

**Conservation genetics of the isolated Ojibway/LaSalle  
Complex massasauga rattlesnake population.**

**Prepared for the Massasauga Recovery Team**

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## Brief Background

The Ojibway/LaSalle Complex population of massasauga rattlesnakes (*Sistrurus catenatus catenatus*) of extreme sw. Ontario is one of only four remaining Canadian 'populations' of this species, and is one of the two most imperiled (Weller and Parsons 1991; Weller and Oldham 1993). The two largest and arguably most secure populations (Weller and Oldham 1993) have been characterized genetically using both high-resolution DNA microsatellites (Gibbs *et al.* 1997, 1998) and RAPDs (Lougheed *et al.* 2000), but we lack such data for the other two. Pither (2003) has clearly identified genetic profiling of Ojibway/LaSalle massasauga rattlesnakes as critical to informed decisions regarding management and recovery of this population (Part IV Recommendations – Point 8), noting that minimally such data will allow us to test for inbreeding. This study seeks to address this deficiency.

Genetic characterization of the Ojibway/LaSalle population requires the use of high-resolution molecular markers – much research and development has already occurred. Gibbs *et al.* (1997, 1998) provide massasauga rattlesnake-specific sequences for six nuclear DNA microsatellite primer pairs that will be used for the study proposed here. More recently, Gregory (2001) screened four additional massasauga-specific microsatellite loci, providing ten microsatellite loci to characterize nuclear genetic diversity for the Ojibway Complex populations. While other markers may provide greater insight in future work (e.g. single nucleotide polymorphisms, major histocompatibility complex loci) the tight timelines required for the present work recommend using existing markers.

The limited sample sizes of the Ojibway/LaSalle 'population' preclude some population-level approaches. However, the specimens currently held at the Toronto Zoo (four females collected while gravid plus some offspring), in conjunction with other Ojibway Complex samples held by Lisle Gibbs (Ohio U) and others, do allow us to undertake some useful analyses that can guide conservation efforts. Further, the current 'best-guess' estimates of adult numbers in LaSalle (~50, Pither 2003), suggest that the sample herein may represent a significant proportion of the population.

## Proposed questions to be addressed

1. From the sampled offspring of wild-caught gravid females (some held at the Toronto Zoo and others subsequently released), is there evidence of multiple sires for their offspring?
2. Is there evidence of significant inbreeding in the Ojibway Complex population?
3. How does nuclear genetic diversity of the Ojibway Complex population compare to the other four populations that have been investigated (i.e. is there evidence for a bottleneck effect and reduced genetic diversity in the former)?
4. How distinct is the Ojibway Complex population from other Canadian and US populations that have been assayed?

## Methods

### *Samples*

The 70 tissue/blood samples received for this study derive from wild-caught individuals and offspring born in captivity to wild-caught females (Appendix). Nine of these originated from the Wainfleet location and 61 from the LaSalle population. For the latter, two were duplicate samples (one of the females at the Toronto Zoo had had a blood sample taken in a previous year), and an additional one was the placenta from a gravid female and thus was excluded. Five females (one from Wainfleet and four from LaSalle) were included in the sample with all or some of their offspring. For the LaSalle population, a total of 18 individuals were wild-caught and assumed to be a representative sample. I further assume that these samples represent one demographic unit (although I do not know the precise provenance of individuals OJIB 2, OJIB 3, OJIB 4, OJIB 4R61, OJIB1-93, and OJIB 2R4L). Genetic data for these 18 individuals, combined with data from five other sites from Gibbs et al. (1997), were used for population-level analyses (Table 1). I genotyped all individuals that were sent but for this report have analysed only appropriate subsets of these (i.e. excluding Wainfleet samples and those without detailed locality data).

### *Genotyping*

Species-specific dinucleotide microsatellite primer pairs were developed previously as described by Gibbs et al (1998). A total of ten microsatellites were chosen for the present study from both Gibbs et al. (1997, 1998) and Gregory (2001) – see Table 2.

