Molecular Ecology (2006) **15**, 3755–3767 doi: 10.1111/j.1365-294X.2006.03056.x

Hybridization between mtDNA-defined phylogeographic lineages of black ratsnakes (*Pantherophis* sp.)

H. LISLE GIBBS,* SARAH J. COREY,* GABRIEL BLOUIN-DEMERS,† KENT A. PRIOR‡ and PATRICK J. WEATHERHEAD§

**Department of Evolution, Ecology and Organismal Biology, Ohio State University, Columbus, Ohio 43210-1293, USA, †Department of Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5, ‡Parks Canada, Gatineau, Quebec, Canada K1A 0H3, §Program in Ecology and Evolutionary Biology, University of Illinois, Champaign, Illinois 61820, USA*

Abstract

Phylogeographic analyses using mitochondrial DNA (mtDNA) have revealed many examples of apparently deep historical subdivisions ('phylogroups') within many vertebrates. It remains unclear whether these phylogroups represent independently evolving, adaptively differentiated lineages or groups that show little functional differentiation and, hence, will merge on contact. Here, we use mtDNA sequence data to evaluate the phylogeographic relationships between two of the northernmost populations of black ratsnakes (*Pantherophis obsoletus* complex) in Ontario, Canada and previously analysed populations in the United States. We then use population-level analyses to evaluate the level of adaptive divergence between previously established mtDNA phylogroups. Phylogenetic analyses show that southern Ontario snakes have mtDNA haplotypes that fall within the Central mtDNA phylogroup, as designated by Burbrink *et al*. (2000). In contrast, snakes in eastern Ontario carry either Central or Eastern-specific haplotypes. Within the hybrid region, we found highly variable frequencies of mtDNA haplotypes among isolated sub-populations, no association between variation in cytonuclear (mtDNA) and nuclear (microsatellite DNA) markers, no difference in survival or reproductive success among snakes with different mtDNA haplotypes, and no effect of mate similarity in mtDNA on female clutch size. These results argue that the Eastern and Central phylogroups have merged in this region, likely due to a lack of adaptive differentiation between individuals in each lineage. Hence, in these snakes, phylogeographic structure in mtDNA is more a reflection of historical isolation rather than adaptive divergence. The observed reticulation between lineages and lack of evidence for hybrid disgenesis also bears on the classification of these lineages as distinct species.

Keywords: black ratsnake, hybridrization, mtDNA phylogroups, *Pantherophis obsoletus* complex, phylogeography

Received 17 April 2006; revision accepted 9 June 2006

Introduction

A major result from phylogeographic analyses based on mitochondrial DNA (mtDNA) is the repeated identification of deep historical subdivisions (hereafter mtDNA phylogroups made up of distinct mtDNA haplotypes) within many vertebrate taxa (Avise 2000). The evolutionary significance of these mtDNA lineages, however, remains largely unresolved. In particular, it is unclear how frequently these mtDNA phylogroups represent independently evolving lineages that have developed substantial amounts of adaptive differentiation vs. those that show little differentiation in functional variation (Avise *et al*. 1998). Addressing this question is important because it bears on the usefulness of mtDNA phylogeographic structure as a criterion for species recognition (Goldstein & DeSalle 2000; Templeton 2001; Wiens & Penkrot 2002; Sites & Marshall 2003) and for designating conservation units (Avise 2000; Crandall *et al*. 2000; Moritz 2002).

Correspondence: H. Lisle Gibbs, Fax: 614 292 2030; E-mail: gibbs.128@osu.edu

One widely used method for addressing whether mtDNA can be used to define adaptively distinct lineages is to investigate patterns of variation present in other genetic markers in zones of secondary contact between mtDNA phylogroups (cf. Barton & Hewitt 1985, 1989). This can include: (i) estimating associations between mtDNA variation and variation in other traits (e.g. morphology) which are more likely to reflect adaptive variation (cf. Crandall *et al*. 2000), and (ii) documenting the shape of the clines shown by multiple loci across a contact zone which allows inferences about the nature of selection acting against hybrids between phylogroups and, hence, the level of gene flow between clades (Barton & Hewitt 1985; Hewitt 1988; Harrison 1993). Although valuable, each of these indirect approaches has weaknesses. These include assumptions that variation in non-mtDNA characters (e.g. morphology) is both heritable and under selection and, for hybrid zone analyses, the need to infer process (e.g. levels of gene flow and type and degree of selection) from spatial patterns of genetic variation alone.

A more direct approach is to use the results of population studies to estimate directly the relative fitness of individuals with different mtDNA haplotypes and to assess the reproductive success of 'hybrid' pairs made up of males and females belonging to different phylogroups. This approach has been commonly used to study the degree of reproductive isolation between previously designated species (cf. Howard *et al*. 1998), but has rarely been used to assess the isolation of individuals from different mtDNA lineages. Significant differences in survival and reproduction between individuals with different haplotypes would suggest associated differences in adaptive variation, at least in the common environment where the hybrid population is present. In contrast, no fitness difference would indicate little or no adaptive differentiation. Likewise, reduced success of hybrid mtDNA pairs would also suggest adaptive differences, whereas no difference in reproductive success would suggest little functional differentiation.

Here, we use a combination of phylogeographic and hybrid zone analyses to assess the degree of adaptive differentiation between recently described mtDNA lineages within the black ratsnake complex (*Pantherophis* sp. — Utiger *et al*. 2002). Recently, Burbrink *et al*. (2000) used mtDNA sequence information to examine relationships among morphologically defined subspecies of the black ratsnake (*Pantherophis obsolete sensu lato*). They identified three well-supported mtDNA lineages that did not conform to any of the currently accepted subspecies, but instead had distinct longitudinal (Eastern, Central, and Western) distributions in eastern North America (Fig. 1). These results, combined with the presence of subtle and overlapping differences in external morphology led Burbrink (2001) to suggest that these lineages represent distinct species.

Burbrink *et al*. (2000) examined samples from US populations only despite the fact that the range of the species

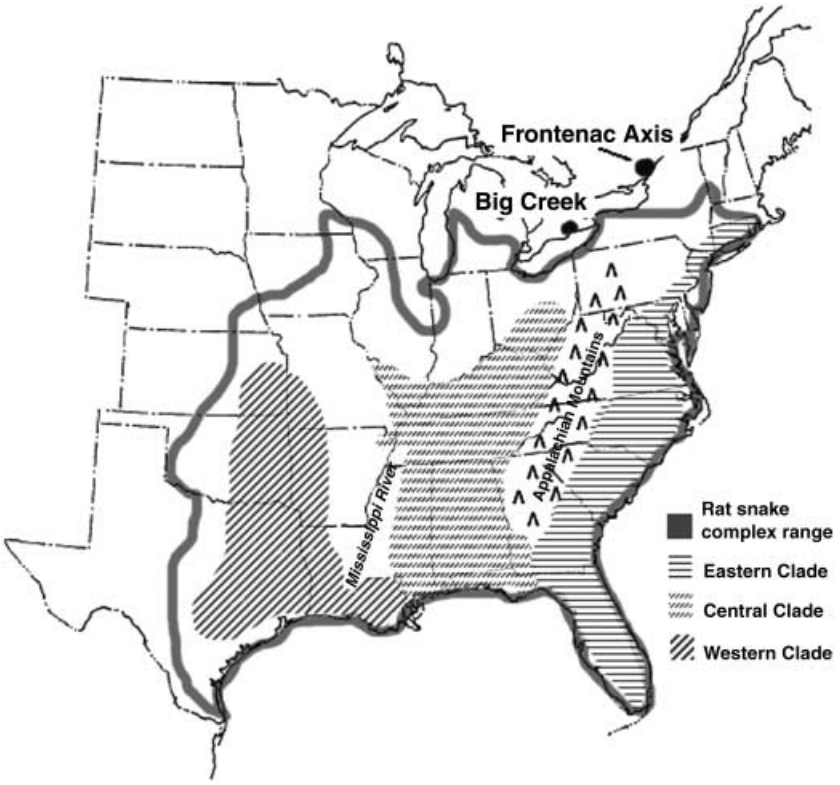

Fig. 1 Distribution of Eastern, Central, and Western ratsnake haplotypes identified by Burbrink *et al*. (2000) relative to the overall range of *Pantherophis obsoleta* in North America and the location of the two Canadian populations analysed in this study (modified from Burbrink *et al*. (2000)).

