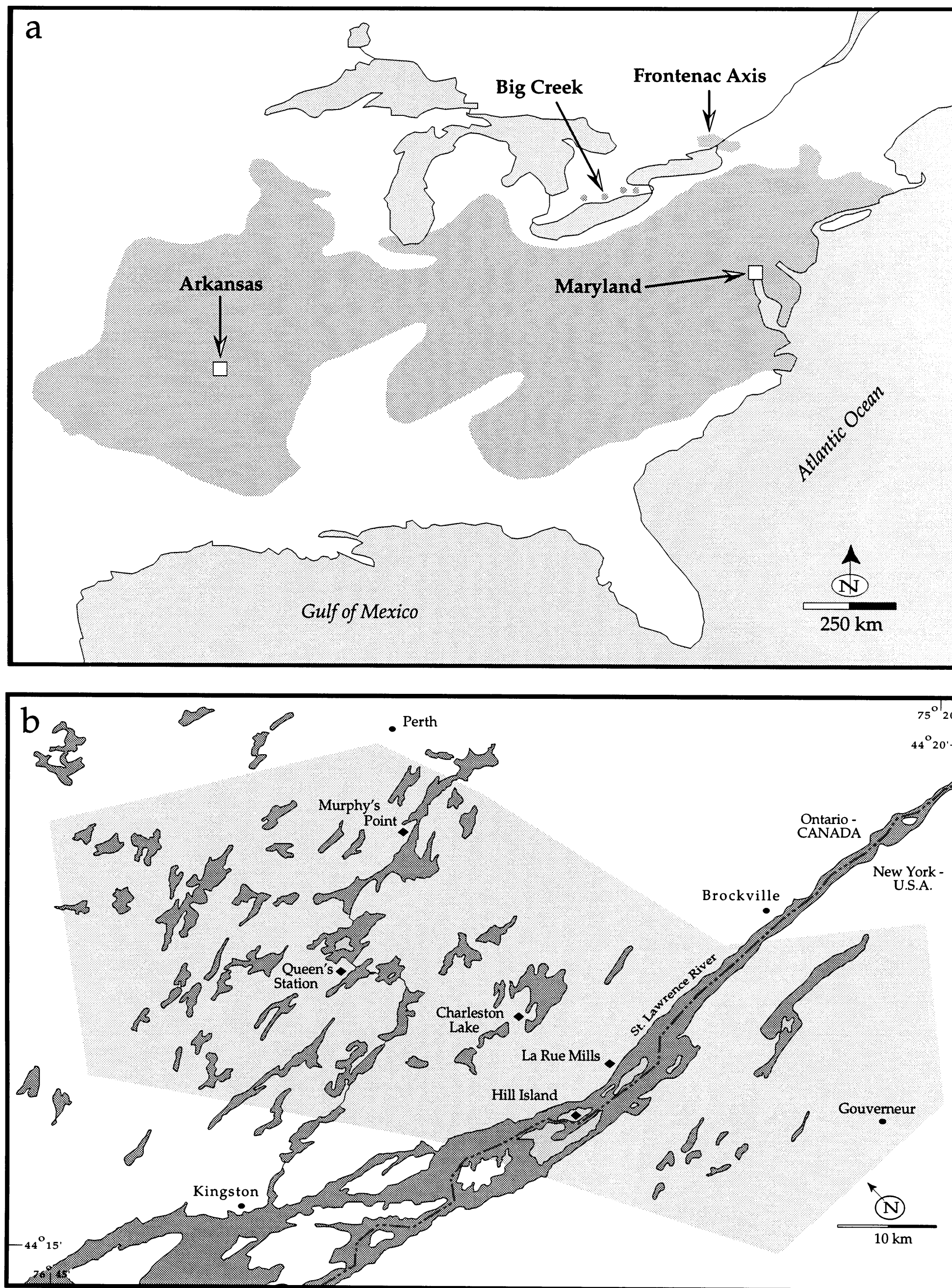

Figure 1. General distribution of the black rat snake (shaded area) and the relative locations of the four study populations in Canada and the U.S. including Big Creek, Frontenac Axis, Maryland, and Arkansas (a), and general range of the Frontenac Axis population (shaded area) located in eastern Ontario and northern New York and the relative locations of subpopulations (Murphy's Point, Queen's Station, Charleston Lake, La Rue Mills, and Hill Island) sampled across the region (b).

Methods

Study Populations and Sample Collection

We sampled 210 individual black rat snakes from regional populations, sub-populations within a regional population, and communal hibernacula within local subpopulations as described above. At the coarsest scale, the 210 individuals represent four distinct regional populations, distributed across the species' range, including two isolated populations in Ontario (Big Creek, Frontenac Axis) and two non-isolated populations from the U.S. (Maryland and Arkansas; Figs. 1a & 2).