All hot PCR reactions were performed in an Applied Biosystematics GeneAmp 9700 thermocycler or an MJ Research Gradient Cycler as follows. Forward primers were end-labelled using  $\gamma$ - $P^{33}$  ATP in a reaction containing 0.12  $\mu$ L of sterile double-distilled water, 0.05  $\mu$ L of forward primer (10  $\mu$ M), 0.05  $\mu$ L of T4 polynucleotide kinase (Fermentas PNK 10 units), 0.03 mL of Buffer A (Fermentas PNK buffer) and 0.05  $\mu$ L of  $\gamma$ - $P^{33}$  ATP (10  $\mu$ Ci/ $\mu$ L). Reactions were run at 37°C for 30 minutes and then at 65°C for 10 minutes to halt kinase activity. Labelled primers were used in PCR reaction cocktails with 1-5  $\mu$ L DNA template, 2.70-6.35  $\mu$ L sterile double-distilled water, 1  $\mu$ L PCR Buffer (25 mM MgCl<sub>2</sub>, 50 mM Tris pH 8.0, 50 mM Tris pH 8.8, 500 mM KCl, 4 mg/ml BSA, and 0.1% gelatin), 0.5  $\mu$ L dNTPs (10mM), 0.5  $\mu$ L reverse primer (10  $\mu$ M), 0.25  $\mu$ L forward primer (10  $\mu$ M), 0.1  $\mu$ L Taq (5 units) and 0.3  $\mu$ L end-labelled forward primer (10  $\mu$ M). Cycling parameters were an initial denaturation at 94°C for 2 minutes, 30 cycles of 92°C for 30 seconds, 55-62°C (depending upon the primer being used) for 20 seconds, 72°C for 20 seconds and a final extension at 72°C for 5 minutes.

Five  $\mu$ L of stop solution was added to the PCR product and samples were heated to 90°C before being loaded into a 6.5% polyacrylamide gel. Previously run DNA sequences were loaded in each gel to facilitate consistent scoring of the alleles. The gels were electrophoresed using a standard PAGE gel rig in 0.5X TBE buffer (0.45M

Tris-Borate, 0.01M EDTA) at 65 watts for 2.5 hours. Completed gels were transferred to blotting paper, vacuum dried, and exposed to Progene Scientific Autoradiography X-ray film for 6-7 days. For each individual and locus, the genotype was scored independently by two observers from the resulting autoradiographs.

### *Statistical Analyses*

Using the program Kinship (Version 1.2; Queller and Goodnight 2000), relatedness between pairs of offspring within the four LaSalle broods was estimated using the regression method of Queller and Goodnight (1989) correcting for population allele frequencies calculated from the genotypes of sample of 18 wild-caught individuals. For each brood, I also tested the hypothesis that each offspring was a full sib (against the null of half sibship) using a likelihood ratio test with significance of the ration determined from a dataset of 10,000 full-sib pairs simulated from population allele frequencies.

For each population sample, summary statistics (expected and observed heterozygosities, number of alleles) and estimates of pair-wise  $F_{ST}$  were estimated, and tests for heterozygote deficiencies, and differences in genic and genotypic distributions, were performed in GENEPOP (Version 3.4; see Raymond and Rousset, 1995) with details of analytical methods contained therein.

## **Results, interpretations and recommendations for recovery plans**

Two of ten surveyed loci, Scu16 and Scu126, gave anomalous results (e.g. some offspring genotypes with apparently neither maternal allele for parentage analysis). Barring sample mislabelling or mix-up, which I think unlikely given that the genetic data used in parentage analysis do make sense for the remaining eight polymorphic loci and two duplicate samples had identical multilocus genotypes, and recognizing the possibility of a non-amplifying null allele, I eliminated these loci from ALL analyses.

### ***Extra-pair Paternity?***

- **Results:** Analyses of offspring genotype arrays suggest that two of four broods were sired by more than one male. This conclusion is based on presence of > 2 inferred paternal alleles from locus Scu137 in broods from female U-03 and Q-02 (see Table 3). I thus hypothesize that broods from the other two females (P-02 and L-00) are singly sired.
- **Recommendations:** Given limited housing for a captive population (and assuming that offspring can be sexed), I recommend that, where possible, you retain one male and one female from each brood. For multiply-sired broods it would be best to retain half sibs, one female and one male. This will allow for maximum flexibility in a captive breeding program. For females U-03 and P-02 only two offspring were retained for the captive population so genetic profiles obviously do not inform their selection. For female L-00,

assuming a single sire fathered all of her offspring, all offspring are full sibs and any female and male can be selected. For offspring of female Q-02 I recommend that you select the female-male pair that has the lowest pair-wise relatedness in Table 4, and thus presumably this highest probability of being half sibs.

