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Phylogeography of *Bufo fowleri* at its northern range limit

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Abstract

Many of the species that recolonized previously glaciated areas in the Great Lakes basin of North America over the past 10–12 000 years exhibit genetic evidence of multiple invasion routes and present-day secondary contact between deeply divergent lineages. With this in mind, we investigated the phylogeographical structure of genetic variability in Fowler's toads (*Bufo fowleri*) at the northern edge of its distribution where its range encircles the Lake Erie basin. Because *B. fowleri* is so closely tied to habitats along the Lake Erie shoreline, we would expect to find clear evidence of the number of invasions leading to the species' colonization of the northern shore. A 540 bp sequence from the mitochondrial control region was amplified and analysed for 158 individuals from 21 populations. Interpopulation sequence variation ranged from 0% to 6%. Phylogenetic analysis of p-distance using the neighbor-joining method revealed two deeply divergent (6% sequence divergence) mtDNA lineages (Phylogroup 1 and 2), possibly arising as a result of secondary contact of populations that entered the region from two separate glacial refugia. However, the phylogeographical pattern was not simple. The populations at Long Point, on the north shore of Lake Erie, clustered with the population from Indiana Dunes on Lake Michigan to form Phylogroup 2 whereas all other *B. fowleri* populations examined from both sides of Lake Erie constituted Phylogroup 1. Furthermore, mtDNA sequences from the related species *Bufo americanus*, obtained from populations outside the range of *B. fowleri*, clustered with mtDNA haplotypes of *B. fowleri* Phylogroup 1, indicating the possibility of partial introgression of mitochondria from one species to the other.

Keywords: amphibian, *Bufo fowleri*, mtDNA, phylogeography, postglacial recolonization

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Introduction

Holman (1995) proposed that species of amphibians and reptiles initially re-entered what is now Ontario, Canada, postglacially from present day Indiana and Ohio, USA, across the area between present day Lakes Huron and Erie. Additional recolonizations from the north and east would have been delayed (< 10 000 years before present) until the receding Champlain Sea to the east revealed the St Lawrence lowlands (Holman 1995). The genetic signature of two postglacial routes of recolonization is evident in the Spring Peeper (*Pseudacris crucifer*) (Austin *et al*. 2002) and the Spotted Salamander (*Ambystoma maculatum*) (Zamudio & Savage 2003). Both species display deep interpopulation genetic divergences not predicted by the geographical separation of contemporary populations. Other species, such as *Bufo fowleri*, with very different temperature requirements (McKenney *et al*. 1998), would have likely arrived afterwards, as a secondary (*sensu* Holman 1992) invader. There is fossil evidence to support the hypothesis of primary (*Pseudacris* and *Ambystoma*), and secondary (*B. fowleri*) invasion of postglacial North America (Holman 1992; Holman 1995). Since southern Ontario has been shown to be a zone of secondary contact for two primary invaders, it is therefore reasonable to ask whether such as zone exists for a secondary invader such as *B. fowleri.*

Contemporary populations of *B. fowleri* have a precarious hold on the northern Lake Erie shoreline. They are isolated from each other by shoreline development, agriculture and other nonviable habitats. They are further isolated from populations on the southern shoreline by many kilometers of the open waters of Lake Erie. Where there is population isolation with occasional dispersing individuals connecting, or founding, geographically disjunct populations there is

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likely to be phylogeographical structure (Avise 2000). Thus, the geographical subdivision of genetic variability is related to the scale of the dispersal ability of the species (Avise 2000). If the individuals of a species can disperse relatively far, then many populations are likely within an area of panmixia, or genetic neighborhood (Wright 1951), and we would not expect to see the accumulation of small mutations that delineate a lineage on a unique trajectory. However, if the individuals do not move long distances then it becomes more likely that we would find geographical structuring of genetic variability. Amphibians, for example, are expected to be consigned by their biology and behavior to have relatively isolated populations associated with discrete habitats such as breeding ponds (Sinsch 1990; Blaustein *et al.* 1994; Rowe *et al.* 2000). Amphibians are therefore likely to exhibit phylogeographical structure on relatively small spatial scales on the order of kilometers. Geographic structuring of genetic variability has been shown in the true toads of the genus *Bufo*. *Bufo calamita* exhibits small but significant geographical substructuring of microsatellite DNA with pairwise between pond distances ranging from 0.5 to 16 km (Rowe *et al.* 2000). *Bufo woodhousei* populations spaced between 150 km and 250 km apart exhibits phylogeographical structure at the ND1 region of mtDNA (Masta *et al.* 2003). Although island populations of *Bufo bufo* showed no significant isolation by distance among islands separated by less than 10 km (Seppa & Laurila 1999), urban and rural populations of *B. bufo* did exhibit significant isolation at a scale of approximately 30 km, when the additional isolating effects of roads were taken into account (Hitchings & Beebee 1998). Populations of *B. bufo* separated by 5–15 km of pasture and small urban developments were estimated to have more than two migrants per generation on average (Scribner *et al.* 1994). Populations of *B. fowleri* did not demonstrate isolation-by-distance when populations were separated by maximums of approximately 35 kilometers in Mississippi (Hranitz & Diehl 2000) or 120 km in Ontario (Green 1984) when genetic variation was assayed using allozymes.