The Big Creek population occurs north of Lake Erie in southwestern Ontario (ca. 42°75′N, 80°50′E). Neighboring populations (Skunk's Misery, Oriskany, Niagara) are

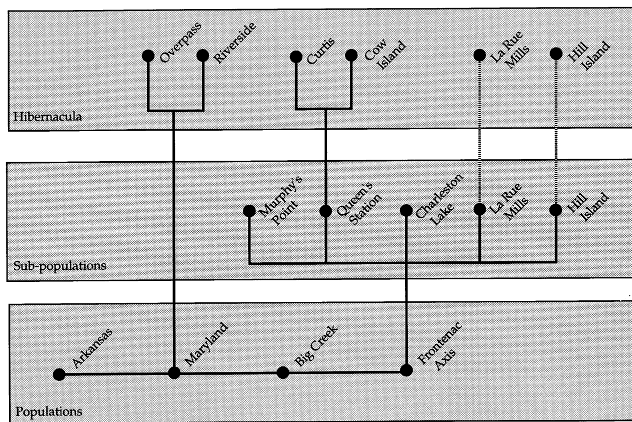

Figure 2. Schematic representation of the hierarchical relationship among samples of black rat snakes used in genetic analyses. Samples collected from La Rue Mills and Hill Island hibernacula were used at two levels in the hierarchical analyses.

all a minimum of 50 km away with largely unsuitable habitat (intensive agriculture) in between.

The disjunct Frontenac Axis population occurs in eastern Ontario and northern New York state, bridging the St. Lawrence River at the east end of Lake Ontario (ca. 44°50′N, 76°50′E, see Figs. 1a & b). The nearest population is found ca. 120 km to the south near Syracuse, New York (A. R. Briesch, personal communication). The fact that black rat snakes are absent from naturalists' surveys of upper New York around the turn of the century (e.g., De Kay 1842) suggests that the disjunction of the Frontenac Axis population from southwestern Ontario populations may have preceded European settlement and land-clearing.

The Maryland population was sampled in the vicinity of the city of Joppatowne (39°25′N, 76°22′E; Fig. 1). This is a non-isolated population occurring within the mid-eastern core of the species' range. Black rat snakes are abundant in some parts of the Joppatowne study site, an urban landscape bisected by (semi-)natural habitats (old field, riparian forest).

Finally, the Arkansas population sample was obtained from both the Ozark and Ouachita National Forests (ca. 35°50′N, 93°50′E) in western Arkansas (J. H. Withgott, pers. commun.). As with the Maryland population, the Arkansas population may be regarded as coming from the core of the species' range.

Snakes were hand-captured and sampled opportunistically throughout both the Big Creek (1991–1993) and Arkansas (1993) study areas. In contrast, snakes obtained from Frontenac Axis (1991–1993) and Maryland (1992–1993) were collected at only a few specific localities, including communal hibernacula. This feature of our sampling protocol provided the opportunity to sub-divide hierarchically the Frontenac Axis and Maryland regional population samples, allowing us to investigate within-population genetic structure. Specifically, the Frontenac Axis population sample is composed in part of individuals obtained from five (sub-population) localities ("Murphy's Point Provincial Park," "Queen's University Biological Station," "Charleston Lake Provincial Park," "La Rue Mills," and "Hill Island" Figs. 1b & 2). The Queen's Station sample can be further sub-divided since it is composed in part of individuals associated with either the "Curtis" or "Cow Island" hibernaculum (inter-hibernacula distance = 1.2 km). La Rue Mills and Hill Island are also hibernacula samples (inter-hibernacula distance = 17 km; Figs. 1b & 2), and therefore, for the purpose of analyses, these samples were regarded at two hierarchical scales (i.e., representative of local sub-populations *and* communal hibernacula). The Maryland samples were also collected from two adjacent hibernacula (i.e., "Overpass" and "Riverside"; inter-hibernacula distance = 1.6 km, Fig. 2).

Once a snake was captured we drew ca. 100 μL of blood from a caudal vessel using a 0.5 cc insulin syringe fitted with a 28-gauge needle. Blood samples were immediately mixed with 800 μL of lysis buffer (Seutin et al. 1991) and refrigerated until DNA extraction. In all cases, snakes were permanently marked (i.e., clipping of caudal scutes or PIT-tagging) upon capture to eliminate the possibility of resampling the same individual on subsequent occasions.

DNA Extraction and RAPD Amplifications

Genomic DNA was extracted from blood samples using standard techniques (Sambrook et al. 1989). Approximately 400 μL of the lysis buffer and blood solution was suspended in additional lysis buffer to a total volume of 4 mL, and rocked gently overnight at 37°C until no clots remained. Samples were then digested with proteinase K, extracted 2–3 times with a 70:30 mix of phenol:chloroform, and once with chloroform. The DNA was then precipitated by adding 1/10 volume of 3M sodium acetate (pH 5.5), and two volumes of 100% ethanol, spooled out using a Pasteur pipette, washed in 70% ethanol, and then allowed to dry for 5 minutes. It was then re-dissolved in 0.3–1.0 mL of TE depending on the yield of DNA, and gently rocked overnight at 37°C. We quantified the amount of DNA using a Hoefer TKO 100 fluorometer. A test aliquot of DNA was digested and the concentration and quality of the sample DNA assessed by comparison with known standards of snake DNA. If the estimates of concentration were inaccurate, then the samples were allowed to re-dissolve at 37°C and the quantification procedure was repeated.