### ***Evidence for Inbreeding?***

- **Results:** I found no compelling evidence for inbreeding based on tests for heterozygote deficiency (compared to the Hardy-Weinberg expectation) – see Table 5. Only one of eight polymorphic loci (Scu26) showed significant deficiency of heterozygosity after Bonferonni correction (Rice 1989), whereas the expectation is that inbreeding should affect all loci similarly. Overall, there was no significant heterozygote deficiency ( $p = 0.0595$ ) although this is only marginally non-significant and less than the conservative  $\alpha$ -level of 0.10 sometimes recommended for conservation studies. One can argue that this and other findings given below are, in part, due to limited sample size ( $n=18$ ) but Springfield ( $n=21$ ) and Cicero ( $N=25$ ) do show significant inbreeding overall with sample sizes that are not that much greater than those for LaSalle. Moreover, the 18 individuals used in the present study probably comprise a significant portion of the total population size of the Ojibway/LaSalle Complex (Pither 2003). Note also that in other populations (Gibbs et al. 1997) heterozygote deficiencies were attributed to previously undiagnosed, fine-scale populations structure rather than inbreeding in the strict sense.
- **Recommendations:** Given that there exist more samples already collected, I would recommend that these be genotyped and pooled with existing data to provide a more robust test for the presence of inbreeding (and indeed for other genetic analyses of population diversity and differentiation). Additional samples from population surveys also will augment these analyses and increase our ability to test for the signature of inbreeding. Ultimately, it is premature to consider population translocation strategies like those undertaken by Madsen et al. (1999; see also Madsen et al. 1996) before the addition of these data (see Summary below).

### ***Within-Population Genetic Diversity?***

- **Results:** Irrespective of finding no significant evidence for inbreeding overall, our measures of genetic diversity are lower than in any of the other five surveyed populations (e.g. expected heterozygosity and number of alleles). Moreover, although some of this disparity in diversity might be attributed to larger geographic sampling area of the samples in the study by Gibbs et al.

(1997), Gregory (2001) examined subpopulations within Killbear Provincial Park at a scale more comparable to Ojibway/LaSalle and these diversity estimates remain larger. Andre (2003) examined diversity in three microsatellite loci (Scu01, Scu05, and Scu07) for five population samples in Illinois, Indiana and Ohio and here too estimates of Ojibway/LaSalle for these same three loci, with few exceptions, were lower. While we do not know what historical diversity existed in this population it is probable that the recent precipitous population decline and smaller size of the LaSalle population has resulted in loss of genetic diversity through both the effect a population bottleneck and genetic drift.

- Recommendations: All samples, to my knowledge, derive from the LaSalle site (with the exceptions of the samples noted in the methods). Genotypes of individuals from the other known occupied site (Spring Garden) will give us a more completed picture of genetic diversity existing within the Ojibway/LaSalle Complex. Repatriation efforts, if they proceed, should incorporate genotypes from both of sites to increase the diversity of re-introduced population. Similarly, further retention of individuals for captive breeding should focus on maximizing the retention of already existing genetic diversity – incorporating neonates from different females and if possible from Spring Garden.

### ***Differentiation Among Populations?***

- Results: Because we cannot yet reliably determine homology of alleles between the published dataset of Gibbs et al. (1997) and the data gathered for the present study (a five sample panel of previously sampled individuals proved insufficient for this purpose), I cannot directly estimate among population differentiation. However, based on work in other populations (i.e. finding significant differentiation in microsatellites over distances < 50 kms and in some instances suggested to be < 10 kms.; see Gibbs et al. 1997 and Gregory 2001) it is highly probable that the Ojibway/LaSalle population represents a distinct component of neutral genetic diversity for this species in Canada and globally. Analyses for data for one locus (Scu07) for which I am reasonably certain of homology of allele sizes supports this contention as LaSalle was significantly different from all other populations in both allelic ( $p$  for all 5 pair-wise comparison  $\leq 0.0034$ ) and genotypic frequencies ( $p$  for all 5 pair-wise comparison  $\leq 0.014$ ), with pair-wise  $F_{ST}$  between LaSalle and other samples (all > 0.044) at least as large as for other comparisons.
- Recommendations: I recommend that we run a larger panel of samples (minimum 20 individuals) from previously published work to clarify homology of alleles so that the two data sets may be more thoroughly compared. Regardless, based on my assertions above, I think that the Ojibway/LaSalle