The variability, or resolution, of the genetic region used determines the spatial scale within which genetic structure is expected (Scribner *et al.* 1994; Parker *et al.* 1998). For example, in toads, rapidly changing microsatellites display geographical structure on a scale of a kilometer or less (Rowe *et al.* 2000), while more slowly mutating regions of the mitochondria [ND1 (Masta *et al.* 2003), $tRNA^{Ile}$, $tRNA^{Met}$, $tRNA^{Gln}$, and ND2 — (Macey *et al.* 1998)], 16S and cytochrome *b* (Mulcahy & Mendelson 2000), and 12S (Liu *et al.* 2000) show geographical structure on a scale of hundreds of kilometers. Allozymes, which are under the control of nuclear genes with generally very conservative rates of mutation, do not show any geographical structuring to genetic variation at scale of hundreds of kilometers (Green 1984). Among mitochondrial markers, the D-loop or control region is not constrained by function and is thus a rapidly evolving locus useful for delineating moderately divergent lineages (Goebel *et al.* 1999). We selected this locus with the expectation that it would expose phylogeographical structure within the Lake Erie watershed where tens and hundreds of kilometers separate contemporary populations.

Using sequence data from the highly variable control region of the mitochondria of *Bufo fowleri* we tested the hypothesis that as southern Ontario is a likely zone of secondary contact between two glacial refugia, there would be a large genetic divergence evident between some *B. fowleri* populations along the northern shoreline of Lake Erie. Such deep divisions would not be predicted by contemporary geographical distance. This area has only been free of glacial ice for approximately 10 000 years and therefore the majority of phylogeographical structuring among contemporary populations would likely be shallow.

Materials and methods

Populations were sampled at sites from across the northern edge of the Fowler's toad distribution (Fig. 1, Appendix 1). Additional *B. fowleri* samples were obtained from Ross and Union Counties in Ohio and Leigh County in Pennsylvania, USA, courtesy of S. Masta. For comparison, *Bufo americanus* individuals were sampled from Long Point Ontario, Niagara County Ontario and Montreal Quebec, Canada. For adult toads, toe clips were preserved in the field in 70% ethanol and total DNA extractions were completed following Fetzner (1999). For juveniles, tissue samples from the heart, liver and skeletal muscle were frozen and maintained at −80 °C and total DNA extractions were completed following the methods described by Fetzner (1999) and Chase *et al.* (1998). Control region mtDNA was amplified using published primers (Goebel *et al.* 1999). Thermocycling conditions (Robocycler) for double-stranded DNA (dsDNA) amplification began with 39 cycles each of which each had

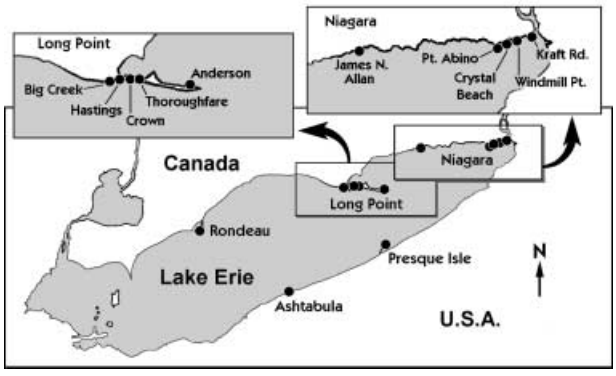

Fig. 1 Lake Erie, showing the sampling locations of *Bufo fowleri* populations.

a 30-s denaturation at 94 °C, a 45-s annealing at 52 °C, and a 1.5-min extension at 72 °C. A 10-min extension at 72 °C followed the final cycle. Reaction mixtures for polymerase chain reaction (PCR) contained 50 mM KCl, 10 mM Tris–HCl, pH 9.0, 0.1% Triton X-100, 0.4 mM of each oligonucleotide, 2.5 mM $MgCl_2$, 0.5 U/100 µL *Taq* DNA polymerase (Sigma), and 0.1 µM each primer in a reaction volume of 25 µL. A negative control was included for all PCR reactions. Amplified DNA was examined on 2.5% agarose gel and then cleaned with the Qiaquick PCR purification kit (Qiagen). Cleaned DNA was sequenced using Amersham Biosciences DYEnamic ET Terminators Chemistry Cycle Sequencing System with Thermo Sequenase II DNA Polymerase.