extends into Canada. Elsewhere, Burbrink (2001) proposed that Canadian ratsnake populations (Fig. 1) are all part of the Central clade. Because these populations had not been sampled, their actual phylogeographic affinities are unknown. Analysis of these populations is of interest for three reasons. First, establishing the phylogenetic relationships between these populations could shed light on post-Pleistocene colonization patterns in eastern Northern America by populations from distinct refugia through inferences about how populations that are geographically distinct but genealogically related could be established (cf. Austin *et al*. 2004). Second, because these populations (particularly those within the Frontenac Axis) have been the subjects of detailed study (Prior *et al*. 1997; Lougheed *et al*. 1999; Blouin-Demers *et al*. 2002, 2005), the opportunity also exists to assess fitness differences between mtDNA phylogroups in any hybrid populations that are identified. Lougheed *et al*. (1999) have also shown differentiation in microsatellite loci between populations that, based on their geographic locations, may belong to separate mitochondrial lineages. This differentiation raises the possibility that there may be significant genome-wide differentiation among lineages with possible fitness consequences. Finally, ratsnakes are classified as threatened by the Committee on the Status of Endangered Wildlife in Canada (Prior & Weatherhead 1998) and one of the extant populations (Big Creek) is of particular conservation concern because of low population numbers and continuing habitat degradation (Prior & Weatherhead 1998). Therefore, phylogeographic analyses will aid in determining whether these two populations should be considered distinct conservation units (cf. Crandall *et al*. 2000).

In this study, we use mtDNA sequence variation to assess phylogenetic relationships of individuals from the two largest remnant Canadian ratsnake populations [Big Creek (BC) and Frontenac Axis (FA)] in relation to populations in the United States. We complement the genetic analyses with morphological analyses of snakes from both populations. We then document patterns of mtDNA variation among subpopulations of snakes in a newly described hybrid zone located in the FA region and use previously obtained data on survival and reproduction (Blouin-Demers *et al*. 2005) to explore whether there are differences in the survival and reproduction of snakes belonging to different mtDNA phylogroups. Finally, we determine the extent of cytonuclear disequilbirum (cf. Lamb & Avise 1986) between mtDNA and nuclear DNA variation in this zone.

Materials and methods

Samples

We used DNA extracted from blood samples collected from adult snakes that had been analysed in previous studies (see Prior *et al*. (1997), Lougheed *et al*. (1999) and Blouin-Demers *et al*. (2005) for details about sampling procedures and locations). For the initial mtDNA analysis we sequenced almost all of the cytochrome *b* gene (see below) from 15 individuals from the BC population and from 16 individuals from a range of subpopulations within the FA region including Chaffeys' Lock ($n = 4$), LaRue Mills ($n = 3$), Charleston Lake Provincial Park ($n = 3$), Hill Island ($n = 4$) and Murphy's Point Provincial Park ($n = 2$) (see Lougheed *et al*. 1999 and Prior *et al*. 1997) for specific subpopulation locations). To obtain additional information on the mtDNA phylogroup identity of individuals in the FA region, we also assayed the haplotype of an additional 264 individuals previously studied by Lougheed *et al*. (1999) and 81 individuals studied by Blouin-Demers *et al*. (2005) using newly developed phylogroup-specific primers (see below).

mtDNA variation

We used the primers L14910 and H16064 (Burbrink *et al*. 2000) to amplify most of the mtDNA cytochrome *b* gene (1086 bp) from samples of genomic DNA. We also conducted preliminary analyses of variation in control region I using primers described in Burbrink *et al*. (2000), but decided to focus on cytochrome *b* because of the higher level of phylogenetically informative variation present in this gene (Burbrink *et al*. 2000). The cytochrome *b* gene was amplified from template DNA in 30 μL reactions consisting of 100 ng genomic DNA, 1X PCR buffer (Invitrogen), 2 mM $MgCl_2$, 200 μM of each dNTP, 0.4 μM of each primer, and 2.5 U *Taq* (Invitrogen). Reactions were run in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) with a 7-min denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 55° for 30 s, then extension at 72 °C for 3 min, and a final elongation temperature of 72 °C for 7 min. PCR products were checked on an agarose gel and then precipitated using a Polyethylene Glycol/ETOH protocol, dried using a Speed-Vac and resuspended in 50 μL of ddH_2O. Cycle sequencing of the purified PCR products was carried out using external and internal primers from Burbrink *et al*. (2000) and the Big Dye (version 3.0) Cycle Sequencing Kit (Applied Biosystems). Cycle-sequenced products were cleaned using Sephadex columns and nucleotide sequences determined using an ABI 3100 Genetic Analyser.

As described below, our analysis of cytochrome *b* sequences indicated that only two of the phylogroups described by Burbrink *et al*. (2000) were present in our samples: those from the Central and Eastern clades. To develop a more rapid and economical way of determining the phylogroup identity of a given individual, we compared alignments of all available distinct cytochrome *b* haplotypes from these clades (from Burbrink *et al*. 2000 and our results

[see below]) with the goal of identifying sites containing nucleotide substitutions that were fixed between Central and Eastern phylogroups. We identified two such sites at positions 347 and 350 of the cytochrome *b* gene: all Central haplotypes were fixed for a C and T, respectively, at these positions, whereas all Eastern haplotypes were fixed for T and C, respectively, at the same sites. Following the approach described by Sommer *et al*. (1992), we designed two phylogroup-specific primers whose 3′-ends were centred on these variable sites [Central-specific primer (Eob 336 CF): 5′-CAACAGCCTTCTTTGGCTAT-3′; Eastern-specific primer (Eob 366 EF): 5′-CAACAGCCTTCTTTGGTTAC-3′]. Each of these forward primers was combined with a newly designed reverse primer (Eob 599 R: 5′-GGGATTT-TATCAATATCTGA-3′) to conduct a phylogroup-specific PCR assay. Briefly, each individual was amplified in separate reactions with each set of primers (Eob 336 CF and Eob 559, and Eob 336 EF and Eob 559). Presence of a product for a given phylogroup-specific primer set resulted in the classification of that sample as belonging to that phylogroup, while the absence of a band for the other primer set served as a negative control. Reaction conditions were optimized using samples whose phylogroup identity had already been determined by direct sequencing (see above). The 10 μL reaction mixture consisted of 0.85 μL BSA (10 mg/mL), 5.35 μL H_2O, 0.85 μL 10× Buffer, 0.45 μL dNTP's (10 μM), 0.45 μL of each primer (10 μM), 0.7 μL $MgCl_2$ (50 μM), 0.05 μL *Taq* (5 U/μL), and 0.7 μL DNA (50–100 ng/μL). Cycling parameters were 94 °C for 7 min, then 35 cycles of 94 °C for 40 s., 61 °C for 20 s., and 72 °C for 1 min, with a final extension of 72 °C for 5 min. Amplifications were run on 1.5% agarose gels to visualize presence or absence of a band.

For unknown reasons, the amplification assay described above failed or was ambiguous for 19 samples. For these samples we amplified the almost complete cytochrome *b* gene using the procedures described above and digested the fragment with the restriction enzyme BssSI (New England BioLabs). This enzyme has a restriction site (C′ACGA_G) beginning at position 185 that is found in all Central haplotypes but which is not present in any Eastern haplotypes.