population should be managed as a distinct genetic/demographic entity relative to other populations. I also emphasize that the ecological uniqueness of the Ojibway/LaSalle compared to other Canadian populations (tallgrass prairie) raises the possibility of adaptation to distinctive features of local environment and this too recommends immediate and independent conservation action.

## Summary

The genetic data contained herein and perhaps available through analyses of additional individuals, together with continued survey/demographic work *in situ* provide a unique perspective in conservation, and potentially recovery, of the tallgrass prairie population of massasauga rattlesnakes (and indeed remnants of Ontario tallgrass prairie itself).

I think that work scheduled for this year (2004) should focus on: 1. intensive survey/demographic work on the LaSalle and Spring Garden sites (ensuring that all individuals are pit-tagged and sampled for genetic analyses, their locations precisely recorded, and phenotypic manifestations of inbreeding depression noted; see Austin 2004), 2. standardizing individual identification numbers so that there is no fear of duplicate or mixing of samples which obviously would confound test of inbreeding, population genetic diversity and differentiation, and 3. collating all data for all existing and newly-found individuals to include (together with other data like sex and age) pit-tag number, individual identification number, pedigree information and genotype. Given small population size of the Ojibway/LaSalle Complex population an appropriately targeted effort incorporating the recommendations above could result in data (including genotypic) for a significant portion of the total population. This would provide opportunities to address questions related to mating system and asymmetry of mating success (e.g. with all or most adult males we should be able to infer which males most likely sired which offspring), or fine-scale population structure within the Ojibway/LaSalle Complex. Results of analyses on an augmented genetic dataset together with survey/recapture data from a 2004 field season should inform decisions on translocation and management for 2005.

## Literature cited

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**Table 1:** Sites and samples sizes used for population level analyses (for all but LaSalle, sampling details may be found in Gibbs et al. 1997).

<b>Site Name</b>	<b>Details</b>	<b>Sample Size</b>
Bruce Peninsula	Upper portion of the Bruce Peninsula – most from Bruce Peninsula National Park.	42
Beausoleil	Beausoleil Island part of Georgian Bay Islands National Park, Ontario.	32
Killbear	Killbear Provincial Park on eastern shores of Georgian Bay, Ontario.	81
LaSalle	LaSalle woodlot within the Ojibway Prairie Complex in extreme sw. Ontario (Windsor).	18
Springfield	Samples from 3 geographically isolated sites in west-central Ohio, USA.	21
Cicero	Cicero Swamp Wildlife Management Area near Syracuse, New York, USA.	25

**Table 2:** Microsatellite loci and primer pairs used in current analyses for annealing temperatures and other details see the original sources indicated.

<b>Locus Name</b>	<b>Primer Pair (5' to 3')</b>	<b>Source</b>
Scu01	F: GTCAACACTTGTGTTCTGC R: CTGTATTAAGTTGTTTTGTTCA	Gibbs et al. (1998)
Scu05	F: GACATTGCTGAACAGACTAT R: TTGTGTAGCATAGTGAAACA	Gibbs et al. (1998)
Scu07	F: CTTTGTGCTATTTTTCCACC R: GCCAAAAAAGTAAAATATGAGC	Gibbs et al. (1998)
Scu11	F: AATCAGCATGTGGCTTAAATC R: GCTGCTTGGCTACATATGC	Gibbs et al. (1998)
Scu16	F: TATGGGAATCTGGCTTTCTC R: AACTGATTCATATCTGCACTGC	Gibbs et al. (1998)
Scu26	F: GAAATTGGTGGGAAGAGACCTG R: GTCCAGGATATGAGGGATCTG	Gibbs et al. (1998)
Scu106	F: AAGAGCCAATACTGGGGTCC R: CTGGATGGCGAATCCAAAC	Gregory (2001)
Scu125	F: GTCAACCCATCACGACTC R: TTTCTCTGCTATGCAATATCC	Gregory (2001)
Scu126	F: ATCACTTCACACTTGTTTCATACTG R: AACCATCTTTAATGCCTCTG	Gregory (2001)
Scu136	F: ACATAATTTGAAGGTGCCTATT R: AGAATTCTGGGAGTCAAAGAC	Gregory (2001)