Sequences were aligned and manipulated using CLUSTALW version 1.4 (Higgins *et al*. 1994) DNAsp v3.5 (Rozas & Rozas 1999), and BIOEDIT version 5.0 (Hall 1999). Phylogroups were examined through haplotype clustering using neighbor-joining (NJ; Saitou & Nei 1987) Minimum Evolution (ME; Rzhetsky & Nei 1993) and maximum parsimony (MP; Fitch 1971) in MEGA2 version 2.1 (Kumar *et al*. 2001). Genetic distance was calculated using p-distance and Kimura two-parameter (Kimura 1980) across all populations using 1000 bootstrap replications. The genetic diversity at each population was calculated using ARLEQUIN version 2.0 (Schneider *et al*. 2000) for nucleotide diversity (Tajima 1983; Nei 1987) and haplotype diversity (Nei 1987).

Geographic structuring was further evaluated using the analysis of molecular variance (AMOVA) options in ARLEQUIN version 2.0. The AMOVA tested whether genetic variation between groups, between populations within groups and within populations is significant. Using the AMOVA approach, we tested various alternative hypotheses suggested by geography, history and NJ-tree visualizations. The p-distance and Kimura two-parameter were used as measures of genetic distance for the AMOVA, and p-values were determined using 1000 permutations. Φ statistics (analogous to *F*-statistics) were calculated using ARLEQUIN.

Combining the tree-making approach with a frequency distribution of pairwise genetic distances methods can illustrate where hybridization has occurred (Liebers *et al*. 2001), and allow other inferences regarding the population history of these NJ-revealed sites. A unimodal frequency distribution suggests that the population has recently gone through a bottleneck or a founding event. A distinct, bimodal distribution of distances (spanning 0.2 p-distance or more) would indicate the presence of a deeply divergent mtDNA haplotype in the population (Liebers *et al*. 2001). The observed frequency distribution of pairwise genetic distance was tested against the expected Poisson distribution (Slatkin & Hudson 1991) (generated for the observed mean) using χ^2 maximum likelihood in GENSTAT version 7.1 (Trust 2003).

Previously published allozyme frequencies (Green 1981; Green 1984) for seven variable loci (*6PGD*, *CK1*, *EST1*, *GP2*, *IDH1*, *SOD* and *LDH1*) were reanalysed using the AMOVA approach to determine whether there was concordance between mtDNA and nDNA phylogeographical structure. Three populations at Long Point (Hastings, Crown and Thoroughfare), and four populations in the eastern basin of Lake Erie (Dunnville, Rock Point, Windmill Point and Point Abino) were included in the allozyme analysis.

Results

A 540-bp PCR product was amplified for 158 individuals from 21 populations. The product corresponds to region 880–1460 of the control region in *Xenopus laevis* (Roe *et al*. 1985). Reference sequences have been deposited in GenBank (Accession numbers AY529737–AY529865).

Polymerase chain reaction products were obtained from mitochondrial rich tissue (muscle and toe clip containing muscle), displayed no sequence ambiguity and ghost bands were never observed in post-PCR agarose gel electrophoresis, suggesting that these were not nuclear pseudogenes (Bensasson *et al*. 2001). Indeed, to date no numts have been found in Amphibia (Bensasson *et al*. 2001).

Aligned sequences contained an average of 82 gaps with a resultant sequence size of 622 bp where 109 of those sites were variable. The transition to transversion ratio (ts/tv) was 1.8.

Analyses using different distance methods were equivalent, and p-distance is presented here, as it is the more simple measure (Nei & Kumar 2000). Toads at all sites were characterized by low genetic diversity (Table 1). In only two cases was the same haplotype recorded from more than one population. With this exception, different haplotypes were found in each location or population sampled.

All tree-building algorithms (NJ, ME and MP) produced identical phylogroups, demonstrating both a shallow (sequence divergence of < 1%) and a deep (sequence divergence of 6%) division (Fig. 2). Shallow divisions correspond to significant geographical subdivision of mtDNA variability for *B. fowleri* populations in the Lake Erie watershed (Table 2). Toads from Rondeau, Ontario, and Ashtabula, Ohio, were identical (mean pairwise $F_{ST} = 0$) and comprised a south-west Phylogroup 1b. Toads collected in Ontario from populations between the mouth of the Grand River and the Niagara River formed Phylogroup 1a. Toads from Presque Isle, Pennsylvania, on the southern shore of the eastern basin of Lake Erie were next most similar to Phylogroup 1a and formed Phylogroup 1c. Animals from Long Point, Ontario, clustered together to form Phylogroup 2b and were more similar to animals from Indiana, southern Ohio and southern Pennsylvania (Phylogroup 2a) than any animals on Lake Erie. All *B. americanus* individuals clustered with Phylogroup 1. This phylogroup