Phylogenetic analyses

To determine the phylogenetic affinities of our cytochrome *b* sequences with those of Burbrink *et al*. 2000), we conducted a Bayesian inference of their phylogenetic relationships using MRBAYES version 3.1 (Huelsenbeck & Ronquist 2001). We first used the program DT-MODSEL (Minin *et al*. 2003) to identify objectively an appropriate nucleotide substitution model using a decision theory framework. This method uses a Bayesian information criterion that incorporates relative branch error as a performance measure in a decision theory framework to choose simultaneously among a set of possible substitution models. Its advantage over other methods of model selection based on likelihood ratio tests (e.g. MODELTEST — Posada & Crandall 2001) is that DT-MODSEL selects models that are simpler yet provides estimates of branch lengths that are more accurate than those chosen by other methods (Minin *et al*. 2003). Using the selected model, we then conducted Bayesian analyses using random starting trees run for 5×10^6 generations and sampled every 100 generations. Base frequencies and other substitution model parameters were estimated directly from the data. Burn-in frequency was set to the first 25% of the sampled trees. Direct examination of the sampled log-likelihood values showed that values had reached a stationary equilibrium by this point. All trees preceding this cut-off were discarded when calculating posterior nodal probabilities, mean log-likelihood scores, and a summary phylogeny including estimates of branch lengths.

Morphological analyses

Burbrink (2001) described external morphological characters that showed subtle differences between ratsnake mtDNA phylogroups found in the United States. To examine the relationship between morphological and mtDNA variation in Canadian ratsnakes, we recorded the 24 distinguishing characters between *Pantherophis spiloides* (= Central mtDNA clade) and *Pantherophis alleghaniensis* (= Eastern mtDNA clade) listed by Burbrink (2001) on nine females and 10 males from the FA population and on three females and 10 males from the BC population that had been located in the collections of the Royal Ontario Museum and the Canadian Museum of Nature (Appendix 1). Our aim was to determine whether the morphology of our two populations mirrored the findings of the genetic analyses which indicates that one population consists of only snakes with *spiloides* mtDNA while the other contains snakes with both *spilodes* and *alleghaniensis* specific mtDNA (see below). Although these sample sizes are modest, they represent all the specimens of Canadian ratsnakes that we were able to locate in museum collections. We measured ratios of the following characters relative to head length (HL): rostral height (RH/HL), rostral width (RW/HL), parietal length (PL/HL), anterior parietal width (PW/HL), anterior frontal width (FWA/HL), medial frontal width (FW/HL), posterior frontal width (FWP/HL), frontal length (FL/HL), prefrontal length (PRFL/HL), anterior prefrontal width (PRFWA/HL), posterior prefrontal width (PRFWP/HL), anterior internasal width (INWA/HL), posterior internasal width (INWP/HL), internasal rostral contact (INR/HL), supralabial length (LL/HL), eye diameter (EYE/HL), anterior genial length (AG/HL), posterior genial length (PG/HL), anterior nasal length (ANL/HL), posterior nasal length (PNL/HL), preocular width (PROW/HL), and loreal height (LHT/HL).

We also calculated the ratio of tail length to snout-vent length (T/SV) and counted the number of subcaudal scales (SC). We could not measure any characters related to dorsal blotches because all of our specimens were uniformly black. All characters are defined in detail in Burbrink (2001).

Although the mean values of the distinguishing characters differed significantly between the two proposed species, Burbrink (2001) documented overlap among them. Therefore, for each measurement, we recorded whether the value was characteristic of Central or Eastern clade snakes based on the mean values reported for males and females of each clade by Burbrink (2001). When a value could not be deemed characteristic of a species unambiguously (when the value fell between the reported means of the two clades for that sex), the value was left unassigned. We then tallied the values unambiguously characteristic of each clade for each individual and assigned the individual to the clade for which it had the most characteristic values.

Population level analyses

Our initial analyses showed that populations of ratsnakes in the Frontenac Axis consisted of individuals with both Central and Eastern haplotypes. To determine if any cytonuclear disequilbirum (cf. Lamb & Avise 1986) was present between mtDNA and nuclear DNA variation, we examined patterns of variation in mtDNA phylogroup (determined here) and six microsatellite loci (determined by Lougheed *et al*. (1999)) for 232 individuals from three of the subpopulations (Chaffey's Lock, Murphy's Point Provincial Park and Hill Island) studied by Lougheed *et al*. (1999). Two populations (LaRue Mills and Charleston Lake) analysed by Lougheed *et al*. (1999) were excluded because they had < 10 individuals with a particular haplotype (see Fig. 4). For each subpopulation sample, we divided snakes into two classes based on haplotype and then assessed the degree of differentiation in microsatellites between classes by: (i) calculating F_{ST} using FSTAT 2.3 (Goudet 1995) and testing whether the value was significantly different from zero, and (ii) using an exact test within GENEPOP (Raymond & Rousset 1995) to test for differences in allele frequencies between phylogroups. These analyses were restricted to single subpopulations to avoid confounding the effects of coincident differentiation in both mtDNA and microsatellites (Lougheed *et al*. 1999) between subpopulations. We considered other methods of assessing cytonuclear disequilibrium (e.g. Basten & Asmussen 1997) but the large number of alleles present at individual microsatellite loci meant that there are too many genotypic classes for meaningful statistical analyses to be conducted.

Next, we assessed the possibility that mtDNA phylogroup was a marker for lineages with different adaptations that would be reflected in fitness differences among snakes belonging to each lineage in a common environment. To be clear, we are not testing whether there is a direct effect of mtDNA haplotype on fitness per se, but rather whether neutral variation in mtDNA is correlated with other adaptive changes that might have occurred during the course of independent evolution of the Central and Eastern lineages. To assess the relationship between mtDNA haplotype and survival, we used snake length (measured as SVL — see Blouin-Demers 2003) as a surrogate measure of age in these indeterminately growing animals (see Blouin-Demers *et al*. 2002 for quantitative estimates of the relationship between SVL and age for this population) and asked if there was a significant difference in the size of snakes belonging to different phylogroups. Any difference would suggest fitness differences in relation to survival between phylogroups. For this analysis we used 177 snakes (73 females and 104 males) from the five FA subpopulations described by Lougheed *et al*. (1999) that had been genotyped for mtDNA and had been measured at least once over a 3-year period (1993–1995). We acknowledge that there may have been small effects of growth on length that could affect a comparison of snakes measured in different years, but consider these to be unimportant given the expected lifespan (up to 30 years) and slow growth of these long-lived animals (Blouin-Demers *et al*. 2002).

Finally, we examined the possible effects of haplotype on individual reproductive success using the results of a parentage study in the Chaffey's Lock subpopulation by Blouin-Demers *et al*. (2005). They used 10 microsatellite DNA loci to identify 34 fathers for 114 of 375 (30%) hatchlings from 34 clutches with known mothers. Multiple paternity was common, occurring in 88% of clutches. We determined the mtDNA haplotype of breeding females and assigned males and then asked the following questions: (i) Was there any evidence of a disproportionate representation of individuals with a particular haplotype among snakes who bred or were the proportions statistically indistinguishable from background haplotype frequencies? (ii) Among individuals who bred, was there any difference in reproductive success [measured as clutch size (females) or number of assigned young (males)] among snakes with different haplotypes after controlling for individual size (which correlates with success in both sexes (Blouin-Demers *et al*. (2005))? Lastly, to determine if there was any effect of haplotype similarity among mates on success (cf. evidence for hybrid disgenesis), we estimated the proportion of identified male sires that had the same mtDNA haplotype as the mother of a given litter. We then used a MANOVA to determine if there was a positive relationship between this value and female clutch size after controlling for female size. Because not all sires were identified for all hatchlings (see Blouin-Demers *et al*. 2005), this analysis assumed that the haplotypes of identified males were representative of all fathers for a particular clutch.

