A Comparison of RAPD Versus Microsatellite DNA Markers in Population Studies of the Massasauga Rattlesnake

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We compared genetic differentiation among populations of the threatened massasauga rattlesnake (*Sistrurus c. catenatus*) using two types of nuclear molecular markers: randomly amplified polymorphic DNA (RAPD) markers and microsatellites. Analyses of molecular variance (AMOVA) and G_{ST} and F_{ST} analyses indicated that levels of among-population differentiation between regional populations (>100 km) were comparable for both markers. However, microsatellites were superior in population assignment tests and at discerning fine-scale genetic differentiation between subpopulations separated by tens of kilometers. These results argue that both types of markers are suitable for defining broad-scale genetic structures in snake populations and can provide important inputs into conservation initiatives of focal taxa. However, our analyses suggest that microsatellites are better for detecting structure at limited spatial scales.

In conservation biology, use of high-resolution DNA markers can illuminate issues of demographic history, population connectivity, breeding structure, and introgression, and thus can provide input into strategies for conservation and management of target organisms (Haig 1998; Moritz 1994; Parker et al. 1998). Two classes of molecular markers that have received much recent attention in studies of genetic diversity in natural populations are randomly amplified polymorphic DNA (RAPD; e.g., Gibbs et al. 1994; Haig et al. 1994, 1997; Kimberling et al. 1996; Prior et al. 1997) and microsatellite DNA loci (e.g., Bushar et al. 1998; Gibbs et al. 1997, 1998; Taylor et al. 1997). The usefulness of these two marker types extends to many other biological applications, including parentage studies (e.g., Estoup et al. 1995; Fowler et al. 1998; Hadrys et al. 1993), QTL mapping of genes responsible for phenotypic attributes (e.g., Dunemann et al. 1999), studies of sperm precedence (e.g., Hooper and Siva-Jothy 1996), and morph (Lushai et al. 1997) or cultivar identification (e.g., Tessier et al. 1998).

Despite this general interest, relatively few field-based studies have directly compared the patterns of variation and differentiation detected by each type of marker using the same individuals and comparable statistical procedures (but see, e.g., Ross et al. 1999). Thus it is unclear whether these different markers are comparable for estimating of various genetic parameters, particularly those quantifying differentiation among populations. Obviously these markers are not equally applicable to all situations and their relative merits and disadvantages have been summarized elsewhere (cf. Parker et al. 1998). For example, the technical ease and lower costs of developing RAPDs may make them better for some applications (Karp et al. 1997), including conservation studies where fast and inexpensive genetic assays of population structure are required. However, because RAPDs exhibit dominance, their use in testing for departures from Hardy–Weinberg equilibrium or for estimating inbreeding is precluded (Hadrys et al. 1992). Ultimately it would be valuable to determine if microsatellites and RAPDs are equally effective at detecting patterns of differentiation that develop over similar time scales. If coincident patterns are detected for a given set of samples using either type of marker then the results obtained from both are directly comparable. However, differences would suggest that one marker type may be more appropriate for detecting isolation over short time scales, which has implications for the use of either type of marker for defining demographically independent management units (cf. Moritz 1994).

In this study we explore this issue through the examination of genetic population structure of a threatened North

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American snake, the eastern massasauga rattlesnake (Viperidae, Crotalinae, *Sistrurus c. catenatus*). In recent years the distribution of the eastern massasauga has become increasingly fragmented and it has been locally extirpated in many areas (Greene and Campbell 1992). This is probably due to a combination of factors including habitat destruction (Greene and Campbell 1992), natural vegetational succession and climatic change (Johnson 1995; Weller and Oldham 1993), and relatively narrow habitat requirements (Reinert and Kodrich 1982; Weatherhead and Prior 1992). Recent evidence from microsatellite DNA markers suggests that (1) regional, isolated populations are significantly differentiated (samples derived from Ohio, New York, and Ontario), and (2) even geographically continuous populations may exhibit fine-scale genetic population structure over distances of less than 2 km (Gibbs et al. 1997, 1998). Our objective was to build on these results by directly comparing various methods for estimating levels of population differentiation and to evaluate the probability of correct population assignment using RAPDs and previously published microsatellite data. Ultimately our goal was to see whether differences arising from separate analyses of these two molecular markers might impact on conservation and management decisions for this and, by extension, other threatened or endangered taxa.