To generate RAPD profiles from the black rat snake DNA we used 20, 10 base pair primers (A1–A20) from the Operon Technologies Primer Kit A in PCR amplifications. Amplification reactions were performed in 25 μL volumes containing 10 mM Tris (pH 8.3), 50 mM KCl,

2 mM $MgCl_2$, 0.001% gelatin, 10 mM dNTP (Pharmacia), 5 μM primer (Operon), 0.5 units of Taq DNA polymerase (Perkin Elmer Cetus), 25 ng of genomic DNA, and dH_2O. Negative controls in which water was substituted for DNA were also run to check for the possibility of contamination. The reaction was overlaid with mineral oil and amplified in a Perkin Elmer Cetus Model 480 DNA Thermal Cycler programmed for 45 cycles of 1 minute at 94°C to denature, 1 minute at 36°C for annealing of primer, and 2 minutes at 72°C for extension. After the final cycle, samples were held at 4°C prior to analysis. Amplification products were separated according to size on 1.2% agarose gels run in 1 × TBE, stained with 35 μL ethidium bromide (10 mg/mL) in 700 ml 0.5 × TBE, de-stained for 30 minutes in water and visualized under ultraviolet light. A 1 Kb DNA ladder was run alongside RAPD products on each gel in order to confirm the sizes/identities of fragments. Black and white photographic negatives were taken of the gels and the profiles of individuals were scored for the presence/absence of fragments for each primer by projecting the photographic negative image on a white screen.

Primer Selection and Fragment Scoring

Initially, we surveyed all 20 (A1–A20) of the Operon primers for a sample of 20 black rat snakes. Based on clarity and resolution of the profiles produced, we chose five of these primers (A1, A5, A7, A10, and A20) which were then used to generate profiles for a larger sample of individuals. To quantify the consistency with which DNA bands of the five primers used could be reproduced, we ran two separate amplifications for the same 20 individuals. After blind scoring the presence (+) or absence (−) of specific bands in these 20 individuals we calculated a "repeatability" score for each locus based on the consistency with which a particular band was either present or absent in the two sets of amplifications. For example, if a particular band was consistently present or consistently absent between amplifications in all 20 individuals, then that locus was given a repeatability score of 1.0. In contrast, if 10 of 20 individuals were scored differently for a particular locus on the two amplifications, then the locus would be assigned a repeatability score of 0.50. Only those loci that exhibited complete reliability (i.e., repeatability score of 1.0) were subsequently used for analyses.

Statistical Analysis of RAPD Profiles

Fragment patterns generated by the five RAPD primers were analyzed both directly as phenotypes and by estimating allele frequencies at corresponding loci, enabling more traditional genetic analyses. As a preliminary test of genetic structure we conducted an analysis of molecular variance (AMOVA). The AMOVA is equivalent to a classical analysis of variance (ANOVA) in that it computes (molecular) variance components from a matrix of squared (molecular) distances between pairs of observations (i.e., RAPD phenotypes). We calculated pairwise Euclidean distance measures between RAPD phenotypes using the software program RAPDistance (Armstrong et al. 1994) and then ran our AMOVAs with WINAMOVA (Excoffier et al. 1992). The significance of the variance component estimates were computed by non-parametric permutation procedures in WINAMOVA.

The WINAMOVA allows for a three-level hierarchical partitioning of genetic variation. Therefore, in our first set of analyses the regional populations of Frontenac Axis, Big Creek, and Maryland were pooled to form an "eastern" group and Arkansas served as a "western" group (Figs. 1a & 2). As such, we were able to partition phenotypic variation among individuals within populations, among regional populations within groups, and among groups (east vs. west). Population sample sizes for these analyses were as follows, Big Creek (n = 15), Frontenac Axis (n = 29), Maryland (n = 30), and Arkansas (n = 26). Though as many as 139 samples from the Frontenac Axis population were available to us, we elected to use only a randomly selected sub-sample of 29 at this level of analysis in order to avoid potentially confounding effects of widely disparate samples sizes (e.g., Lynch & Milligan 1994) and because WINAMOVA limits the number of samples for analysis to 100.

Our second AMOVA analysis assessed genetic structure within the Frontenac Axis population. Here we pooled the sub-population samples of Murphy's Point and Queen's Station to form a "Northern" group and Charleston Lake, La Rue Mills, and Hill Island to form a "Southern" group (Figs. 1b & 2). As above, we were then able to partition phenotypic variation among individuals within sub-populations, among sub-populations within groups, and among groups (North vs. South). Because of WINAMOVAs limitations on sample size, our sub-population sample sizes were as follows: n = 23 for Murphy's Point (a random sample of the 33 available); n = 21 for Queen's Station (a random sample of the 48 available); n = 13 for Charleston Lake, n = 27 for La Rue Mills, and n = 16 for Hill Island. We assessed the possibility that the results of these AMOVAs (and subsequent analyses of allele frequencies) might be influenced by sampling effects by comparing results generated during five replicate analyses. For each replicate analysis we used unique random samples to represent Frontenac Axis (for population AMOVA) and Murphy's Point and Queen's Station (for sub-population AMOVA).