Table 1 Distribution of mtDNA cytochrome *b* haplotypes among Frontenac Axis and Big Creek ratsnake populations. C refers to a haplotype that falls within the Central clade as defined by Burbrink *et al*. (2000) whereas E refers to the Eastern clade (see Fig. 2). Haplotype C4 has identical sequence to a haplotype found by Burbrink *et al*. (2000) in Ohio (Genebank Accession Number: AF283643) whereas sequences for C1–C3, C5, C6 and E1-E3 have been deposited under numbers DQ538337-538344. Subpopulation abbreviations are: CH (Charleston Lake), LR (LaRue Mills), MP (Murphy's Point), CL (Chaffey's Lock), HI (Hill Island) (see Fig. 4)

Population	Haplotype	No. of individuals	Subpopulations
Frontenac Axis	C1	1	HI
	C2	1	CL
	C4	8	CH, LR, HI, CL
	E1	4	HI
	E2	1	CL
	E3	1	CL
Big Creek	C3	5	
	C4	8	
	C5	1	
	C6	1	

Results

Phylogenetic relationships of mtDNA sequences

We detected nine distinct haplotypes among the cytochrome *b* sequences generated from 31 snakes from the BC and FA populations combined (Table 1). The most common haplotype (C4) was found in both populations and was identical to a sequence previously reported by Burbrink *et al*. (2000) for a snake from Ohio (GenBank Accession no. AF283643) that belonged to the Central clade. All other haplotypes were distinct from those previously reported by Burbrink *et al*. (2000) and were restricted to one or the other population. Interestingly, while haplotypes from the BC population were similar to each other (range of raw percent divergence between distinct haplotypes (p): 0.082–0.184%), haplotypes in the FA population fell into two distinct groups consisting of those that were either similar ($p \leq 0.50\%$ divergent) or divergent ($p \geq 2.94\%$) from each other.

Phylogenetic analysis of these sequences using Bayesian analysis demonstrates that this pattern arises because BC haplotypes are phylogenetically similar to each other and cluster with the other Central clade sequences reported by Burbrink *et al*. (2000) with a high degree of clade support, whereas the FA haplotypes consist of two distinct groups: those clustering with other Central haplotypes and those that group with other Eastern clade haplotypes (Fig. 2). Our interpretation is that the ancestors of snakes in the BC population originated from a Central clade population in the United States whereas the FA population represents a contact zone formed from ancestors from both Central and Eastern populations that has subsequently become isolated from both ancestral source populations.

Morphological results

Analyses of morphology provide qualitative support for the phylogenetic patterns revealed by mtDNA. We had a single FA individual for which the values characteristic of each species were tied. Excluding this snake, 11 individuals (61%) from FA had a majority of characters typical of Central clade individuals whereas seven individuals (39%) were classified as being from the Eastern clade (Fig. 3). Among the BC snakes, 12 individuals (92%) were classified as from the Central clade and a single individual (8%) was classified as from the Eastern clade (Fig. 3). However, χ^2 analyses reveal that only a small number of individuals show significant ($P < 0.05$) deviations from equality in terms of relative numbers of characters that have values that are diagnostic of Central vs. Eastern clade snakes (Fig. 3): among snakes from the Frontenac Axis only 3/19 (15.7%) showed significantly more characters representative of a particular taxa (2 Central and 1 Eastern) while 4 of 13 (30.7%) individuals from Big Creek showed significantly more characters with Central-specific values. Thus, while this small number of individuals supports the pattern suggested by mtDNA of a Central-type population at Big Creek and a hybrid population in the Frontenac Axis, most individuals are in fact morphologically ambiguous. This ambiguity appears to result from the low resolution afforded by morphological characters as a way of distinguishing between lineages.

Distribution of Central and Eastern mtDNA haplotypes among Frontenac Axis subpopulations

Genotypic data from a larger number of snakes confirms the presence of a hybrid zone in the Frontenac Axis between Central and Eastern mtDNA haplotypes: among the 264 snakes that were genotyped from five subpopulations, there were similar numbers of individuals with Central ($N = 146$ (55.3% total) vs. Eastern mtDNA haplotypes ($N = 118$ (44.7%)) ($\chi^2 = 2.97$; d.f. = 1; $P = 0.085$). However, among subpopulations, there is significant ($\chi^2 = 49.7$, d.f. = 4; $P < 0.001$) variation in the relative frequency of different haplotypes with the relative frequency of individuals with a Central haplotype ranging from close to one (97%: LaRue Mills) to a low of 27% (Murphy's Point) (Fig. 4).

Lack of association between mtDNA and nuclear DNA variation

We found no evidence for any cytonuclear association in any of the three subpopulations (Chaffey's Lock, Murphy's Point Provincial Park, Hill Island) where sample sizes were

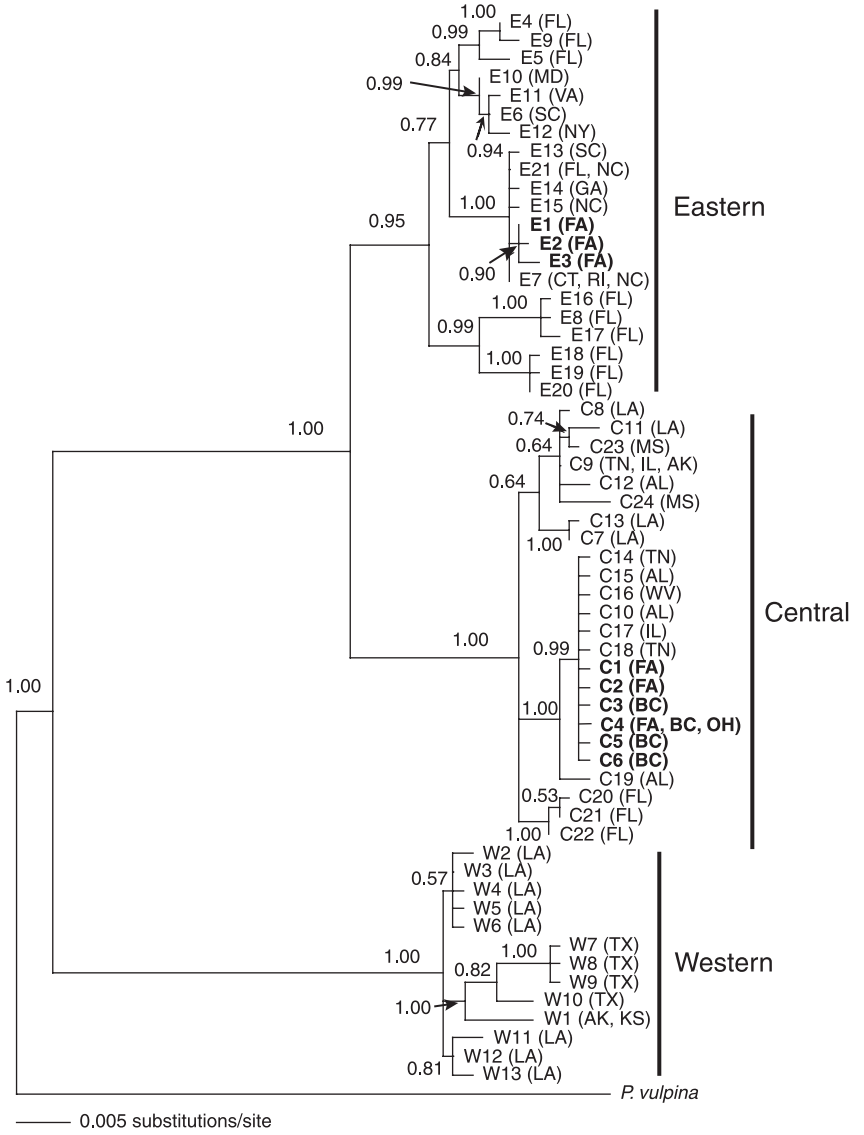

Fig. 2 A consensus tree from the Bayesian analyses of combined mtDNA cytochrome *b* sequences from Burbrink *et al*. (2000) (identified by clade membership and location [state] — also see Appendix II) and sequences from the two Ontario ratsnake populations (identified in bold by clade membership and location [FA = Frontenac Axis and BC = Big Creek]) shown in Fig. 1. Based on the results from DT-MODSEL the nucleotide substitution model used was the HKY+I+G model. Probabilities of support (> 50%) for particular nodes are shown at the base of the node in question. East, Central and West labels define lineages previously identified by Burbrink *et al*. (2000). Overall mean lnL for this tree was –3420.34. Tree is rooted using the *P. vulpina* sequence reported by Burbrink *et al*. (2000).

sufficiently large ($n > 10$ per haplotype category) to conduct analyses: F_{ST} values quantifying levels of microsatellite differentiation between Central and Eastern snakes were all very small (0–0.008) and not significantly different from zero (all $P > 0.05$) and exact tests comparing overall allele frequency distributions were also not significant ($P \geq 0.52$). This suggests high levels of introgression between the nuclear genomes of snakes from each mtDNA lineage since coming into contact in the FA region.