Materials and Methods

Sample Collection

This project is part of an ongoing study of local and regional genetic population structure in snakes (Gibbs et al. 1994, 1997, 1998; Lougheed et al. 1999; Prior et al. 1997; Prosser et al. 1999). Between 1991 and 1994 blood samples (50–100 ml) were taken from hand-captured adult massasauga rattlesnakes in both Ontario and the Unites States. Blood was immediately mixed with 800 ml of lysis buffer (Seutin et al. 1991) and stored at 4°C until extraction. All snakes were equipped with either a passive integrated transponder or were marked by caudal scute clipping to prevent resampling of the same individuals. Samples derive from each of four isolated regional populations: Bruce Peninsula, Ontario, which separates Georgian Bay from Lake Huron (n = 24; 81°26′–81°39 W and 45°09′–45°16′ N); Beausoleil Island, Ontario, on the east side of Georgian Bay (n = 25; 79°50′–79°54′ W and 44°50′–44°54′ N); Cicero Swamp, New York (n = 21; 75°59′–76°05′ W and 43°07′–43°10′ N); and Springfield, Ohio (n = 19; 83°43′–84°03′ W and 39°49′–40°03′ N). Although sampling design is not completely hierarchical, within each of two regions (Bruce and Ohio) the samples can be further subdivided into two "subpopulations" separated by, at most, a few tens of kilometers: within Bruce (Emmett Lake n = 7, and Cyprus Lake n = 8); within the Ohio samples (Wright Patterson n = 7, Cedar n = 8). These within-region samples are small and exclude a number of individuals from the respective regional samples because those individuals were captured elsewhere within those regions. Additional accounts of the sample locations are given by Gibbs et al. (1997).

DNA Extraction and DNA Profiling

Total genomic DNA was extracted using either of the standard phenol/chloroform methods (Sambrook et al. 1989) and quantified using a fluorometer. Details are provided in Prior et al. (1997) and Gibbs et al. (1998).

Initial screening was performed using 10 individual DNA samples and twenty 10 bp primer pairs (A1–A20) from Operon Technologies Primer Kit A. Methods for amplification and visualization of RAPDs follow Prior et al. (1997). Based on clarity and resolution of RAPD profiles from this screening, we chose five of the Operon primers (A4, A10, A12, A17, and A20) to assay variation among all sampled individuals. To evaluate repeatability of bands in these profiles we performed two separate amplifications for all sampled individuals. We used only those marker bands for which we had 100% repeatability for subsequent statistical analysis [see Prior et al. (1997) for details].

Data for six microsatellite loci derive from a published study (Gibbs et al. 1997) and laboratory methods are reported therein. For this study we omitted one population from the microsatellite study for which we do not have RAPD data (Killbear) and included only individuals for which we have data for both markers.

Assessment of Population Structure

For RAPDs, Lynch and Milligan (1994) advise that unbiased estimates of genetic parameters are best achieved by only including loci where the null phenotype exceeds a frequency of $3/N$ (where N is the sample size for each population). Therefore in subsequent analyses we analyze a "complete" RAPD dataset (including all loci) and a "subset" [following the pruning rule suggested by Lynch and Milligan (1994)].

Intrapopulational genetic diversity was evaluated using POPGENE version 1.21 (Yeh et al. 1997); for RAPDs we calculated Shannon's information index (I; King and Schall 1989) and Nei's (1973) genetic diversity (h), and for microsatellites, I and Nei's (1973) expected heterozygosity (H_{exp}). For each RAPD and microsatellite locus, we tested for heterogeneity of allele frequencies across all populations using G^2 tests in POPGENE. To evaluate the degree of genetic subdivision among populations, we used POPGENE to calculate Nei's (1987) coefficient of gene differentiation, G_{ST}, for RAPD loci and Wright's (1951, 1978) F_{ST} for microsatellites. From these values we also derived estimates of gene flow (Nm).

To examine the relative apportioning of variation among and within regions, we performed an analysis of molecular variance (AMOVA; see Excoffier et al. 1992) using WINAMOVA (Excoffier et al. 1992) for RAPDs, and ARLEQUIN version 1.1 (Schneider et al. 1997) for microsatellites. For RAPDs, such hierarchical analyses are possible because AMOVA is based on a matrix of squared Euclidean distances between "chromosomes" (= individual multilocus RAPD phenotypes) and thus does not require full genotypic information. Statistical significance of variance components and correspondingly Φ_{ST} (the analogue of the conventional F_{ST}) were assessed using null distributions generated via random permutation of individuals.