Table 1 Measures of genetic diversity for Lake Erie Fowler's toad populations

Phylogroup	Site	Sample size (n)	Number of haplotypes	Polymorphic sites (S)	Mean number of pairwise differences between haplotypes (d)	Gene diversity ($\hat{H}$)*	Sequence diversity (π)†
1a (Niagara)	Crystal Beach	6	6	36	15.81	0.909	0.007
	James N Allan	11	11	50	14.038	0.93	0
	Kraft Road	10	9	48	15.102	0.918	0
	Point Abino	13	13	57	19.915	0.929	0.001
	Windmill Point	10	10	63	23.538	0.905	0.002
1b	Presque Isle	10	10	63	14.692	0.906	0.012
1c	Ashtabula	4	4	29	17.167	1	0
	Rondeau	11	11	45	14.707	0.914	0
2b (Long Point)	Anderson	10	11	67	20.868	0.915	0.001
	Big Creek	11	11	71	19.141	0.921	0.012
	Crown	10	10	90	28.678	0.911	0.001
	Hastings	10	10	59	15.007	0.91	0.001
	Thoroughfare	11	11	40	13.945	0.917	0.001

*$\hat{H} = n/n - 1(1 - \Sigma p_i^2)$; †$\pi = n_d/n$.

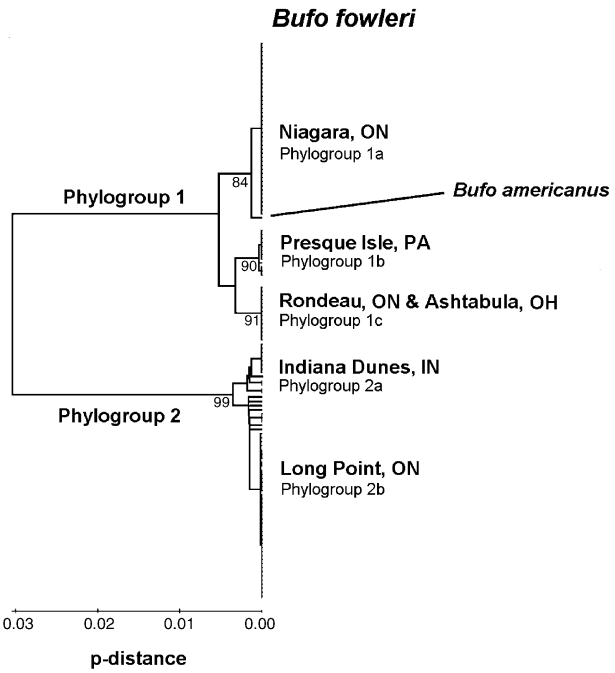

Fig. 2 Neighbor-joining tree of sequence divergence (p-distance) from control region mtDNA of *Bufo fowleri* of populations from the northern edge of their distribution. Bootstrap values > 80% are shown.

structure explained 55% of the variation with a nearly equivalent proportion found within populations (40%). Populations within phylogroups were significantly (but minimally, *c.* 4.5%) differentiated (Table 2). Pairwise frequency distributions of genetic distance were different from the expected Poisson distribution in all but Phylogroup 1c (Fig. 3).

There was no concordance between the mtDNA and nDNA phylogeographical structure. An AMOVA using allozymes frequencies revealed that the variance between Phylogroups 1 and 2 ranged from 0 to 10% ($P < 0.001$), whereas variance among populations within phylogroups was higher (5.4–85.71%, $P < 0.001$). Variance within populations was very high (35.57–96.77%, $P < 0.001$).

Discussion

A deep phylogeographical division, such as we have found within *B. fowleri* in the Lake Erie basin, has been explained in other species as the genetic signature of secondary contact between two previously isolated postglacial lineages (Vallianatos *et al.* 2001; Austin *et al.* 2002; Zamudio, Savage 2003). But the pattern is not so clear in these toads (Fig. 4) and other explanations may be entertained.

The deep sequence divergence in Lake Erie watershed populations of *B. fowleri* may result from incomplete lineage sorting leading to the paraphyletic retention of ancestral haplotypes. This would indicate two colonizations of southwestern Ontario (Austin *et al.* 2002; Holman 1995), one from the Midwest evident in Phylogroup 2 and another from the northeast, following the receding Champlain Sea, evident in Phylogroup 1. However, this scenario is complicated by the contemporary spatial arrangement of populations. There are currently Phylogroup 1 populations of *B. fowleri* both to the east and to the west of Long Point, which is Phylogroup 2. The hypothesis of paraphyly is thus complicated, since it invokes either the missed (or unsuccessful) colonization of Long Point by Phylogroup 1 mtDNA, or the initial colonization of Lake Erie from individuals to the east and the subsequent founding of the western basin populations via the Central basin south shore (approximately Pennsylvania).