**Table 3:** Minimum number of paternal alleles contributed to offspring for 8 assayed microsatellite loci for broods of each of four gravid LaSalle females taken into captivity. N indicates number of offspring genotyped. Locus designations are from Gibbs et al. (1997) and Gregory (2001) – see also Table 2. Minimum number of inferred sires (No. Sires) from inferred offspring paternal alleles (i.e. if number of alleles for any locus  $\leq 2$  then only one sire is required to explain array of offspring genotypes; assuming no mutation, if number of alleles for any locus  $> 2$  and  $\leq 4$  then two sires are required). Note that these inferences are based on a single, variable locus Scu137. Scu11 was monomorphic but is here included for completeness.

Female	N	Locus								No. Sires
		Scu01	Scu05	Scu07	Scu11	Scu26	Scu106	Scu125	Scu137	
U-03 Zoo ID ? "Ursala"	10	2	2	2	1	2	2	2	4	2
Q-02 Zoo ID 38522	14	1	2	2	1	2	2	2	3	2
P-02 Zoo ID 38596 "Polly"	2	1	1	1	1	1	1	2	2	1
L-00 Zoo ID 38597 "Lana"	12	2	2	1	1	2	2	2	2	1

**Table 4:** Relatedness (a regression measure of relatedness proposed by Queller and Goodnight (1989) between offspring of female Q-02. Values range from  $-1$  to  $1$ . Values less than zero imply that a pair of individuals is less closely related to each other than would be expected by chance, given the frequency of alleles for each locus in the reference population (Queller and Goodnight 1989). Values in bold indicate pairs of offspring that I identified as full sibs using the likelihood ratio test described in the text and an  $\alpha$ -level of  $0.05$ .

Offspring ID	38745	38746	38747	38748	38749	38750	38751	38752	38753	38754	38755	38756	38757	38758
<b>38745</b>	*													
<b>38746</b>	0.488	*												
<b>38747</b>	0.488	<b>1.000</b>	*											
<b>38748</b>	0.269	0.649	0.649	*										
<b>38749</b>	0.616	0.506	0.506	0.234	*									
<b>38750</b>	0.147	0.209	0.209	0.166	0.143	*								
<b>38751</b>	<b>0.607</b>	0.459	0.459	0.221	0.459	0.082	*							
<b>38752</b>	0.346	0.297	0.297	0.166	0.598	0.191	0.032	*						
<b>38753</b>	<b>0.700</b>	0.443	0.443	0.000	0.666	0.573	<b>0.520</b>	0.433	*					
<b>38754</b>	0.382	0.203	0.203	-0.153	0.658	<b>0.415</b>	0.340	0.420	<b>0.738</b>	*				
<b>38755</b>	0.261	0.521	0.521	0.097	0.520	<b>0.493</b>	0.221	0.318	0.569	<b>0.780</b>	*			
<b>38756</b>	0.358	0.284	0.284	0.262	0.283	0.100	0.295	0.413	0.144	0.291	0.451	*		
<b>38757</b>	0.346	0.275	0.275	0.493	0.327	0.250	0.280	-0.210	0.168	0.350	0.521	0.468	*	
<b>38758</b>	0.215	0.539	0.539	<b>0.859</b>	0.193	0.023	0.160	0.530	-0.062	-0.226	-0.002	<b>0.458</b>	0.083	*

**Table 5:** Genetic diversity (expected heterozygosity -  $H_e$ , observed heterozygosity -  $H_o$  and number of alleles -  $N_a$ ) for each locus and averaged for each of six 'populations'.