In addition, we estimated allele frequencies at the corresponding RAPD loci using the software RAPDFST 3.0 (Black 1995) in order to conduct standard genetic analyses of population structure. In all species studied to date, most RAPD polymorphisms are due to the presence of a dominant (+) and a recessive (−) allele at the

same locus (e.g., Bowditch et al. 1993; Apostol et al. 1995). The dominant allele determines the presence of the band such that +/+ and +/− individuals have the (+, present) phenotype and −/− individuals have the (−, absent) phenotype. As in previous studies (e.g., Haig et al. 1994), we assumed that all the loci scored had this property and that all population samples were in Hardy-Weinberg equilibrium, so that our estimate of the frequency of q of the recessive allele (−) equaled the square root of the frequency of the null phenotype (−). Calculation of Wright's (1978) F_{ST} (among-population differentiation) was carried out in RAPDFST, using the weighted mean frequency of alleles formula. This method expresses F_{ST} as the ratio of the observed variance in the frequency of an allele among sub-populations relative to its maximum variance in the total population. Following this, a χ^2 value was calculated to determine if the estimate of F_{ST} differed from zero (i.e., indicative of significant population differentiation). We also applied Wright's (1978) qualitative guidelines for interpretation of F_{ST} values. That is, an F_{ST} of 0–0.05 = little differentiation, an F_{ST} of 0.05–0.15 = moderate differentiation, an F_{ST} of 0.15–0.25 = great differentiation, and an F_{ST} above 0.25 = very great differentiation. Estimates of effective migration rate were determined using Wright's (1931) formula, $Nm = (1-F_{ST})/(4F_{ST})$ where: N = deme size and m = migration rate among demes, assuming current equilibrium between migration and genetic drift. We then used the allele frequency data to generate measures of genetic distance (D; Nei 1978) between populations and estimates of mean (expected) heterozygosity within populations with the software BIOSYS-1.7 (Swofford & Selander 1989).

Lynch and Milligan (1994) recently provided a set of guidelines for the analysis of population genetic structure using RAPD markers. In order to ensure unbiased estimates of population genetic parameters they advise researchers to restrict their analyses to the use of RAPD loci having alleles that occur at only moderate frequency across the populations under study. Specifically, only loci with alleles exhibiting an observed frequency of less than $1 - (3/n)$ should be used (where n = the population sample size). Consequently, we analyzed both "complete" (use of all loci) and "restricted" (use of loci with frequency less the $1 - (3/n)$) RAPD datasets and considered both sets of results. However, we found few quantitative and no qualitative differences between the two. Therefore, because of their more conservative nature, we present only the results of our analysis of restricted datasets.

To put our results in context we summarized published data on snake population genetics. In addition to studies reviewed by Dessauer et al. (1987), we found nine other studies that reported measures of heterozygosity and genetic distance among snake populations based on protein variability.

Results

RAPD Profiles

Forty bands were produced by the five RAPD primers, with 6 to 11 individual bands being generated by each primer (mean = 8.00 ± 2.09 SE). Band repeatability was relatively high overall, with the 40 bands exhibiting the same state (+,+ or −,−) between two independent RAPD-PCR amplifications pooled across 20 snakes on average 91.39% (± 1.89 SE) of the time. However, only 16 (40%) of the 40 bands showed 100% reliability. Of those, 14 exhibited variability among individuals at the population level and these bands (or "loci") formed the "complete" datasets (see above). Applying the criteria of Lynch and Milligan (1994) for pruning RAPD loci (observed frequency less than $1 - (3/n)$) necessitated that we eliminate 7 (loci 1, 3, 5, 6, 10, 11, and 14; Table 1) of the original 14 loci. Therefore, data derived using the remaining 7 loci formed our "restricted" datasets on which we report here.

Regional Population Structure

We identified two population-specific RAPD markers. First, 10 members of the Arkansas population were the only individuals to express the (+) allele at locus 7. Second, a single individual from Arkansas was the only snake that exhibited the (−) allele at locus 14 (Table 1).

AMOVA results based on RAPD phenotypes demonstrated significant genetic differences among regional groups (East vs. West), isolated populations (Frontenac Axis, Big Creek, Maryland, Arkansas), and individuals within each of the four populations (in all cases $p < 0.001$). Of the total genetic diversity, 12% was attributable to regional differences, ca. 8% to population differences within these regions, and 80% to individual differences among rat snakes within populations. Five replicate AMOVAs, using unique random samples of 29 individuals to represent Frontenac Axis, yielded little quantitative and no qualitative differences from these original results. Thus, sampling effects do not appear to have been significant at this spatial scale.

This general pattern of population genetic structure was clarified by our analysis of allele frequency data generated by RAPDFST (Black 1995). Estimated allele frequencies at RAPD loci varied among regional populations (Table 1); mean among-population differentiation (F_{ST}) was 0.266 ± 0.062 SE (Table 2). In other words, an important component (26.6%) of the overall genetic variation was due to among-population divergence (Big Creek vs. Frontenac Axis vs. Maryland vs. Arkansas). Conversely, 73.4% of allelic variation was found within the isolated regional populations. Both the statistical ($\chi^2 = 50.54$, df = 14, $p < 0.001$) and qualitative significance of the total F value are indicative of considerable genetic

Table 1. Phenotypic band (and allelic) frequencies among 14 RAPD loci for four populations of black rat snakes.