Lack of fitness differences between snakes with Central and Eastern mtDNA haplotypes

The lack of association between nuclear and mtDNA variation and the large variation in the relative frequencies of Central and Eastern haploytpes among genetically isolated subpopulations (Fig. 4) suggests that mtDNA variation may be neutral and largely influenced by nonselective evolutionary processes such as drift. Consistent with this prediction, there were no significant association between phylogroup identity and measures of individual survival and reproduction. Specifically, based on a multiple ordinal regression analysis, there was no association between mtDNA haplotype and either snake size (SVL) (Wald $\chi^2 = 2.94$, d.f. = 1, $P = 0.087$) or sex (Wald $\chi^2 = 1.57$, d.f. = 1, $P = 0.22$) (power = 0.130) although the trend was for snakes with Eastern haplotypes to be slightly larger, hence older (mean SVL for E type snakes: 1108.1 mm ± 27.1 SE [$n = 86$]; C type: 1044.9 ± 28.9 [$n = 65$]).

For individuals studied by Blouin-Demers *et al*. (2005), there was no significant association between phylogroup identity and whether individuals bred or, if they did, how successful they were. With respect to breeding opportunity, there was no difference in haplotype ratios among individuals

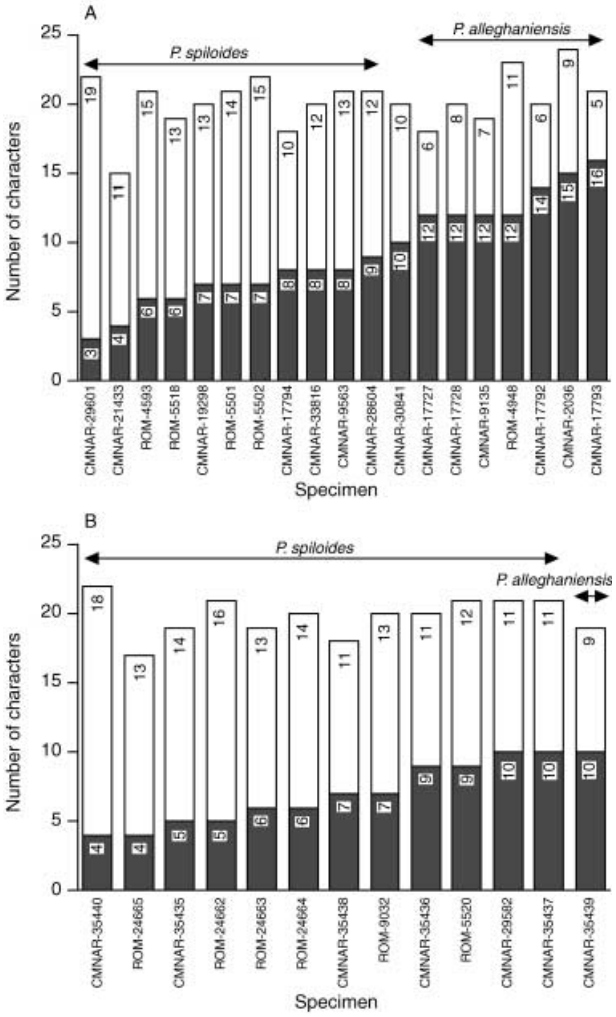

Fig. 3 Number of characters out of 24 unambiguously characteristic of Central clade (= *P. spiloides*) and Eastern clade (= *P. alleghaniensis* for all specimens from the Frontenac Axis (a) and from Big Creek (b). Arrows show the assignment of individuals to species based on the identity of a majority of characters.

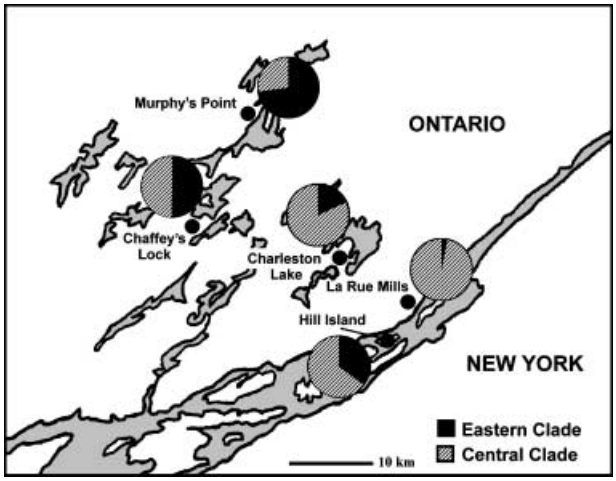

Fig. 4 Relative frequencies of Central and East mtDNA haplotypes in isolated subpopulations of ratsnakes in the Frontenac Axis region. The numbers of snakes genotyped in each subpopulation is as follows: Murphy's Point, $n = 55$; Chaffey's Lock, $n = 122$; Charleston Lake, $n = 22$; LaRue Mills, $n = 32$; Hill Island, $n = 33$.

that bred (males who sired ≥ 1 offspring: 19 C: 29 E; females bearing > 1 offspring: 11 C: 22 E) and background ratios for this subpopulation (60 C: 62 E) estimated from samples analysed by Lougheed *et al*. (1999) (males: $\chi^2 = 1.28$, d.f. = 1, $P = 0.26$; females: $\chi^2 = 2.63$, d.f. = 1, $P = 0.11$). This indicates that individuals with a particular mtDNA type did not have a greater chance of obtaining a mate, which contributes to variance in individual reproductive success in this population, at least among males (Blouin-Demers *et al*. 2005). Among snakes that bred, there was no association between haplotype and reproductive success for either males or females after controlling for size (ANCOVA with individual SVL as a covariate: males: $F = 0.49$, d.f. = 1, $P = 0.49$; females, $F = 0.28$, d.f. = 1, $P = 0.59$). Finally, there was no effect of the similarity in mtDNA between a female and her mates on her litter size holding female size constant (ANCOVA with female SVL as a covariate: $F = 0.69$, d.f. = 1, $P = 0.35$). We conclude that snakes with different haplotypes have similar reproductive success in a common environment, and that there is no evidence that mating between males and females with different haplotypes lead to reduced fecundity.

Discussion

Phylogeography

We found that a hybrid population of ratsnakes contained individuals with distinct mtDNA haplotypes, thereby adding to a growing list of recent studies documenting contact zones between maternal lineages of reptiles and amphibians in eastern North America (Church *et al*. 2003; Clark *et al*. 2003; Zamudio & Savage 2003; Austin *et al*. 2004). The generally accepted explanation for this pattern is that allopatric lineages that had differentiated due to isolation by large–scale biogeographic barriers, such as the Mississippi River and the Appalachian Mountains, came into contact following recent and rapid postglacial expansion northward. This is the scenario proposed by Burbrink *et al*. (2000) for the spread of the mtDNA lineages of ratsnakes through central and eastern North America.

The mtDNA affinities of the two Canadian populations provide clues to the geographic routes that different ratsnake lineages took to colonize the northern-most portion of their range. Based on the distribution of haplotypes shown in Fig. 5 of Burbrink *et al*. (2000), the presence of

only Central haplotypes in the BC population implies that it was founded by snakes that expanded around either the west or east end of Lake Erie from source populations in Ohio, or possibly Pennsylvania. This population continued expanding eastward along the northern shores of Lake Erie and Lake Ontario, eventually contacting snakes from the Eastern phylogroup that had expanded into Canada around the eastern end of Lake Ontario. Based on Fig. 5 of Burbrink *et al*. (2000), the closest extant populations of snakes of the same lineage as those in the Frontenac Axis are in New England or southern New York. Recently, however, von Hasseln (2005) has documented the presence of Eastern haplotypes in ratsnake populations in northern Vermont and northeastern New York. This implies a dispersal event to the north of the Appalachians in the eastern United States into Canada.