Table 2 Analysis of molecular variance of Fowler's toad populations. Ten models representing different groupings of populations were tested and the two models explaining the most variation are presented

Model	Populations in Groups			Variance component	% Variance explained	*P*
I	Phylogroup 2a,	Among groups	Φ_{CT}	0.5536	55.36	< 0.00001
	Phylogroup 1a,	Among populations within groups	Φ_{SC}	0.10201	4.55	< 0.00001
	Phylogroup 1b, Phylogroup 1c	Within populations	Φ_{ST}	0.59914	40.09	< 0.00001
II	Phylogroups 1 and 2	Among groups	Φ_{CT}	0.56095	56.09	< 0.00001
		Among populations within groups	Φ_{SC}	0.26229	11.52	< 0.00001
		Within populations	Φ_{ST}	0.67611	32.39	< 0.00001

Model I is the groups revealed by the neighbor-joining tree in Fig. 2. Model II is a test of the deeper Phylogroups 1 and 2. Φ_{CT} represents the correlation level among randomly chosen haplotypes from one group, compared with the correlation level of pairs of randomly chosen haplotypes from all groups. Φ_{SC} represents the correlation level among randomly chosen haplotypes from one population, in comparison with the correlation level of pairs of randomly chosen haplotypes from that group. Φ_{ST} represents the correlation level among randomly chosen haplotypes from one population, in compared with the correlation level of pairs of randomly chosen haplotypes from all the sampled populations (Schneider *et al*. 2000).