	Population and sample size			
Locus[a]	*Frontenac Axis* 29	*Big Creek* 15	*Maryland* 30	*Arkansas* 26
Locus 1				
+	0.333 (0.208)	0.066 (0.033)	0.800 (0.550)	1.000 (1.000)
−	0.666 (0.792)	0.933 (0.967)	0.200 (0.450)	0.000 (0.000)
Locus 2[b]				
+	0.083 (0.083)	0.133 (0.067)	0.233 (0.117)	0.961 (0.808)
−	0.833 (0.917)	0.866 (0.933)	0.766 (0.883)	0.038 (0.192)
Locus 3				
+	0.958 (0.792)	0.866 (0.633)	0.866 (0.633)	0.730 (0.481)
−	0.041 (0.208)	0.133 (0.367)	0.133 (0.367)	0.269 (0.519)
Locus 4[b]				
+	0.125 (0.063)	0.133 (0.067)	0.266 (0.150)	0.000 (0.000)
−	0.875 (0.938)	0.866 (0.933)	0.733 (0.850)	1.000 (1.000)
Locus 5				
+	0.958 (0.792)	0.933 (0.733)	1.000 (1.000)	1.000 (1.000)
−	0.041 (0.208)	0.066 (0.267)	0.000 (0.000)	0.000 (0.000)
Locus 6				
+	0.916 (0.708)	1.000 (1.000)	0.966 (0.817)	1.000 (1.000)
−	0.083 (0.292)	0.000 (0.000)	0.033 (0.183)	0.000 (0.000)
Locus 7[b]				
+	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.384 (0.212)
−	1.000 (1.000)	1.000 (1.000)	1.000 (1.000)	0.615 (0.788)
Locus 8[b]				
+	0.041 (0.021)	0.000 (0.000)	0.600 (0.367)	0.923 (0.731)
−	0.958 (0.979)	1.000 (1.000)	0.400 (0.633)	0.076 (0.269)
Locus 9[b]				
+	0.333 (0.188)	0.466 (0.267)	0.466 (0.267)	0.076 (0.038)
−	0.666 (0.813)	0.533 (0.733)	0.533 (0.733)	0.923 (0.962)
Locus 10				
+	1.000 (1.000)	1.000 (1.000)	1.000 (1.000)	0.500 (0.288)
−	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.500 (0.712)
Locus 11				
+	1.000 (1.000)	1.000 (1.000)	1.000 (1.000)	0.961 (0.808)
−	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.038 (0.192)
Locus 12[b]				
+	0.375 (0.208)	0.000 (0.000)	0.166 (0.083)	1.000 (1.000)
−	0.625 (0.792)	1.000 (1.000)	0.833 (0.917)	0.000 (0.000)
Locus 13[b]				
+	0.041 (0.021)	0.066 (0.033)	0.000 (0.000)	0.000 (0.000)
−	0.958 (0.979)	0.933 (0.967)	1.000 (1.000)	1.000 (1.000)
Locus 14				
+	1.000 (1.000)	1.000 (1.000)	1.000 (1.000)	0.961 (0.808)
−	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.038 (0.192)

[a] *A "+" indicates the presence of a variable band and a "−" the absence.*
[b] *Loci used to form "restricted datasets."*

differentiation among the four regional populations. That individual F values associated with the majority (57%) of the seven loci comprising our restricted dataset were significant ($\alpha = 0.0036$ for multiple comparisons) suggests that the magnitude of the overall F was not due to differences in allelic frequency at only a few loci.

To identify exactly where genetic disjunctions occurred at the regional scale we calculated pairwise population estimates of F_{ST} and Nei's (1978) genetic distance using RAPDFST and BIOSYS-1.7, respectively. These analyses identified considerable structure between Arkansas and each of the other three populations, indicating great to very great genetic divergence between Arkansas and each of Big Creek, Frontenac Axis, and Maryland (Table 3). Though significant F_{ST} values were also calculated between Maryland and both Big Creek and Frontenac Axis, such differences qualitatively represent only minor genetic divergence (i.e., F_{ST} = 0.05–0.15). Our contrast between Frontenac Axis and Big Creek resulted in a non-significant F_{ST} implying little genetic differentiation between the two populations. Between-population F_{ST} averaged 0.164 ± 0.059 SE over all pairwise comparisons.

Estimates of Nei's (1978) genetic distance among regional populations averaged 0.190 ± 0.080 SE (ranging from 0.004 to 0.440). Though RAPD and protein variabil-

Table 2. Black rat snake population differentiation (F_{ST}) and migration rate (Nm) estimates based on allele frequency data derived using seven variable RAPD loci.*

	Restricted dataset (7 loci)				
Locus	F_{ST}	χ^2	*df*	p	Nm
2	0.479	91.01	3	<0.001	0.3
4	0.045	8.55	3	0.034	5.4
7	0.166	31.54	3	<0.001	1.3
8	0.386	73.34	3	<0.001	0.4
9	0.061	11.59	3	0.008	3.8
12	0.707	134.33	3	<0.001	0.1
13	0.017	3.23	3	0.357	14.4
Means/Totals	0.266	50.54	14	<0.001	0.70

Geographically isolated populations contrasted include Big Creek, Frontenac Axis, Maryland, and Arkansas.

ity data may not be directly comparable we felt it was important to consider our results in the context of other studies that have investigated genetic variation in snakes. Few other studies have detected such strong genetic differentiation (average Nei's distance = 0.044 ± 0.011 SE, n = 10 studies) between snake populations (Prior unpublished data). Most of these studies of protein variability were conducted on relatively smaller geographic scales (i.e., populations separated by 10–100s km rather than 100s–1000s km). The geographically closest pairs of isolated rat snake populations we surveyed (i.e., Big Creek, Frontenac Axis, Maryland—between 450–600 km apart) averaged as little as 1.5% difference, a value of at least the same magnitude (4.4%) as that found in most of the studies we reviewed. As above, replicate analyses using unique samples of individuals to represent Frontenac Axis, yielded little quantitative and no qualitative differences.