The phylogenetic affinities of the two currently isolated Canadian populations also imply that ratsnakes once had a much broader distribution than is currently observed. In particular, there are substantial gaps of hundreds of kilometres between the Frontenac Axis population and putative source populations in southern Ontario and southern New York. Loss of ratsnakes from these regions could be a result of anthropogenic effects on snake populations, although ratsnakes appear to survive well in regions of the United States with large human populations and modified landscapes (Conant & Collins 1991). Also, historical herpetological collections in this area suggest that these gaps preceded European settlement (Prior 1997). However, it is possible that at the northern limit of their range, slower growth and greater ages at maturity (Blouin-Demers *et al*. 2002) make ratsnake populations less resilient to anthropogenic disturbances that increase mortality.

Evolutionary significance of mtDNA phylogroups

Our genetic and population-based results provide strong evidence that mtDNA phylogroups in this species do not identify evolutionary lineages that have undergone adaptive differentiation. Both results support the idea that since coming into contact, snakes from ancestral Central and Eastern populations have hybridized freely due to a lack of fitness costs to mating between individuals from different phylogroups. We find this result particularly compelling because it represents hybridization between the two northernmost populations of each lineage that, presumably, have had the longest time to undergo differentiation in allopatry of any population. Despite this opportunity, no evidence for adaptive differentiation was detected.

Direct assessment of the survival and reproductive success of ratsnakes with different haplotypes in a presumably common environment shows no evidence for fitness differences between lineages identified on the basis of mtDNA variation. Further, a direct assessment of possible costs of mating between mates with dissimilar mtDNA types shows that litter size is not related to the degree of similarity between a female's mtDNA and that of the males siring her young. Note that nearly all the eggs laid by females in captivity successfully hatched (Blouin-Demers *et al*. 2005), so there were no apparent viability effects associated with different genetic mating combinations. We recognize that our measures of fitness were short-term and indirect and may not have been sufficiently refined to detect small differences in success between phylogroups if they exist. Size is only a crude measure of age in these snakes (Blouin-Demers *et al*. 2002) and our measures of reproductive success do not incorporate measures of success over the entire lifespan of individuals. Nonetheless, these measures are typical of those used to assess costs of hybridization in vertebrate contact zones (cf. Bronson *et al*. 2005) and should be sufficient to detect pronounced fitness costs if present.

These findings provide evidence for the mechanism ('cost-free' mating between Central and Eastern individuals) that has lead to the genetic patterns we observe presently. A counter argument to this claim is that the lack of observed fitness differences is a consequence rather than a cause of the high levels of hybridization that we observe. This begs the question of how such high levels of hybridization could occur unless there was only a limited degree of adaptive differentiation between Central and Eastern phylogroups that were clearly isolated before contact (see below). For this reason, we feel that the observed lack of differences in survival and reproduction leading to cost-free mating between individuals in different phylogroups best represents the situation that existed when the groups first came in contact in this hybrid zone.

The lack of differentiation in microsatellites among phylogroups within three hybrid populations provides direct evidence for a lack of genome-wide differentiation among snakes belonging to different mtDNA lineages within the Frontenac Axis region. This pattern could either be the result of a long-standing lack of differentiation among nuclear genes that predates the onset of hybridization in the Frontenac Axis region, or it could be a consequence of hybridization that has occurred in this region. We favour the post-contact hybridization explanation because microsatellite data point to significant differentiation in portions of the nuclear genome in East and Central populations away from the zone of contact. There is highly significant differentiation in microsatellites between pure Central (Big Creek) and pure Eastern populations (Maryland — all individuals analysed by Lougheed *et al*. (1999) were confirmed as having Eastern haplotypes using the techniques described above [Gibbs, unpublished data]) — the F_{ST} for this comparison was 0.149 and significantly different from zero (Lougheed *et al*. 1999). These results suggest that there has been substantial differentiation in both mtDNA and nuclear markers within different ratsnake lineages, but

that this differentiation has probably occurred through drift at neutral loci (e.g. microsatellites) and not as a result of selection on functional loci that affect fitness.

Although we have documented hybridization through detailed studies at a single location, other indirect evidence suggests that hybridization may occur repeatedly between Central and Eastern lineages throughout their ranges. Specifically, Burbrink *et al*. (2000) documented the co-occurrence of both phylogroups in the southern range of these snakes near the Apalachicola River that is consistent with (although does not demonstrate) hybridization in this region. In the northern parts of the range of both lineages, Burbrink (2001) found that, unlike other regions, snakes were morphologically indistinct and suggested this may be due to hybridization. Despite apparently repeated hybridization, the lineages have nonetheless remained distinct enough to form reciprocally monophyletic lineages that are geographically isolated throughout much of their ranges. Our results suggest that the distinctness of these lineages may be maintained as a result of the slow diffusion of lineage-specific genetic variants due to limited dispersal across a recent contact zone (Hewitt 1988), but additional analyses of the clinal variation in lineage-specific nuclear DNA markers across such a zone are required to test this idea. Also, as acknowledged by Burbrink (2001), more detailed sampling of these snakes in potential contact zones (e.g. the northern Appalachians) is needed to determine how geographically segregated these lineages actually are.

Studies of hybrid zones in other snakes suggest that the introgression observed between Central and Eastern lineages of ratsnakes has been unusually rapid and/or complete, possibly because of a lack of significant differentiation between lineages before coming into contact. For example, DNA-based studies of other postglacial snake hybrid zones have revealed greater association between differentiation in mtDNA and nuclear DNA markers than was detected in this study, suggesting more limited introgression between mtDNA phylogroups. In particular, Carlsson *et al*. (2004) found that in Fenoscandinavian adders (*Vipera berus*) differentiation in both microsatellite and RAPD nDNA markers mirrored the same east–west split observed by Carlsson & Tegelström 2002) for mtDNA. Of greater relevance is that individuals with ambiguous assignment scores based on both nDNA markers were most commonly sampled from the same contact zones that had been identified on the basis of mtDNA variation, suggesting limited introgression between nuclear genomes of different phylogroups, although the degree of introgression varied among contact zones. Analysis of microsatellite variation among the ratsnake populations reported here using a similar assignment test program (STRUCTURE — Pritchard *et al*. 2000) revealed a very different result (Gibbs, unpublished data): there was no evidence for admixture in microsatellites among individuals in the hybrid (FC) population when compared to microsatellite variation in two possible source populations (BC [C phylogroup] and Maryland [E phylogroup — see above]). Rather, the most strongly supported hypothesis was the presence of three distinct populations ($K = 3$) with no admixture between them. This implies that the introgression in the FA population occurred sufficiently long ago that drift has had the opportunity to act to produce significant divergence between the hybrid and source populations such that no individuals with 'ambiguous' genotypes remain in the hybrid FA population.

Limited hybridization has also been documented between the congeneric Baird's ratsnake (*Panterophis bairdi*) and black ratsnakes (*Panterophis obsoletus* — presumably belonging to the Western clade of Burbrink *et al*. (2000) based on location) in west Texas, based on allozyme and morphological data (Lawson & Lieb 1990). Lawson & Lieb (1990) argued that there was little evidence for extensive introgression in these snakes and that there was probably substantial reproductive isolation between these species. This may be due to greater differentiation between *bairdi* and Western clade ratsnakes than between Central and Eastern forms discussed here. Based on Burbrink *et al*. (2000), differentiation between Western and *P. bairdi* mtDNA variation (cytochrome *b* and control region combined) was approximately three times greater (minimum and maximum divergences between combined data: 7.65–9.06%) than that between Eastern and Central clades (1.60–2.76%).