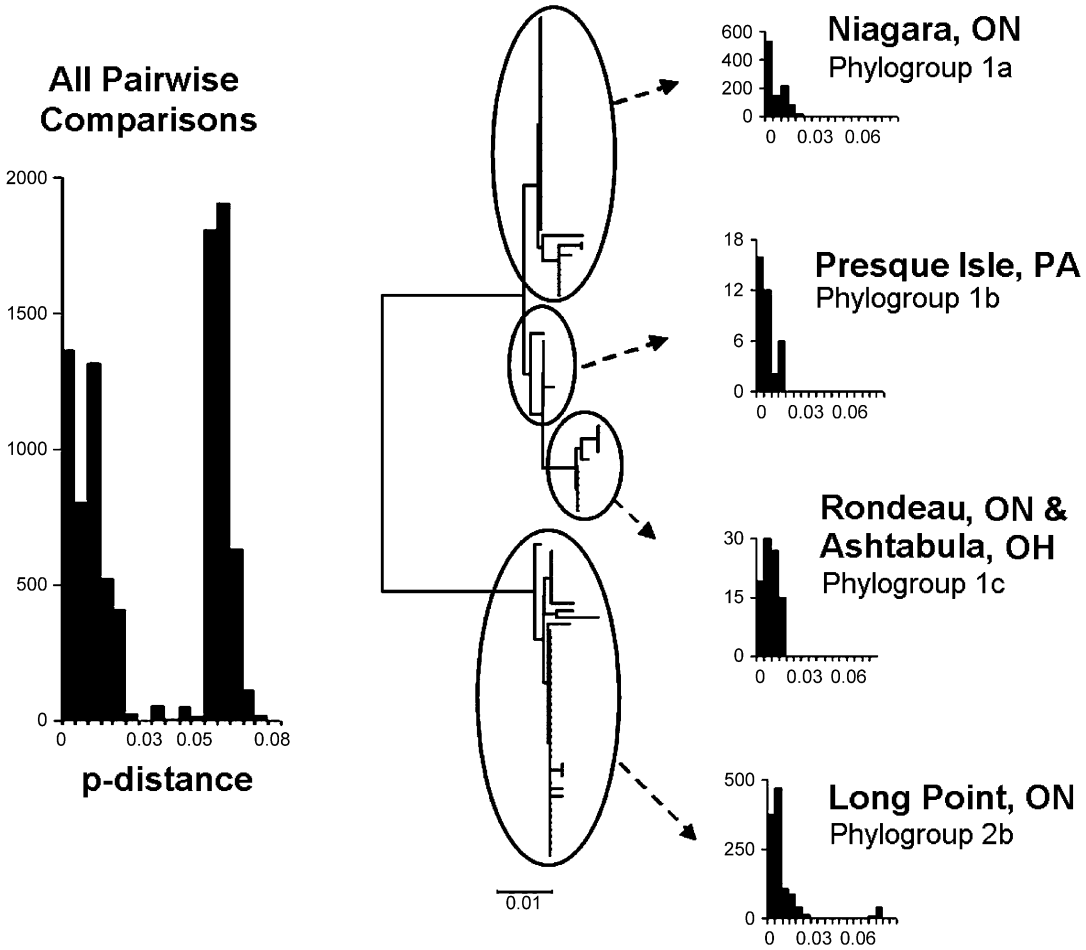

Fig. 3 Comparative illustration of mtDNA phylogeography for *Bufo fowleri* on Lake Erie. On the right is the paired neighbor-joining tree and frequency histogram for all Lake Erie populations. On the left are the paired images of the neighbour-joining tree and the frequency histogram of pairwise distances for that branch. Frequency distribution *x*-axis is in increments of 0.5% sequence divergence (p-distance). Phylogram scale bars show p-distance.

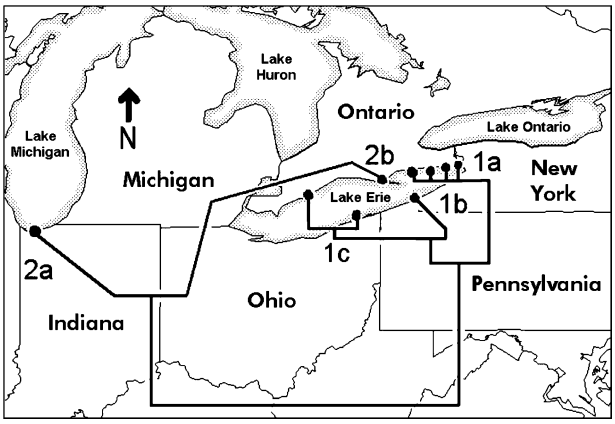

Fig. 4 Comparative map of mtDNA phylogeography for *Bufo fowleri* showing the divisions of phylogroups into Phylogroups 1a, 1b, 1c, and 2a and 2b. The geographical location of each population is the tip of the appropriate branch of the neighbour-joining tree. Branch length is not proportional to distance.

Alternatively, the pattern of mtDNA distribution might reflect past hybridization with closely related *B. americanus*. The two species are known to hybridize (Green 1984) and introgression coincident with a small population bottleneck could produce this genetic signature. Hybridization is frequent, and populations are known to fluctuate in size (Green 1997). Fixation of introgressed mtDNA of one species within individuals from populations of another species is not new and has been demonstrated in salmonid fishes (Wilson & Bernatchez 1998), and hylid frogs (Lamb & Avise 1986).

Consider a population much like contemporary Long Point, i.e. intermixing *B. americanus*, *B. fowleri* and occasional F_1 hybrids. In this scenario, interspecific mating of female *B. americanus* and male *B. fowleri* produced hybrids. After this mating, and the metamorphosis of the F_1 generation, there was an extreme population bottleneck. The bottleneck reduced the numbers of all individuals and there was no selective advantage to either species or the hybrids. Afterwards, the *B. americanus* female/*B. fowleri* male F_1 female hybrids (intermediate between the species in breeding temperature preference and timing) would be most likely to find and breed with *B. fowleri* males (Green & Parent 2003). If founding populations at Lake Erie sites were small, and/or remained small for several generations, fixation could occur quite rapidly (Avise & Saunders 1984). This simple chronology could lead to the fixation of the *B. americanus* mtDNA in an otherwise *B. fowleri* population.

Reducing groups in the hierarchical AMOVA to putative *B. americanus* mtDNA (Phylogroup 1) and putative *B. fowleri* mtDNA (Phylogroup 2) can indirectly test the hypothesis of past hybridization as an explanation for the phylogroup structure we see in Lake Erie *B. fowleri*. The resultant group structure was strongly significant and explained a large portion (56%) of the observed variation (Table 2).

Another line of evidence for hybridization is apparent when each branch of the neighbor-joining tree is paired with the frequency histogram of pairwise genetic distances for that branch (Fig. 3). Here, a right-sided tail, and bimodal shape of the distribution indicates the presence of the mtDNA of the other putative parent species. At Long Point, where the majority of the mtDNA is Phylogroup 2 the presence of a second mode shows a very small proportion of Phylogroup 1 mtDNA. For all other populations within Canada a second mode would indicate the presence of Phylogroup 2 mtDNA. The absence of this second mode is immediately clear for all Phylogroup 1 populations (Fig. 3).