Population	Scu01	Scu05	Scu07	Scu11	Scu16	Scu26	Scu106	Scu125	Scu137	Mean <sup>1</sup>
Bruce Peninsula	$N = 41$ $H_o = 0.805$ $H_e = 0.879$ $p = 0.0086$ $N_a = 14$	$N = 40$ $H_o = 0.300$ $H_e = 0.587$ $p < 0.0001$ $N_a = 5$	$N = 42$ $H_o = 0.714$ $H_e = 0.836$ $p < 0.0001$ $N_a = 10$	$N = 42$ $H_o = 0.619$ $H_e = 0.709$ $p = 0.0079$ $N_a = 15$	$N = 42$ $H_o = 0.548$ $H_e = 0.512$ $p = 0.7518$ $N_a = 4$	$N = 42$ $H_o = 0.667$ $H_e = 0.703$ $p = 0.4088$ $N_a = 6$	na	na	na	$H_o = 0.621$ $H_e = 0.743$ $p < 0.0001$ $N_a = 10$
Beausoleil	$N = 32$ $H_o = 0.686$ $H_e = 0.811$ $p = 0.0008$ $N_a = 15$	$N = 32$ $H_o = 0.719$ $H_e = 0.654$ $p = 0.6084$ $N_a = 4$	$N = 31$ $H_o = 0.613$ $H_e = 0.768$ $p = 0.0404$ $N_a = 9$	$N = 31$ $H_o = 0.032$ $H_e = 0.032$ $N_a = 2$	$N = 31$ $H_o = 0.258$ $H_e = 0.419$ $p = 0.0460$ $N_a = 2$	$N = 32$ $H_o = 0.719$ $H_e = 0.748$ $p = 0.3028$ $N_a = 7$	na	na	na	$H_o = 0.554$ $H_e = 0.602$ $p = 0.0012$ $N_a = 7.4$
Killbear	$N = 80$ $H_o = 0.663$ $H_e = 0.872$ $p < 0.0001$ $N_a = 16$	$N = 79$ $H_o = 0.595$ $H_e = 0.726$ $p < 0.0001$ $N_a = 12$	$N = 79$ $H_o = 0.671$ $H_e = 0.812$ $p < 0.0001$ $N_a = 9$	$N = 79$ $H_o = 0.633$ $H_e = 0.677$ $p < 0.0001$ $N_a = 9$	$N = 81$ $H_o = 0.469$ $H_e = 0.487$ $p = 0.0031$ $N_a = 5$	$N = 80$ $H_o = 0.188$ $H_e = 0.257$ $p < 0.0001$ $N_a = 5$	na	na	na	$H_o = 0.550$ $H_e = 0.669$ $p < 0.0001$ $N_a = 10.2$
LaSalle	$N = 18$ $H_o = 0.611$ $H_e = 0.697$ $p = 0.1006$ $N_a = 6$	$N = 17$ $H_o = 0.471$ $H_e = 0.429$ $p = 0.8458$ $N_a = 2$	$N = 18$ $H_o = 0.500$ $H_e = 0.716$ $p = 0.0239$ $N_a = 5$	$N = 18$ $H_o = 0$ $H_e = 0$ $N_a = 1$	na	$N = 18$ $H_o = 0.056$ $H_e = 0.398$ $p < 0.0001$ $N_a = 4$	$N = 18$ $H_o = 0.611$ $H_e = 0.692$ $p = 0.9079$ $N_a = 4$	$N = 18$ $H_o = 0.611$ $H_e = 0.512$ $p = 0.0141$ $N_a = 2$	$N = 18$ $H_o = 0.717$ $H_e = 0.778$ $p = 0.8282$ $N_a = 6$	$H_o = 0.328$ $H_e = 0.448$ $p = 0.0595$ $N_a = 3.6$
Springfield	$N = 21$ $H_o = 0.429$ $H_e = 0.819$ $p < 0.0001$ $N_a = 9$	$N = 21$ $H_o = 0.429$ $H_e = 0.621$ $p = 0.0273$ $N_a = 5$	$N = 20$ $H_o = 0.650$ $H_e = 0.813$ $p = 0.0048$ $N_a = 9$	$N = 20$ $H_o = 0.750$ $H_e = 0.857$ $p = 0.1145$ $N_a = 9$	$N = 21$ $H_o = 0.400$ $H_e = 0.656$ $p < 0.0001$ $N_a = 4$	$N = 20$ $H_o = 0.500$ $H_e = 0.828$ $p = 0.0142$ $N_a = 9$	na	na	na	$H_o = 0.552$ $H_e = 0.616$ $p < 0.0001$ $N_a = 8.2$
Cicero	$N = 25$ $H_o = 0.560$ $H_e = 0.795$ $p = 0.0109$ $N_a = 8$	$N = 25$ $H_o = 0.520$ $H_e = 0.521$ $p = 0.3547$ $N_a = 5$	$N = 25$ $H_o = 0.613$ $H_e = 0.768$ $p = 0.0024$ $N_a = 4$	$N = 25$ $H_o = 0.840$ $H_e = 0.683$ $p = 0.9576$ $N_a = 5$	$N = 25$ $H_o = 0.400$ $H_e = 0.656$ $p = 0.0149$ $N_a = 4$	$N = 25$ $H_o = 0$ $H_e = 0$ $N_a = 1$	na	na	na	$H_o = 0.507$ $H_e = 0.553$ $p = 0.0097$ $N_a = 4.6$