For all pairwise comparisons between populations, we calculated pairwise Φ_{ST} using WINAMOVA (RAPDs) and ARLEQUIN (microsatellites). Statistical significance was again derived using a permutation test. Estimates of gene flow (Nm) between pairs of populations were calculated according to Wright (1951). We used this same approach for assessing the possibility of fine-scale genetic differentiation within our defined populations [i.e., within the Bruce population (between Emmett and Cyprus), and for the Ohio population (between Wright and Cedar)].

Probability of Identity

We carried out a more direct comparison of the resolution of these two types of molecular markers by using a population "assignment" test developed by Patkeau et al. (1995). For microsatellites, the assignment test evaluates the probability that a particular individual's genotype would occur in each of the examined populations. The

Table 1. For each locus, values of G_{ST} (Nei 1973) and F_{ST} (Wright 1978) for RAPDs and microsatellites

RAPDs			Microsatellites		
Locus[a]	G_{ST}	*Nm*	Locus	F_{ST}	*Nm*
OPA 04–1*	0.018	27.0	Scμ 01	0.118	1.87
OPA 04-2*	0.032	15.1	Scμ 05	0.171	1.21
OPA 04-5*	0.016	31.0	Scμ 07	0.129	1.68
OPA 04-7*	0.047	10.1	Scμ 11	0.177	1.16
OPA 04-8*	0.0063	78.6	Scμ 16	0.208	0.95
OPA 04-11*	0.16	2.6	Scμ 26	0.261	0.71
OPA 10-1*	0.011	42.9	Overall	0.173	1.20
OPA 10-2*	0.20	1.9			
OPA 12-1*	0.015	32.3			
OPA 12-3*	0.089	5.1			
OPA 17-1*	0.048	10.0			
OPA 17-5	0.15	2.8			
OPA 17-7	0.25	1.5			
OPA 20-1	0.27	1.4			
OPA 20-2	0.18	2.2			
Overall (all loci)	0.16	2.6			
Overall (subset)	0.058	23.33			

Shown also are estimates of gene flow (*Nm*). For RAPDs overall values are calculated across all loci and for a subset of loci restricted according to the rules outlined in Lynch and Milligan (1994). See text for details.

[a] Loci for restricted subset are indicated by an asterisk.

individual is assigned to the population in which the individual's genotype has the highest expected frequency based on the observed distributions of alleles (Patkeau et al. 1995, 1997). When performed for all individuals, a probability of correct assignment for each nominal population can be calculated. This method assumes Hardy–Weinberg equilibrium and no linkage disequilibrium among loci. For RAPDs, we used a modification of this method (using the presence or absence of an amplified band rather than allele frequencies; see Haig et al. 1997). To calculate assignment probabilities we used the online program Assignment Calculator (Brzustowski 1998) for microsatellites, and a program written in ThinkPascal® for RAPDs.

Results

Summary of Results of RAPD Profiling

For the present analyses we retained a total of 15 RAPD loci which showed 100% repeatability across amplification trials (see Table 1). For four RAPD loci, rare RAPD phenotypes were restricted to single populations: Cicero, OPA 04-1; Bruce, OPA 04-5; Beausoleil, OPA 04-7; Beausoleil, OPA 12-1. However, genetic differences among populations appear largely due to frequency differences of amplified fragments rather than to complete absence or presence of particular bands. Eleven loci had null frequencies above $3/N$ for all populations, and these were used for our restricted dataset (Table 1).

Intrapopulation Variability and Population Structure

Nei's gene diversity across loci for the complete RAPD dataset ranged from 0.132 (Cicero) to 0.211 (Ohio) and from 0.155 (Bruce) to 0.180 (Cicero) for the subset. Mean expected heterozygosity across microsatellite loci ranged from 0.531 (Cicero) to 0.689 (Bruce). Ranges of mean values for Shannon's information index for the three datasets were: across all RAPD loci, 0.201 (Cicero) to 0.314 (Bruce); for the RAPD subset, 0.219 (Beausoleil) to 0.249 (Bruce); for microsatellites, 1.019 (Cicero) to 1.501 (Ohio). The rank order of these diversity estimates did not appear to be consistent across datasets, although formal testing of this observation is inappropriate with only four populations. Not surprisingly, average variability detected for the six microsatellites greatly exceeded that detected by assayed RAPD loci (e.g., average I across populations for RAPDs was four to five times that for microsatellites).