Sub-population Structure

We also found evidence of genetic structure among sub-populations within the isolated Frontenac Axis population. The AMOVA results at this scale demonstrated significant genetic differences among sub-populations within the north/south groups (12.96% of total variance, $p < 0.001$), but not between the north/south groups themselves (1.00% of total variance, $p = 0.771$). Significant variation (86.04% of total variance, $p < 0.001$) was also detected among individuals within sub-populations. Thus, of the total genetic diversity accounted for at this spatial scale, ca. 13% was attributable to local sub-population differences, and ca. 86% to individual differences within subpopulations. Five replicate AMOVAs, using unique

Table 3. Estimates of genetic differentiation (F_{ST}), genetic distance, and migration rate (Nm) between pairs of regional populations; sub-populations within the Frontenac Axis; and neighboring communal hibernacula of black rat snakes.

		Restricted dataset (7 loci)[a]		
Pairwise comparison	*Distance (km)*	F_{ST}[b]	*Genetic distance*	Nm
Regional populations				
Big Creek vs. Arkansas	~1500	0.323*	0.440	0.5
Frontenac Axis vs. Arkansas	~1900	0.273*	0.339	0.7
Maryland vs. Arkansas	~1700	0.287*	0.317	0.6
Big Creek vs. Maryland	590	0.039*	0.020	6.1
Frontenac Axis vs. Maryland	560	0.041*	0.022	5.8
Frontenac Axis vs. Big Creek	465	0.019	0.004	12.9
Mean values	~1119	0.163	0.190	4.4
Sub-populations within Frontenac Axis				
Murphy's Pt vs. Hill Island	50	0.037*	0.021	6.4
Murphy's Pt vs. La Rue Mills	45	0.035*	0.017	6.9
Queen's Station vs. La Rue Mills	40	0.009	0.000	26.3
Queen's Station vs. Hill Island	40	0.017	0.008	14.2
Murphy's Pt vs. Charleston Lk	35	0.056*	0.017	4.2
Murphy's Pt vs. Queen's Station	25	0.038*	0.019	6.4
Queen's Station vs. Charleston Lk	25	0.038*	0.016	6.3
Charleston Lk vs. Hill Island	20	0.031	0.008	7.9
La Rue Mills vs. Hill Island[c]	18	0.034*	0.011	7.2
Charleston Lk. vs. La Rue Mills	15	0.081*	0.023	2.8
Mean values	31	0.038	0.014	8.8
Communal hibernacula				
La Rue Mills vs. Hill Island	18	0.034*	0.011	7.2
Overpass vs. Riverside	1.6	0.039*	0.026	6.1
Curtis vs. Cow Island	1.2	0.006	0.000	39.5
Mean values	1.4	0.022	0.013	22.8

[a] *Estimates are based upon seven variable RAPD loci.*
[b] *Asterisks denote significant differentiation between sample pairs.*
[c] *Values for comparison between La Rue Mills vs. Hill Island are included in calculation of sub-populations means but not hibernacula means.*

random samples to represent Murphy's Point and Queen's Station, yielded little quantitative and no qualitative differences from these original results.

The RAPD allele frequency data also revealed moderate differentiation among sub-populations of rat snakes (overall $F_{ST} = 0.058 \pm 0.022$ SE). By this estimate, ca. 6% of allelic variation in the Frontenac Axis is found among sub-populations. Thus, gene flow (*Nm*, Wright 1931) among local sub-populations 24–34 km apart is relatively high (i.e., $Nm > 1$), or at least sufficient to preclude major genetic divergence. Pairwise comparisons supported this interpretation, with *F* values and genetic distance measures generally indicative of moderate to little genetic differentiation or isolation among sub-populations (Table 3). Sub-population F_{ST} averaged 0.038 ± 0.007 SE. Based on protein variability, King and Lawson (1995) documented similar measures of divergence (0.032 ± 0.005 SE) among water snake (*Nerodia sipedon*) sub-populations. That some of the most widely separated subpopulation pairs (e.g., Queen's Station vs. La Rue Mills and Hill Island, 40 km apart) exhibited little differentiation further demonstrates the lack of distinct subdivision across much of the Frontenac Axis. Nevertheless, we also found evidence of moderate divergence between some sub-populations. For example, Charleston Lake versus La Rue Mills yielded an $F_{ST} = 0.081$, despite being separated by a relatively short geographic distance (15 km). It is perhaps not insignificant that a major four-lane highway occurs between these two local sub-populations.

Genetic distances between rat snake sub-populations across the Frontenac Axis averaged 0.014 ± 0.002 SE. As noted above, several studies of protein variability in snakes have derived similar estimates of genetic distance among "populations" (e.g., Merkle 1985; Paik & Yang 1987; Hedges 1989).

Finally, replicate analyses using unique samples of individuals to represent Murphy's Point and Queen's Station, yielded minor quantitative and no qualitative differences in our results.

Inter-Hibernacula Structure

Genetic structure was also evident between some neighboring hibernacula (Table 3). Analysis of RAPD allele frequency data identified a significant, though minor, divergence between La Rue Mills and Hill Island. Given the geographic distance separating them, it is highly unlikely that members of these two hibernacula have direct contact with one another (Fig. 1b). Thus, current gene flow between La Rue Mills and Hill Island would involve snakes using hibernacula between these two sites. As a result, La Rue Mills and Hill Island exhibit genetic distance and gene flow estimates representative of sub-population structure and more in keeping with comparisons made at that spatial scale (Table 3).