1 – Mean calculated across five loci assayed for all six population samples only: Scu01, Scu05, Scu07, Scu11, and Scu26.

**Appendix:** Sampling and identification details of individuals genotyped for the present study. Those samples (putatively unrelated, wild-caught) used in the Ojibway/LaSalle Complex population-level analyses are indicated in the last column.

SAMPLE ID	OTHER ID.	GEOGRAPHIC LOCALITY OR ORIGIN	Sex	Date of samples (mm/dd/yyyy)	Description	OTHER	Population Analysis?
Sca 34	OJIB 2	Ojibway?	unk	?	Blood and scat from wild snakes	Received DNA	✓
Sca 35	OJIB 3	Ojibway?	unk	?	Blood and scat from wild snakes	Received DNA	✓
Sca 36	OJIB 4	Ojibway?	unk	?	Blood and scat from wild snakes	Received DNA	✓
Sca 160	OJIB 4R61	Ojibway?	unk	?	Blood and scat from wild snakes	Received DNA	✓
Sca 161	OJIB 1-93	Ojibway?	unk	?	Blood and scat from wild snakes	Received DNA	✓
Sca 162	OJIB 2R4L	Ojibway?	unk	?	Blood and scat from wild snakes	Received DNA	✓
I-00	-	LaSalle Woods (SiteA)	M	5/15/2000	Wild	blood	✓
J-00	-	LaSalle Woods (SiteA)	M	11/8/00	Toronto ID 38934	blood	✓
K-00	-	LaSalle Woods (SiteA)	F	8/21/200	Wild	blood	✓
N-01	-	LaSalle Woods (SiteA)	M	7/9/01	?	blood	✓
O-02	-	LaSalle Woods (SiteB)	F	5/21/2002	?	blood	✓
R-02	-	LaSalle Woods (SiteA)	M	8/23/2002	?	blood	✓
MJ	55	Wainfleet	F	Aug-03	Mother: MJ-01 to MJ-07	blood	
MJ-01	56	Wainfleet	unk	8/30/2003	offspring of MJ	blood	
MJ-02	57	Wainfleet	unk	8/30/2003	offspring of MJ	blood	
MJ-03	58	Wainfleet	unk	8/30/2003	offspring of MJ	blood	
MJ-04	59	Wainfleet	unk	8/30/2003	offspring of MJ	blood	
MJ-05	60	Wainfleet	unk	8/30/2003	offspring of MJ	blood	
MJ-06	61	Wainfleet	unk	8/30/2003	offspring of MJ	NOT SENT	
MJ-07	62	Wainfleet	unk	8/30/2003	offspring of MJ	blood	
YAG1	Little Anne Yagi 63	Wainfleet	F	3/9/2003	Adult Female non-gravid	blood	
T-03	Tom	Washington St.,Lasalle	unk	6/13/2003	Wild	blood	✓
U-03	Ursala	LaSalle Woods (SiteA)	F	8/19/2003	Wild	blood	✓
U-03 (placenta)	Ursala	LaSalle Woods (SiteA)	F	-	placenta from Ursala	placenta	