Results of G^2 tests after sequential Bonferonni correction (Rice 1989) indicated statistically significant heterogeneity of band frequency for 6 of 15 assayed RAPD loci (OPA 04-11, OPA 10-2, OPA 17-5, OPA 17-7, OPA 20-1, OPA 20-2). Tables 1 and 2 present the results from the different approaches used to apportion variation in RAPDs and microsatellites into within- and among-population levels. G_{ST} for RAPD loci ranged from 0.0063 (OPA 04-8) to 0.27 (OPA 20-1), with an average across all loci of 0.16 and a corresponding *Nm* of 2.6 (Table 1). Average G_{ST} across the subset of RAPD loci was 0.058 ($Nm = 23.3$). Analysis of the microsatellite data suggested an overall level of among-population differentiation similar to that of the complete RAPD dataset (average $F_{ST} = 0.173$, $Nm = 1.20$), with values of F_{ST} ranging from 0.118 (Scm 01) to 0.261 (Scm 26) for individual loci (Table 1).

Regardless of which dataset or marker was used, the AMOVAs indicated that a significant proportion of total genetic variation was attributable to among-population differentiation (Table 2). For RAPDs, restricting the analyses to the subset of 11 loci raised the among-population component only slightly from 14.20% to 15.04%. For microsatellites, approximately 20% of the total variation was comprised of the among-population component and the remainder to within population differences.

For all data partitions, all regional pairwise Φ_{ST} comparisons indicated significant genetic differentiation ($P < .0001$). Mean pairwise Φ_{ST} for the complete RAPD dataset was 0.141, with values ranging from 0.089 (Bruce-Beausoleil) to 0.255 (Beausoleil-Cicero). For the RAPD subset, mean Φ_{ST} was 0.159 (range 0.080 for Cicero-Ohio to 0.316 for Beausoleil-Cicero). Mean pairwise Φ_{ST} for microsatellites was 0.198, with the minimum estimate for Bruce-Beausoleil (0.080) and the maximum for Beausoleil-Cicero (0.266). Across all datasets, estimated values of *Nm* were less than 3, and for Beausoleil-Cicero comparisons consistently values were less than 1. For two additional comparisons (Beauso-

Table 2. Results from analyses of molecular variance

Level	df	SS	MS	Variance	Total (%)	*P*
RAPDs (all loci included)						
Among	3	16.465	5.488	0.195	14.20	<.001
Within	85	99.939	1.176	1.176	85.80	—
RAPDs (subset of loci)						
Among	3	12.879	4.293	0.154	15.04	<.001
Within	85	74.132	0.872	0.872	84.96	—
Microsatellites[a]						
Among	3	65.598	21.866	0.451	19.57	<.001
Within	174	322.767	1.855	1.855	80.43	—

Probability values are based on a null distribution from 1000 bootstrap replications.

[a] Based on the F_{ST} distance method.

Table 3. Comparison of the probability of correct assignment using microsatellite genotypes and RAPD phenotypes

	Bruce	Beausoleil	Cicero	Ohio
RAPDs (all loci)				
Bruce (n = 24)	0.29 (7)	0.33 (8)	0.25 (6)	0.125 (3)
Beausoleil (n = 25)	0.08 (2)	0.72 (18)	0.12 (3)	0.08 (2)
Cicero (n = 21)	0.24 (5)	0.095 (2)	0.67 (14)	0.00 (0)
Ohio (n = 19)	0.05 (1)	0.05 (1)	0.16 (3)	0.74 (14)
Microsatellites				
Bruce (n = 24)	0.92 (22)	0.41 (1)	0.00 (0)	0.41 (1)
Beausoleil (n = 25)	0.04 (1)	0.96 (24)	0.00 (0)	0.00 (0)
Cicero (n = 21)	0.00 (0)	0.00 (0)	1.00 (21)	0.00 (0)
Ohio (n = 19)	0.00 (0)	0.00 (0)	0.05 (1)	0.95 (18)

Along the left side is listed the nominal population (and associated sample size) and along the top the population into which individuals were assigned. For each row is shown the proportion of the nominal population assigned to each of the four sampled populations (actual number classified is indicated in parentheses). See text for details.

leil-Ohio and Cicero-Ohio), estimates of *Nm* using microsatellites were less than 1.