Implications for systematics and conservation

The fact that the Central and Eastern ratsnake mtDNA lineages appear to merge on contact has implications for the proposed reclassification of the Central and Eastern clades of *Panterophis obsoleta* into two distinct species, *Panterophis spiloides* and *Panterophis alleghaniensis*, respectively (Burbrink 2001). To be clear, we do not feel that the results of this study are a strong test of the scheme proposed by Burbrink (2001). In our view, this would require phylogenetic analyses of data from multiple nuclear loci using samples collected throughout the range of these taxa, and particularly from putative contact zones north and south of the Appalachian Mountains, combined with detailed studies of reproductive and ecological interactions between Central and Eastern snakes in such zones. Nonetheless, our results identify phenomena (reproductive exchangeability and phylogenetic reticulation) that are relevant to evaluating the applicability of two widely used species concepts, the biological (BSC) and general lineage species (LSC) concepts (for recent discussion of these concepts see Coyne & Orr 2004). In particular, our results show a high degree of genetic exchangeability between these lineages and, hence, lineages do not appear to be reproductively isolated. If additional studies of contact zones in the US show similar

patterns to those observed in Canada, this would argue that Central and Eastern phylogroups are not separate reproductive entities and, hence, should not be classified as separate species under the BSC.

Evidence is stronger that the Central and Eastern phylogroups form separate geographically distinct lineages and, hence, qualify as species under the LSC (de Queiroz 1998). However, our results demonstrate that reticulation between these lineages can and does occur and, based on indirect evidence for hybridization in other regions, may occur frequently where these lineages come into contact. This weakens the claim that these lineages are strongly isolated from each other, although their distinctiveness based on mtDNA demonstrates that they have clearly experienced separate evolutionary histories for part of their existence. As mentioned above, analyses of additional samples with additional markers will clarify the frequency and consequences of these reticulation events.

In terms of conservation status, the two extant ratsnake populations in Ontario are listed as threatened by the Committee on the Status of Endangered Wildlife in Canada (Prior & Weatherhead 1998). Whereas this study demonstrates that each population has a distinct evolutionary history, based on a genetic exchangeability criterion (cf. Crandall *et al*. 2000), they do not appear to represent two distinct evolutionarily significant units and should not be managed as such. Nonetheless, other criteria (e.g. population viability and size, imminent threats) may require that each population warrants separate conservation status.

Acknowledgements

We especially thank Mary Gartshore for providing samples from the Big Creek region, the numerous people who have assisted with the long-term ratsnake project in the Chaffey's Lock subpopulation, Steve Lougheed for comments, José Diaz for help in the laboratory, Jeff Good for help with the phylogenetic analysis, Kristin Mylecraine for statistical assistance, R. MacCulloch (Royal Ontario Museum) and M. Steigerwald (Canadian Museum of Nature) for lending us specimens and staff at the Canadian Museum of Nature (M. Steigerwald and I. Deguise) for lending their laboratory facilities for the morphological analyses and for excellent logistical support. We also thank the Queen's University Biological Station for logistical support, Ohio State University and the University of Illinois for funding the laboratory work and the Canadian Wildlife Foundation, Carleton University, the Endangered Species Recovery Fund, the Natural Sciences and Engineering Research Council (Canada), the Ontario Ministry of Natural Resources, Parks Canada, and the University of Ottawa for long-term financial support of the field work.

References

Austin JD, Lougheed SC, Boag PT (2004) Discordant temporal and geographic patterns in maternal lineages of eastern North American frogs, *Rana catesbeiana* (Ranidae) and *Pseudacris crucifer* (Hylidae). *Molecular Phylogenetics and Evolution*, **32**, 799–816.

Avise JC (2000) *Phylogeography*. Harvard University Press, Cambridge, Massachusetts.

Avise JC, Walker D, Johns GC (1998) Speciation durations and Pleistocene effects on vertebrate phylogeography. *Proceedings of the Royal Society of London, Series B, Biological Sciences*, **265**, 1707–1712.

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, **16**, 113–148.

Barton NH, Hewitt GM (1989) Adaptation, speciation, and hybrid zones. *Nature*, **341**, 497–503.

Basten CJ, Asmussen MA (1997) The exact test for cytonuclear disequilibrium. *Genetics*, **146**, 1165–1171.

Blouin-Demers G (2003) Precision and accuracy of body-size measurements in a constricting, large-bodied snake (*Elaphe obsoleta*). *Herpetological Review*, **34**, 320–323.

Blouin-Demers G, Prior KA, Weatherhead PJ (2002) Comparative demography of black rat snakes (*Elaphe obsoleta*) in Ontario and Maryland. *Journal of Zoology*, **256**, 1–10.

Blouin-Demers G, Gibbs HL, Weatherhead PJ (2005) Genetic evidence for sexual selection in black ratsnakes (*Elaphae obsoleta*). *Animal Behaviour*, **69**, 225–234.

Bronson CL, Grubb TC Jr, Sattler GD *et al.* (2005) Reproductive success across the black-capped chickadee (*Poecile atricapillus*) and Carolina (*P. carolinensis*) hybrid zone in Ohio. *Auk*, **122**, 733–746.

Burbrink FT (2001) Systematics of the eastern ratsnake complex (*Elaphe obsoleta*). *Herpetological Monongraphs*, **15**, 1–53.

Burbrink FT, Lawson R, Slowinski JB (2000) Mitochondrial DNA phylogeography of the North American ratsnake (*Elaphe obsoleta*): a critique of the subspecies concept. *Evolution*, **54**, 2107–2114.

Carlsson L, Söderberg L, Tegelström H (2004) The genetic structure of adders (*Vipera berus*) in Fenoscandia: congruence between different kinds of markers. *Molecular Ecology*, **13**, 3147–3152.

Carlsson L, Tegelström H (2002) The phylogeography of adders (*Vipera berus*) from Fennoscandia. In: *Biology of the Vipers* (eds Schuett G, Hoggren MH, Douglas ME *et al.*), pp. 1–9. Eagle Mountain Publishing, Sandy, Utah.

Church SA, Kraus JM, Mitchell JC *et al.* (2003) Evidence for multiple Pleistocene refugia in the post-glacial expansion of the eastern tiger salamander, *Ambystoma tigrinum tigrinum*. *Evolution*, **57**, 372–383.

Clark AM, Moler PE, Possardt EE *et al.* (2003) Phylogeography of the timber rattlesnake (*Crotalus horridus*) based on mtDNA sequences. *Journal of Herpetology*, **37**, 145–154.

Conant R, Collins JT (1991) *A Field Guide to Reptiles and Amphibians: Eastern/Central North America*, 3rd edn. Houghton Mifflin, Boston, Massachusetts.

Coyne JA, Orr HA (2004) *Speciation*. Sinauer Associates, Sunderland, Massachusetts.

Crandall K, Bininda-Emonds O, Mace G *et al.* (2000) Considering evolutionary processes in conservation biology. *Trends in Ecology & Evolution*, **15**, 290–295.

Goldstein PZ, DeSalle R (2000) Phylogenetic species, nested hierarchies, and character fixation. *Cladistics*, **16**, 364–384.

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

Harrison RG (ed.) (1993) *Hybrid Zones and the Evolutionary Process*. Oxford University Press, New York.

von Hasseln K (2005) *The phylogeography of Elaphe spp. (North American ratsnake) in New York and Vermont's Champlain Valley*. BA Thesis, Middlebury College, Vermont, USA.

Hewitt GM (1988) Hybrid zones: natural laboratories for evolutionary studies. *Trends in Ecology & Evolution*, **3**, 158–167.

Howard DJ, Reece M, Gregory PC *et al.* (1998) The evolution of barriers to fertilization between closely-related species. In: *Endless Forms: Species and Speciation Events* (eds Howard DJ, Berlocher SH), pp. 279–288. Oxford University Press, New York.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: bayesian inference of phylogenetic trees. *Bioinformatics*, **17**, 754–755.

Lamb T, Avise JC (1986) Direction introgression of mitochondrial DNA in a hybrid population of tree frogs — the influence of mating behavior. *Proceedings of the National Academy of Sciences, USA*, **83**, 2526–2530.

Lawson R, Lieb CS (1990) Variation and hybridization in *Elaphe bairdi* (Serpentes: Colubridae). *Journal of Herpetology*, **24**, 280–292.

