

EFFECTIVE POPULATION SIZES AND TEMPORAL STABILITY OF GENETIC STRUCTURE IN *RANA PIPIENS*, THE NORTHERN LEOPARD FROG

ERIC A. HOFFMAN,^{1,2} FREDERICK W. SCHUELER,³ AND MICHAEL S. BLOUIN¹

¹3029 Cordley Hall, Oregon State University, Department of Zoology, Corvallis, Oregon 97331

³Bishops Mills Natural History Centre, RR#2 Bishops Mills, Ontario, Canada K0G 1T0

Abstract.—Although studies of population genetic structure are very common, whether genetic structure is stable over time has been assessed for very few taxa. The question of stability over time is particularly interesting for frogs because it is not clear to what extent frogs exist in dynamic metapopulations with frequent extinction and recolonization, or in stable patches at equilibrium between drift and gene flow. In this study we collected tissue samples from the same five populations of leopard frogs, *Rana pipiens*, over a 22–30 year time interval (11–15 generations). Genetic structure among the populations was very stable, suggesting that these populations were not undergoing frequent extinction and colonization. We also estimated the effective size of each population from the change in allele frequencies over time. There exist few estimates of effective size for frog populations, but the data available suggest that ranid frogs may have much larger ratios of effective size (N_e) to census size (N_c) than toads (bufonidae). Our results indicate that *R. pipiens* populations have effective sizes on the order of hundreds to at most a few thousand frogs, and N_e/N_c ratios in the range of 0.1–1.0. These estimates of N_e/N_c are consistent with those estimated for other *Rana* species. Finally, we compared the results of three temporal methods for estimating N_e . Moment and pseudolikelihood methods that assume a closed population gave the most similar point estimates, although the moment estimates were consistently two to four times larger. Wang and Whitlock's new method that jointly estimates N_e and the rate of immigration into a population (m) gave much smaller estimates of N_e and implausibly large estimates of m . This method requires knowing allele frequencies in the source of immigrants, but was thought to be insensitive to inexact estimates. In our case the method may have failed because we did not know the true source of immigrants for each population. The method may be more sensitive to choice of source frequencies than was previously appreciated, and so should be used with caution if the most likely source of immigrants cannot be identified clearly.

Key words.—Anuran, genetic structure, microsatellite, *Rana pipiens*, temporal stability, temporal variation, variance effective size.

Received July 15, 2004. Accepted September 9, 2004.

A fundamental goal of population genetics is to understand the relative importance of microevolutionary forces in determining the existing patterns of genetic variation within a species. Consequently, using molecular markers to estimate parameters such as the effective sizes of, and migration rates among, natural populations has become a major focus in the field of evolutionary biology. However, elucidation of past processes and the prediction of future patterns from molecular data requires that a state of equilibrium have developed between genetic drift and gene flow in the populations of interest. Demographic instability can disrupt that equilibrium (Whitlock 1992), rendering single “snapshot” estimates of population structure or population size inappropriate as descriptors of the species (e.g., Tessier and Bernatchez 1999). However, whether population genetic structure is stable over time has been assessed for very few taxa.

The other parameter that is rarely measured for natural populations is the effective population size. The effective population size, N_e , is one of the most important parameters in theoretical and applied population genetics. Yet more than 70 years after Wright (1931) formalized the concept, we still have few highly reliable estimates of N_e in non-laboratory populations, and most of these are for economically important fish or for large mammals (e.g. Wood 1987; Harris and Alendorff 1989; Waples 2002). Few studies have attempted to estimate N_e in frog populations. Most of these actually es-

timated single-season effective number of breeders, N_b , rather than effective population size *per se*. The two most compelling estimates are by Sjogren (1991) and Eastal (1985). From demographic data on a small population of pool frogs (*R. lessonae*; census size a few hundred) Sjogren (1991) estimated the ratio of effective to census sizes, N_e/N_c , to be approximately 0.11. This value is in line with less comprehensive estimates of single-season N_b from other ranid species (Merrell 1968; Berven and Grudzien 1990; Seppa and Laurila 1999). Eastal (1985) measured allozyme allele frequencies in large populations of *Bufo marinus* (thousands of individuals) that had been deliberately introduced to five Hawaiian Islands and to Australia in the 1930's. On the assumption that all islands started out with the same initial allele frequencies, Eastal estimated N_e from current F_{ST} and the number of generations that had passed since colonization, and found the ratio of effective to census sizes, $N_e/N_c \approx 0.001$. This estimate is very low (Nunney 1992, 1996), but is similar to a single-season estimate of N_b/N_c for toad populations in Europe (Scribner et al. 1997). We do not know if these variable estimates represent a fundamental difference between bufonid toads and ranid frogs. We obviously need more estimates of N_e for anurans, and studies on the ecological/demographic factors that control N_e/N_c in this taxon.