In agreement with our understanding of the movement patterns and reproductive ecology of black rat snakes in Ontario, we found no evidence of divergence between the hibernaculum populations of Curtis and Cow Island (Table 3), members of which have overlapping home ranges (Weatherhead & Hoysak 1989). Unexpectedly, the geographically proximate (1.6 km) Riverside and Overpass hibernacula sampled in Maryland were found to be considerably more divergent (Table 3).

Population Diversity

As predicted, the isolated populations of Big Creek and Frontenac Axis were less heterozygous (0.104 ± 0.055 SE and 0.144 ± 0.051 SE, respectively) than the core populations of Maryland and Arkansas (0.213 ± 0.069 SE and 0.162 ± 0.069 SE, respectively), though the differences were not significant (ANOVA; $F = 0.547$, df = 27, $p = 0.655$). Estimates of mean expected heterozygosity per locus for the four regional populations averaged 0.155 ± 0.022 SE, a value which is more than three times the average (0.046 ± 0.004 SE) derived from a review of 33 studies that have investigated protein variation in snakes. Estimates of heterozygosity for the three Frontenac Axis sub-populations averaged 0.188 ± 0.028 SE (Murphy's Point = 0.160 ± 0.067 SE, Queen's Station = 0.160 ± 0.050 SE, Charleston Lake = 0.244 ± 0.067 SE). Analysis of variance indicated no significant differences among the three ($F = 0.086$, df = 41, $p = 0.917$ and $F = 0.448$, df = 20, $p = 0.645$). Heterozygosity estimates for the six communal hibernacula average 0.161 ± 0.017 SE (Curtis = 0.150 ± 0.046 SE, Cow Island = 0.152 ± 0.056 SE, La Rue Mills = 0.089 ± 0.043 SE, Hill Island = 0.166 ± 0.059 SE, Overpass = 0.190 ± 0.061 SE, Riverside = 0.218 ± 0.079 SE), and no significant differences could be detected among them (ANOVA; $F = 0.558$, df = 41, $p = 0.730$). Bellemin et al. (1978) found communally hibernating populations of red-sided garter snakes (*Thamnophis parietalis*) exhibited heterozygosities ranging from 0.011 to 0.028, with a mean of 0.019.

Discussion

Our two general goals were to make an initial assessment of the genetic structure and levels of gene flow in black rat snake populations at three spatial scales and to characterize the genetic diversity of remnant populations found in Ontario relative to populations sampled in the central core of the species' range. Using seven variable RAPD markers, we found evidence of genetic differences between geographically separated populations of black rat snakes. Regional populations appear to be strongly divergent from east to west across the species' range. Gene flow over this distance (1500 km) is insufficient ($Nm < 1.0$) to have kept these regional groups from diverging.

Across shorter distances (500 km) and presumably more recent isolation, regional populations were less genetically divergent. Limited genetic differentiation between Big Creek and Frontenac Axis (1.9%) implies that historically these two populations may have been continuous. The Frontenac Axis population might represent the remnant of a range extension for the subspecies that expanded along the south and (or) north shore(s) of Lake Ontario from the southwestern part of the province (see Fig. 1a) following the last glaciation. The rate of historic gene flow between southwestern and eastern Ontario appears to have been sufficient for Big Creek and Frontenac Axis to retain relatively high genetic similarity, despite their demographic and spatial isolation for at least the past 150 years.

We identified a moderate level of divergence among sub-populations across the Frontenac Axis, suggesting that there is genetic and demographic structure within this isolated population. Habitat heterogeneity and fragmentation, aspects of the species' behavioral ecology (e.g., dispersal patterns), patterns of human activity, and simple geographic distance might all contribute to an interference in the rate of gene flow among sub-populations 25–35 km apart.

Overall, the degree of genetic structure we found for black rat snakes using RAPDs was higher than most previous studies have found for other species using protein electrophoresis. This difference may reflect the methods used rather than biological differences among species. Given that RAPD loci appear to be more variable than protein loci, one should be better able to detect subtle patterns of genetic structure in populations using RAPDs. If so, then our results for black rat snakes suggest that other snake species may be more highly structured genetically than previous studies have indicated. Thus, other species need to be studied using highly variable genetic markers to determine whether genetic substructure is present in relatively small regional populations (i.e., at the scale of 100s rather than 1000s of km).

The range in estimates of genetic distance we found between adjacent hibernacula was unanticipated. Radiotelemetry studies demonstrate that residents of hibernacula that are 1–2 km apart (e.g., Cow Island and Curtis) probably interbreed, implying that effective population sizes may be quite large. The 17 km between La Rue Mills and Hill Island precludes contact between individuals associated with these hibernacula and thus, their relatively greater divergence in comparison to Curtis and Cow Island is not unexpected. The degree of differentiation between La Rue Mills and Hill Island is not unlike that observed at the scale of local sub-populations. By contrast, we found significant divergence between the Overpass and Riverside hibernacula, despite the fact that they were separated by only 1.6 km. Because these two hibernacula exist in what is a predominantly urban landscape (multi-lane highway, residential subdivisions, shopping malls, bowling alleys) snake movements may be restricted, resulting in little opportunity for inter-hibernacula gene flow. Evidence of genetic structure even over fairly short distances (e.g., 2–20 km) implies that gene flow among rat snake populations can be easily disrupted. If so, this pattern could explain the prevalence of subspecies in *Elaphe obsoleta* (5 subspecies currently recognized; Conant & Collins 1991). Habitat interruptions and other ecological discontinuities of seemingly minor extent may be sufficient to isolate snake populations from one another and thus initiate their divergence. This may be particularly true for highly sedentary species and species that center their activities around traditional hibernacula. Human land-use practices have been implicated in the genetic isolation of other herpetofaunal communities (e.g., Reh & Seitz 1990; Madsen et al. 1996).