For RAPDs, the Φ_{ST} estimates between the two Bruce subpopulations suggest no genetic differentiation (complete: $\Phi_{ST} = -0.105$, $P = .999$; subset: -0.127, $P = .999$). However, the same statistic calculated for the Cedar-Wright comparison within the Ohio sample suggests remarkable local differentiation (complete: $\Phi_{ST} = 0.282$, $P < .001$, subset: 0.447, $P < .0001$), exceeding all regional pairwise population comparisons. For microsatellites, the values for the Bruce and Ohio subpopulation comparisons were 0.0613 ($P = .0201$) and 0.203 ($P < .001$), respectively.

Population Assignment Tests for RAPDs and Microsatellites

Results of the population assignment tests are presented in Table 3. Analyses using all RAPD loci performed relatively poorly compared to that with microsatellites, with correct assignment ranging from 29% (Bruce) to 74% (Ohio). Assignment of individuals using the subset of RAPD loci (not shown) was no better than would be expected by chance (i.e., 25%). In contrast, microsatellites assigned individuals to their nominal populations with success that ranged from 92% (Bruce) to 100% (Cicero).

Discussion

RAPDs Versus Microsatellites in Detecting Population Structure

Our most striking result is that most analyses of population structure at the regional level (>100 km) using either class of marker revealed similar overall levels of among-population differentiation. All AMOVA results indicated that approximately one-fifth of total variation may be attributable to differentiation among sampled regions (Table 2), while values of mean G_{ST} (complete RAPD dataset) and F_{ST} (microsatellites) were of comparable magnitudes (Table 1). Together these results suggest that there has been significant isolation and restriction of gene flow among *S. c. catenatus* regional populations, including even those that are relatively close (Beausoleil and Bruce). Patterns of genetic differentiation between pairs of populations also support this interpretation. For example, estimates of gene flow (*Nm*) derived from pairwise Φ_{ST} were all less than three, regardless of which dataset or marker was used. Thus both RAPDs and microsatellites suggested some viscosity of gene flow between populations, although microsatellites suggested somewhat more restricted movement between populations with three *Nm* values less than 1. This possibly reflects the fact that movement of snakes among populations is not possible at present, nor was it in the recent past, due to fragmentation and isolation of habitat. Mean G_{ST} for the restricted RAPD dataset was approximately one-third the magnitude of that for the complete dataset, because the four omitted loci showed highly significant among-population differentiation and correspondingly, the largest values for G_{ST}.

At the microgeographic scale (within populations), analyses of RAPDs and microsatellites both indicated statistically significant differentiation between the two Ohio samples. However, RAPDs failed to detect differentiation between samples within the Bruce population (both Φ_{ST} with negative values), a comparison that was highly significant using microsatellites. This result probably reflects small sample sizes ($n < 10$) and the relatively higher statistical power of microsatellites compared to RAPDs for detecting genetic differences of recent origin.

Similar levels of differentiation are present despite substantial differences in the levels of variability present in each type of marker. Microsatellites are typically much more variable than RAPDs. Different levels of variability across classes of marker reflect a balance between mutation rate and population processes like genetic drift (e.g., Scribner et al. 1994). Microsatellite mutation rates are typically estimated to range between 10^{-3} and 10^{-4} per locus per generation (Dallas 1992; Weber and Wong 1993). For allozyme loci, mutation rates have been suggested to range from 10^{-6} to 10^{-9} per locus per generation (Ayala 1976). Mutation rates for RAPDs probably lie somewhere between these two extremes. Given comparatively higher mutation rates with microsatellites, one would expect that differences among newly isolated populations might accumulate more rapidly and reflect more accurately recent demographic events like bottlenecks (Hedrick 1999; Lehmann et al. 1996). However, the short time span over which these snakes have occupied their postglacial habitats (<10,000 years), combined with their long generation time, may mean that the number of new mutations that could have arisen is small. In addition, the high level of within-population variation for microsatellites may lead to a reduction in estimates of differentiation which are based on ratios of within- versus among-population variation (Charlesworth 1998; Hedrick 1999). Finally, constraints on microsatellite evolution (e.g., biased mutation or selection for certain allele sizes) may downwardly bias estimates of interpopulation differentiation (Lehmann et al. 1996). Combined, these factors may explain why the observed levels of differentiation are similar across markers.