U-A	U-03 (Ursala) - offspring	Toronto	unk	8/30/2003	from brood of 15 - 10 were sampled; released after blood sampling	neonate (U-03)/blood	
U-B	U-03 (Ursala) - offspring	Toronto	unk	8/30/2003	from brood of 15 total from which 10 were sampled; released after blood sampling	neonate (U-03)/blood	
U-C	U-03 (Ursala) - offspring	Toronto	unk	8/30/2003	from brood of 15 total from which 10 were sampled; released after blood sampling	neonate (U-03)/blood	
U-D	U-03 (Ursala) - offspring	Toronto	unk	8/30/2003	from brood of 15 total from which 10 were sampled; released after blood sampling	neonate (U-03)/blood	
U-E	U-03 (Ursala) - offspring	Toronto	unk	8/30/2003	from brood of 15 total from which 10 were sampled; released after blood sampling	neonate (U-03)/blood	
U-F	U-03 (Ursala) - offspring	Toronto	unk	8/30/2003	from brood of 15 total from which 10 were sampled; released after blood sampling	neonate (U-03)/blood	
U-G	U-03 (Ursala) - offspring	Toronto	unk	8/30/2003	from brood of 15 total from which 10 were sampled; released after blood sampling	neonate (U-03)/blood	
U-H	U-03 (Ursala) - offspring	Toronto	unk	8/30/2003	from brood of 15 total from which 10 were sampled; released after blood sampling	neonate (U-03)/blood	
U-I	U-03 (Ursala) - offspring	Toronto	unk	8/30/2003	from brood of 15 total from which 10 were sampled; released after blood sampling - see below	neonate (U-03)/blood	
38935	[#29; U-03 (Ursala) - offspring] - U-I	Toronto	unk	8/30/2003	Sample U-I & one of 38935 or 38936 are duplicates. The other individual is sample U-K	blood/captive	
38936	#30; [U-03 (Ursala) - offspring] - U-K	Toronto	unk	8/30/2003	Sample U-I & one of 38935 or 38936 are duplicates. The other individual is sample U-K	blood/captive	
33906	Morgan	MIS.AN.CO	M	4/7/98	Found in Mississauga--unknown origin	blood/wild	
Q-02		LaSalle Woods (SiteA)	F	8/16/2002	Toronto ID 38522	blood	✓
38745	#1	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38746	#2	Toronto	unk	07/19/2003	Offspring of 38522	blood/captive	
38747	#3	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38748	#4	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38749	#5	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38750	#6	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38751	#7	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38752	#8	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38753	#9	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38754	#10	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38755	#11	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38756	#12	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	

38757	#13	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
38758	#14	Toronto	unk	7/19/2003	Offspring of 38522	blood/captive	
P-02	"Polly"?	LaSalle Woods (SiteB)	F	5/31/2002	Toronto ID 38596	blood	
P-02	P-02/"Polly"?	LaSalle Woods (SiteB)	unk	6/10/03	Toronto ID 38596; Genotyped Twice	blood	✓
38773	#15	Toronto	unk	7/30/2003	Offspring of 38596	blood/captive	
38775	#16	Toronto	unk	7/30/2003	Offspring of 38596	blood/captive	
L-00	"Lola"?	LaSalle Woods (SiteA)	F	7/25/2001	Toronto ID 38597	blood	✓
38776	#17	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38777	#18	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38778	#19	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38779	#20	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38780	#21	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38781	#22	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38782	#23	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38783	#24	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38784	#25	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38785	#26	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38786	#27	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
38787	#28	Toronto	unk	2/8/03	Offspring of 38597	blood/captive	
S-02	Toronto Zoo ID 38834Stuart	LaSalle Woods (SiteA)	M	4/8/03	Wild; Toronto ID 38834 - genotyped twice	blood/wild	✓
S-02	Toronto Zoo ID 38834Stuart	LaSalle Woods (SiteA)	M	8/23/02	Toronto ID 38834	blood	