Lougheed SC, Gibbs HL, Weatherhead PJ *et al.* (1999) Hierarchical patterns of genetic population structure in black rat snakes (*Elaphe obsoleta obsoleta*) as revealed by microsatellite DNA analysis. *Evolution*, **53**, 1995–2001.

Minin V, Abado Z, Joyce P *et al.* (2003) Performance-based selection of likelihood models for phylogeny estimation. *Systematic Biology*, **52**, 674–683.

Moritz C (2002) Strategies to protect biological diversity and the evolutionary processes that sustain it. *Systematic Biology*, **51**, 238–254.

Posada D, Crandall KA (2001) Selecting the best-fit model of nucleotide substitution. *Systematic Biology*, **50**, 580–601.

Prior KA (1997) *Conservation biology of black rat snakes: ecological, demographic, and genetic approaches (*Elaphe obsoleta, *Ontario)*. PhD Thesis, Carleton University.

Prior KA, Weatherhead PJ (1998) Status of the black rat snake, *Elaphe obsoleta obsoleta*, in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa.

Prior KA, Gibbs HL, Weatherhead PJ (1997) Population genetic structure in the black rat snake: implications for conservation. *Conservation Biology*, **11**, 1147–1158.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotypes. *Genetics*, **155**, 945–959.

de Queiroz K (1998) The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminological recommendations. In: *Endless Forms: Species and Speciation Events* (eds Howard DJ, Berlocher SH), pp. 57–75. Oxford University Press, New York.

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

Sites JW, Marshall JC (2003) Delimiting species: a renaissance issue in systematic biology. *Trends in Ecology & Evolution*, **18**, 462–470.

Sommer SS, Groszbach AR, Bottema CDK (1992) PCR amplification of specific alleles (PASA) is a general method for rapidly detecting known single-base changes. *BioTechniques*, **12**, 82–87.

Templeton A (2001) Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology*, **10**, 779–791.

Utiger U, Helfenberger N, Schatti B *et al.* (2002) Molecular systematics and phylogeny of Old and New World ratsnakes, Elaphae AUCT., & related genera (Reptilia, Squamata, Colubridae). *Russian Journal of Herpetology*, **9**, 105–124.

Wiens JJ, Penkrot TA (2002) Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). *Systematic Biology*, **51**, 69–91.

Zamudio K, Savage WK (2003) Historical isolation, range expansion, and secondary contact of two highly divergent lineages in spotted salamanders (*Ambystoma maculatum*). *Evolution*, **57**, 1631–1652.

H.L. Gibbs is an Associate Professor in the Department of Evolution. Ecology, and Organismal Biology (EEOB) at Ohio State University (OSU). His research focuses on the evolutionary and conservation biology of snakes and birds. S.J. Corey is a graduate student in EEOB at OSU with interests in the conservation of reptiles and amphibians. G. Blouin-Demers is an Assistant Professor in the Department of Biology, University of Ottawa who works on the behavioural and physiological ecology of snakes and amphibians. K.A. Prior is a Senior Scientific Advisor with Parks Canada with interests in reptile conservation. P.J. Weatherhead is a Professor in the Program in Ecology and Evolutionary Biology who studies the behavior, ecology and conservation biology of animals, especially snakes and birds.

Appendix I Specimens examined for morphological analyses

Frontenac Axis (19): Frontenac County: CMNAR-9135, CMNAR-17794, CMNAR-9563. Lanark County: CMNAR-30841. Leeds County: CMNAR-17727, CMNAR-17793, CMNAR-2036, CMNAR-33816, CMNAR-17792, CMNAR-17728, CMNAR-29611, CMNAR-19298, CMNAR-28604, CMNAR-21433, ROM-5501, ROM-4593, ROM-5518, ROM-4948, ROM-5502.
Big Creek (*13)*: Haldimand-Norfolk County: ROM-5520, ROM-9032, ROM-24665, ROM-24662, ROM-24663, ROM-24664, CMNAR-29582, CMNAR-35435, CMNAR-35436, CMNAR-35437, CMNAR-35438, CMNAR-35439, CMNAR-35440.

Appendix II Identification numbers for cytochrome *b* haplotypes used in Fig. 2. CAS and LSUMZ numbers are from the legend for Fig. 2 in Burbrink *et al*. (2000)

Haplotype	Identification no.	Sample location	Haplotype	Identification no.	Sample location
E1	Gibbs Eob264	HI (FA — Ontario)	C4	Gibbs Eob258	FA — Ontario
E2	Gibbs Eob518	CL (FA — Ontario)		Gibbs Eob261	FA — Ontario
E3	Gibbs Eob520	CL (FA — Ontario)		Gibbs Eob504	FA — Ontario
E4	LSUMZ 39816	FL		AF283643	OH
	LSUMZ H15884	FL		CAS 208631	OH
E5	AF283603	FL		LSUMZ 44335	IL
	AF283604	FL	C5	Gibbs Eob128	BC — Ontario
E6	LSUMZ H3384	SC	C6	Gibbs Eob130	BC — Ontario
	AF283639	VA	C7	LSUMZ H3246	LA
E7	LSUMZ H15890	CT		LSUMZ H15888	LA
	LSUMZ H15889	CT	C8	LSUMZ 41197	LA
	AF283644	RI		LSUMZ H3306	LA
	LSUMZ 41187	NC	C9	LSUMZ H3206	TN
E8	LSUMZ H3189	FL		LSUMZ H15031	IL
	LSUMZ 39925	FL		LSUMZ H15030	IL
E9	CAS 169468	FL		LSUMZ H14781	AR
E10	LSUMZ 44662	MD	C10	LSUMZ 41189	AL
E11	LSUMZ H3191	VA	C11	LSUMZ H3209	LA
E12	LSUMZ 40444	NY	C12	LSUMZ H3190	AL
E13	AF283641	SC	C13	LSUMZ H3379	LA
E14	LSUMZ 45359	GA	C14	LSUMZ H3376	TN
E15	LSUMZ 41188	NC	C15	LSUMZ H3385	AL
E16	AF283640	FL	C16	LSUMZ 39163	WV
E17	LSUMZ H2229	FL	C17	LSUMZ H14724	IL
E18	AF283605	FL	C18	LSUMZ H2286	TN
E19	CAS 203083	FL	C19	LSUMZ H3345	AL
E20	LSUMZ H3212	FL	C20	LSUMZ H3309	FL
E21	LSUMZ 40943	FL	C21	LSUMZ H3276	FL
C1	Gibbs Eob112	FA — Ontario	C22	CAS 203079	FL
C2	Gibbs Eob503	FA — Ontario	C23	AF283642	MS
C3	Gibbs Eob109	BC — Ontario	C24	LSUMZ H3186	MS
	Gibbs Eob122	BC — Ontario	W1	LSUMZ H14782	AR
	Gibbs Eob123	BC — Ontario		LSUMZ H3388	KS
	Gibbs Eob375	BC — Ontario		LSUMZ H15896	AR
	Gibbs Eob377	BC — Ontario	W2	LSUMZ 42624	LA
C4	Gibbs Eob78	FA — Ontario	W3	LSUMZ 40443	LA
	Gibbs Eob80	FA — Ontario	W4	LSUMZ 41186	LA
	Gibbs Eob88	FA — Ontario	W5	LSUMZ 44451	LA
	Gibbs Eob105	BC — Ontario	W6	LSUMZ H3169	LA
	Gibbs Eob106	BC — Ontario	W7	LSUMZ H1911	TX
	Gibbs Eob108	BC — Ontario	W8	LSUMZ 37499	TX
	Gibbs Eob124	BC — Ontario	W9	LSUMZ 44480	TX
	Gibbs Eob125	BC — Ontario	W10	LSUMZ 39162	TX
	Gibbs Eob126	BC — Ontario	W11	LSUMZ H15892	LA
	Gibbs Eob127	BC — Ontario	W12	LSUMZ H15891	LA
	Gibbs Eob131	BC — Ontario	W13	LSUMZ H3188	LA
	Gibbs Eob243	FA — Ontario	*Pantherophis vulpina*	CAS 184362	OH
	Gibbs Eob257	FA — Ontario	(outgroup)		