Implications for Conservation

Our analysis revealed that a significant component of the total genetic diversity in black rat snakes may be found at the scale of isolated populations. This result indicates that extinction of the Big Creek and Frontenac Axis populations would constitute an important loss of genetic diversity for this species within the eastern half of its range.

From the perspective of conservation of the species within Ontario, our inability to discriminate between Big Creek and Frontenac Axis offers two important implications. First, the apparent lack of genetic structure between southwestern and eastern Ontario might be regarded as evidence that inter-population transfers, aimed at improving the viability of the highly vulnerable Big Creek population, would not result in genetic incompatibles between donors and recipients. However we caution against such an interpretation because RAPD alleles are thought to be neutral genetic markers and it is quite possible that other portions of the genome could be highly differentiated. If so, active mixing of populations could cause "genetic contamination," possibly leading to outbreeding depression (Meffe & Carroll 1994). Second, the lack of genetic distinctiveness of Big Creek and Frontenac Axis implies that the extinction of one of these populations would represent a comparatively minor loss with respect to the diversity of black rat snakes in Ontario. This dispassionate interpretation is akin to the "agony of choice" (e.g., Crozier 1992), in which the evolutionary distinctiveness of taxa is used as a value-weighting factor to direct conservation priorities. Despite the unsavory nature of electing to disregard certain populations as a conservation strategy, pragmatic considerations (financial constraints, logistics) mean that such decisions are likely to be more common in the future and empirical data will be valuable in guiding this process. Nevertheless, we believe it is essential that the results reported here are confirmed with additional genetic

markers (e.g., microsatellites) before any decisions regarding the genetic management of these threatened populations are made. Furthermore, genetic novelty should also be weighed against other considerations (e.g., local values, ecological role; Hunter & Hutchinson 1994) in decisions about conserving local populations.

General theory and most empirical evidence support the expectation that isolated and (or) peripheral populations should exhibit reduced heterozygosity and allelic variation (e.g., Lesica & Allendorf 1995). Nevertheless, following a review of protein variability in 26 populations (mostly *Thamnophis spp.*), Dessauer et al. (1987) concluded that snake populations at the geographic periphery of a species' range, on islands, or in other distributional disjunctions do not generally have lower levels of diversity. In contrast, the two isolated and peripheral Ontario populations of black rat snakes tended to be less heterozygous than either Maryland or Arkansas, though the differences were not significant. Also, Schwaner's (1990) extensive dataset on protein variation in mainland and island populations of tiger snakes (*Notechis scutatus-ater* complex) has revealed a negative correlation between heterozygosity and the degree of isolation (time, distance, or some combination thereof). Schwaner's study is particularly noteworthy in that a strong negative relationship between population heterozygosity and the frequency of scale and skeletal anomalies was found, which may reflect a negative consequence of the loss of genetic diversity. Similarly, observational and experimental work on Swedish adders (*Vipera berus*) supports the view that population isolation, genetic variability, and fitness traits are interrelated in snakes (Madsen et al. 1996). Although there may not be any immediate cause for concern regarding reduced heterozygosity in populations of rat snakes in Ontario, these data (and those for sub-populations and hibernacula as well) represent a benchmark for the assessment of genetic variability in this species and threatened populations of snakes generally.

As much as 13% of the total genetic diversity in the Frontenac Axis population may be attributable to local subpopulation differences. That sub-population divergence may be moderate overall (F_{ST} = 0.058) suggests that local, provincial, and national resource agencies should work toward cooperative management of the entire regional population. In particular, physical or ecological barriers that could impede the natural movements of snakes should be minimized in order to avoid (anthropogenic) substructuring of the Frontenac Axis population. To the extent that similar patterns of genetic structure are found in other populations, appropriately-scaled and decentralized management of threatened snakes may be warranted in many jurisdictions. Furthermore, if the pattern of divergence detected between the two hibernacula in Maryland is largely a function of habitat loss and disturbance causing isolation, then we might expect to see similar or more extreme patterns of divergence among sub-populations of snakes occupying human-dominated landscapes. For example, populations of the internationally threatened eastern fox snake (*Elaphe vulpina gloydi*) and eastern massasauga rattlesnake (*Sistrurus c. catenatus*) are found in remnant habitat patches within highly urban settings in eastern North America.

Data derived using variable RAPD markers suggest that black rat snake populations are genetically structured across a broad range of spatial scales, including divergence among geographically isolated populations, local sub-populations, and perhaps pairs of neighboring hibernacula. The patterns our results revealed in conjunction with our review of the existing literature implies that snakes in general may be highly structured genetically. This information will help direct the conservation of populations of black rat snakes within Ontario to the extent that they provide (1) a means of assessing the relative distinctiveness of remnant populations; (2) an empirical basis for identifying appropriate scales for conservation; and (3) a method of testing the efficacy of management practices. The development of hyper-variable taxon-specific genetic markers (e.g., microsatellites) will be useful for confirming the patterns reported here and may reveal even more genetic structure to populations than we have been able to identify.

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