Judging by the phylogeographical structure within Lake Erie, any bottleneck/hybridization/founding event would likely have occurred prior to the recolonization of the north shore of Lake Erie, otherwise there would not have been enough time for the populations to drift towards the shallow geographical structuring observed. Indeed, the widespread nature of Phylogroup 1 (putative hybridization) suggests that it may be older (Neigel *et al*. 1991), since it is the more pervasive genotype.

Interestingly, for the small number of toads from Indiana Dunes National Lakeshore, on the shore of Lake Michigan (Fig. 4), there was a second mode evident in the frequency histogram, but the outlying individual was not an individual Phylogroup 1, but rather clustered with *Bufo terrestris* (data not shown). Masta *et al*. (2002) describe the Northern, Southern and Eastern clades of *B. fowleri*. Long Point *B. fowleri* individuals cluster with individuals from Masta's *et al*.'s (2002) Northern clade, while *B. terrestris* were shown to be nested within the Southern Clade of *B. fowleri*. We consider that the clustering of *B. fowleri* mtDNA from the southern shore of Lake Michigan with mtDNA from *B. terrestris* likely indicate the overlap of Masta's *et al*.'s (2002) Southern and Northern clades at this locality.

The deep phylogeographical divisions between Long Point (2b) and the north-eastern basin of Lake Erie (1a) revealed by mtDNA are not concordant with allozyme data. Whereas greater than 50% of the observed mtDNA variation was partitioned among the phylogroups, less than 10% of the observed allozyme variation was explained by between-group differences. Here, the majority of variation was distributed among and within populations. If toad dispersal was sex-biased towards males, as in humpback whales (Palumbi & Baker 1994), this is the discordant genetic pattern we would expect, i.e. more philopatric female behavior resulting in greater geographical structuring of a matrilinealy inherited genetic marker (Avise 1995). However, as we have shown elsewhere, there is no sex bias to dispersal in *B. fowleri* (Smith, 2003) and it is therefore an unlikely explanation for the discordance. Differing rates of evolution might also cause this nuclear/organelle discordance

since the effective population size of nuclear genes is four times larger than for mitochondrial (Avise 2000). The so called, three-times rule (Avise 2000) predicts that if a matrilineal tree has required time x to achieve reciprocal monophyly, then, on average, $3x$ time would be required for a nuclear tree to achieve the same shape. Our acceptance of this hypothesis is limited by the fact that populations sampled from Phylogroup 1 for allozymes are only from 1a and we cannot therefore test the shallow phylogroup structure among 1a, 1b and 1c. The deep division between Phylogroup 1 and 2 likely caused result from historic introgressive hybridization, or the 'ghost of hybrids past' (Wilson *et al*. 1998), and we feel that this 'ghost' explains both the nuclear/organelle discordance and the deep phylogeographical division within the Lake Erie watershed.

Our data do not allow for the unequivocal resolution of the competing hypotheses to explain the deep phylogeographical division apparent within the Lake Erie watershed. We consider the hypothesis of secondary contact between two formerly isolated postglacial lineages to be improbable because of the geographical complexity of these hypotheses. In other cases where this hypothesis was accepted there was no evidence of one phylogroup nested between representatives of the other, as we see with *B. fowleri*. Within Lake Erie, we consider the hypothesis of stochastic postfounder hybridization and fixation to be the more parsimonious explanation for the deep phylogeographical structure we have documented here.

The only nuclear loci for which we have information are the allozyme data from Green (1984) where there was no significant difference between our mtDNA Phylogroups 2b and 1a. Indeed, according to Avise's (2000) $3x$ rule, we should not expect the alignment of nuclear and mitochondrial markers until there has been three times the amount of time necessary for reciprocal monophyly of mtDNA. This condition is hard to satisfy in a temperate area which has only been deglaciated for approximately 10 000 years. Nevertheless, Phylogroup 1 and 2 represent two distinct mtDNA lineages and show a high magnitude of sequence divergence between geographically isolated populations. We consider the likelihood of demographic connection between phylogroups on ecological timescales to be highly unlikely. Therefore, Phylogroups 1 and 2 are phylogenetically distinct and deserve high conservation priority (Vane-Wright *et al*. 1991). In a Canadian context, this is particularly true for Phylogroup 2 as it only occurs at one Canadian locality. The shallow phylogroup structure demonstrated within Phylogroup 1 should likely be used to describe management units for the conservation of this species within the Lake Erie watershed. Within Phylogroup 1a there may be enough interpopulation migration to allow site recolonization in the event of a catastrophic decline in abundance. However, Phylogroups 1b and 1c appear much more isolated. A local extinction here would likely be permanent, as we have seen at Point Pelee/Pelee Island (Green 1989). The similarity of the northern Ohio and Rondeau populations is likely due to a founder effect, and not the regular transport of many individuals via over 100 km of open lake water.