Our RAPD data were clearly inferior for populations assignment tests (Table 3), with the best probability for correct assignment using RAPDs (74% for Ohio) markedly lower than the worst probability for microsatellites (92% for Bruce). The dataset pruned according to the criterion of Lynch and Milligan (1994) performed very poorly compared to the full dataset, primarily because all four omitted loci showed statistically significant differentiation (G^2 tests) due to substantial differences in band frequencies across populations. Although RAPDs have proved useful for assignment tests in other contexts (e.g., Haig et al. 1997), greater numbers of dominant compared to codominant markers are generally required to achieve comparable levels of population resolution (Smouse and Chevillon 1998).

Conservation and Management Implications

A significant concern in conservation genetic analysis is whether conclusions derived for a single class of genetic marker are representative of genomewide patterns of variation. Patterns that are found consistently across multiple markers that sample different parts of the genome increase the probability that the patterns are real rather than reflecting features particular to a particular locus or class of marker (e.g., mode of inheritance, mutation process, magnitude of genetic variation) or artifacts of limited sampling of loci (Liu and Furnier 1993; Ross et al. 1999; Scribner et al. 1994). For questions relating to fine-scale genetic structure, differences between the two markers were evident. If differentiation over short distances (<10 km) in both subpopulation comparisons detected using microsatellites accurately reflects the population biology of massasauga rattlesnakes, then the RAPD results are misleading at this scale. Conclusions derived from RAPD analyses alone (albeit with small numbers loci and samples) would have failed to identify demographically independent populations, and hence possible management units (Moritz 1994) at spatial scales of less than 20 km. This finding may be relevant for snakes in general because an increasing number of studies based on microsatellites have documented fine-scale genetic structure in a variety of ecologically distinct species (Bushar et al. 1998; Lougheed et al. 1999; Prosser et al. 1999).

In contrast to results for local levels, our results are similar in important details for both classes of marker at the regional level of analysis. The observation that dominant RAPDs and microsatellites can reveal concordant patterns of population structure, especially but not exclusively at larger geographic scales, has been shown for other taxa. For example, Ross et al. (1999) report that the distribution of genetic variance among three hierarchical levels in fire ants (*Solenopsis invicta*) was similar across four classes of neutral molecular markers including microsatellites and dominant RAPDs. In black rat snakes (*Elaphe o. obsoleta*), Lougheed et al. (1999) found a significant correlation between pairwise F_{ST} values generated for RAPDs and microsatellites across sampling scales. In *Elymus fibrosus* (a species of tribe grass, family Triticeae), although diversity was higher for microsatellites than for RAPDs, there was agreement in the apportionment of genetic diversity within and among examined populations (Sun et al. 1998). Similarly, for massasauga rattlesnakes, separate consideration of RAPDs and microsatellites indicated statistically significant differentiation among regions and similar levels of within-region genetic variation (Table 2). Further, pairwise analyses suggested significant restriction of gene flow among regions ($Nm < 3$) across datasets. Thus at the regional scale, both classes of marker would indicate that regional populations comprise distinct management units and would suggest similar plans of action at the regional and national levels to preserve genetic diversity and define management units within eastern massasauga rattlesnakes.

In summary, results of the present study emphasize that both microsatellites and RAPDs can be successfully employed in, and may give comparable results for, assays of genetic differentiation over relatively larger geographic scales. The drawbacks of RAPDs are well established (viz. dominance); however, we here reiterate that our study suggests that RAPDs are useful in situations where relatively inexpensive first approximations of the genetic landscape of vulnerable or endangered species are desired. Concerns regarding the relatively lower "resolving power" of dominant markers, as we have shown with our assignment tests, are ameliorated by the relative ease with which one can increase the number of assayed RAPD loci. Further, we emphasize that combined RAPD and microsatellite surveys of massasauga rattlesnakes have given us greater confidence that the delineated patterns are real because of the importance of drawing on results from multiple genetic systems (Allendorf and Seeb 2000).

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