Bufus fowleri populations on the north shoreline of Lake Erie have a precarious existence. Our work indicates that their long-term viability is even less certain. The Long Point population is significantly different from the remainder of the Lake Erie populations close enough to provide immigrants that would forestall any local extinction. Toads at this peninsula are a unique lineage and protection reflecting the unique and distinct nature of this population is warranted.

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This study is part of the PhD research of M. A. Smith conducted under the supervision of D. Green at McGill University. M. A. Smith's PhD focused on the spatial ecology of *B. fowleri* at its northern range limit using a broad array of genetic, metapopulation modelling and mark-recapture field experiments. D. Green's research program uses the genetic variation in frogs and toads to decipher the relationships of species, the structure of populations, and mechanisms of evolutionary change.

Appendix 1

Tissue sample collection information

Site description	Latitude	Longitude	*n*	Museum accession number	GenBank accession numbers
Thoroughfare Beach at Long Point, Ontario	42.576184	080.374029	10		AY529737, AY529738, AY529743, AY529742, AY529762, AY529763, AY529764, AY529778, AY529780, AY529808,
Hastings Beach at Long Point, Ontario	42.577043	080.447500	9		AY529760, AY529761, AY529767, AY529766, AY529768, AY529779, AY529806, AY529809, AY529810
Big Creek at Long Point, Ontario	42.573738	080.536957	10	RM 4473–4478	AY529745, AY529746, AY529748, AY529747, AY529749, AY529797, AY529798, AY529799, AY529846, AY529864
Crown Marsh at Long Point, Ontario	42.582536	080.412721	9		AY529739, AY529740, AY529750, AY529744, AY529752, AY529758, AY529759, AY529777, AY529796
Anderson Property, south beach, Long Point tip, Ontario	42.540725	080.105915	10	RM 4479–4493	AY529753, AY529754, AY529756, AY529757, AY529770, AY529755, AY529801, AY529802, AY529803, AY529804
Rondeau Provincial Park, Ontario	42.260483	081.905983	10	RM 4496–4505	AY529775, AY529776, AY529795, AY529794, AY529807, AY529814, AY529815, AY529830, AY529831, AY529845
Windmill Point, Ontario	42.875900	078.999397	9		AY529773, AY529800, AY529818, AY529811, AY529825, AY529861, AY529862, AY529863, AY529771
Port Burwell, Ontario	42.587873	080.403630	3	NMC15771, NMC15777, NMC16914	
Rock Point, Ontario	42.841106	079.547845	1	NMC21953	
Point Pelee, Ontario	41.75726	082.63228	6	NMC543–4, NMC4981–2	AY529865, AY529865
Turkey Point, Ontario	42.67744	080.32874	1	ROM 5586–8	
Point Abino, Ontario	42.51415	079.5545	10		AY529751, AY529836, AY529838, AY529837, AY529839, AY529841, AY529842, AY529844, AY529848, AY529858
James N Allan Provincial Park, Ontario	42.841106	079.547845	14		AY529781, AY529783, AY529788, AY529787, AY529821, AY529849, AY529850, AY529851, AY529852, AY529853

Appendix 1 *Continued*

Site description	Latitude	Longitude	*n*	Museum accession number	GenBank accession numbers
Crystal Beach, Ontario	42.862167	079.053353	6		AY529772, AY529774, AY529816, AY529805, AY529820, AY529854
Kraft Road, Ontario	42.879746	078.951236	10		AY529812, AY529813, AY529819, AY529817, AY529822, AY529823, AY529843, AY529847, AY529855
Indiana Dunes National Lakeshore, Indiana	41.618496	087.208496	11	RM 4468–72	AY529790, AY529792, AY529826, AY529793, AY529828, AY529829, AY529834, AY529835, AY529856, AY529857
Ross County, Ohio	39.3286	083.0598	1	SEM 2306	AF462519
Union County, Ohio	40.3050	083.3745	1	SEM 2307	AF462520
Leigh County, Pennsylvania	40.6144	075.5899	1	SEM 2283	AF462513
Ashtabula, Ohio	41.901303	080.809760	4	RM 4494–5	AY529789, AY529791, AY529833, AY529832
Presque Isle State Park, Pennsylvania	42.10171	080.6402	10		AY529741, AY529769, AY529784, AY529782, AY529785, AY529786, AY529824, AY529827, AY529840, AY529859
Bufo terrestris, Oklahoma	—	—	1	DMG 2305	
Bufo americanus, Ontario	—	—	10	ROM 21664	AF190229
Bufo americanus, Quebec	—	—	1	RM 2681	

RM, Redpath Museum; NMC, Canadian Museum of Nature; ROM, Royal Ontario Museum; SEM, Susan E Masta collection; DMG, David M Green collection.