One approach for estimating N_e that has yet to be applied to anurans is the temporal method. Here, change in allele frequencies is measured in a population that was sampled at two different times. Assuming that the observed change in allele frequencies was caused only by genetic drift, then estimation of the variance effective size over that time period

² Present address: School of Biology, Georgia Institute of Technology, 310 Ferst Drive, Atlanta, Georgia 30332; E-mail: eric.hoffman@biology.gatech.edu.

is straightforward. This approach, originally described by Krimbas and Tsakas (1971), has received considerable attention owing to the ease with which one can now collect molecular genetic data. The availability of DNA from archived historical samples has facilitated these estimates, and recent studies have successfully used historic samples to estimate long term N_e (Hansen et al. 2002 and references therein).

The temporal method was first applied using moment estimators (e.g., Nei and Tajima 1981; Waples 1989; Jorde and Ryman 1995). Likelihood methods have been developed recently (Williamson and Slatkin 1999; Anderson et al. 2000; Berthier et al. 2002), but these can be very computationally intensive. Wang (2001) developed a less computationally intensive pseudomaximum-likelihood method that performed as well as full maximum-likelihood techniques on simulated data. A key assumption of all these techniques is that systematic forces (selection, mutation, and migration) are assumed to be unimportant in changing allele frequencies compared to genetic drift. Wang and Whitlock (2003) developed a likelihood-based estimator that estimates N_e and m (immigration rate) jointly, an advance that removes the assumption that your target population is isolated from all migrational input between the two sampling periods. In this study we estimated N_e in each of five populations of northern leopard frogs, *Rana pipiens*, from New York and Ontario. We compared results from three implementations of the temporal method: Waples (1989) moment method, and the pseudolikelihood methods of Wang (2001) and of Wang and Whitlock (2003).

Because the five populations of *R. pipiens* that we studied were relatively close together, we were also able to investigate whether genetic structure among the populations changed substantially over time. Although there have been many studies of genetic structure in anurans, no study has yet tested whether genetic structure is stable over time in an anuran species. Indeed, only a handful of studies have tested for change in genetic structure over time in any species (e.g., Tessier and Bernatchez 1999; Heath et al. 2002; Hansen et al. 2002). This question is particularly important for frogs because it is not clear to what extent natural populations of frogs exist in dynamic metapopulations with frequent extinction and recolonization, or in stable patches at equilibrium between drift and gene flow (e.g., Berven and Grudzien 1990; Sjogren 1991, 1994; Pope et al. 2000). We would expect substantial change in genetic structure over time under the metapopulation model, but not under the equilibrium model.

In this study we used microsatellite loci to investigate spatial and temporal genetic variation in five populations of *R. pipiens* from five populations in New York and Ontario. First, we compared genetic structure between contemporary and historic samples that are separated by 11 to 15 generations. Second, we estimated the variance effective population size in each population under the assumption of no migrational input (Wang 2001; Waples 1989), and under the assumption that some migration might have occurred (Wang and Whitlock 2003). We discuss these results in terms of what they reveal about the different methods for estimating N_e , and of what they tell us about population genetic structure in anurans.

METHODS

Samples

The northern leopard frog is broadly distributed, occurring from the state of Washington to Nova Scotia, and from Arizona to Great Slave Lake, Northwest Territories, Canada, and Hudson Bay. In western (Hoffman and Blouin 2004a; Leonard et al. 1999) and northern (Seburn and Seburn 1997) areas the species has suffered widespread population declines, but in the upper Midwest and eastern United States and Canada, including the area we sampled, the species remains abundant. *Rana pipiens* breed in the first warm weeks of spring, and tadpoles metamorphose during the summer. Yearling and adult frogs overwinter in permanent or semi-permanent bodies of water and emerge in early spring after snow melt. *Rana pipiens* have well-defined home ranges, and adult frogs tend to remain in a relatively small area within any given year (Dole 1965).

Previous work based on mitochondrial DNA (mtDNA) sequence data identified two genetically distinct groups of *R. pipiens* (Hoffman and Blouin 2004b). All of the populations in this study occur within the "eastern" mtDNA region. We sampled from five sites around Lake Ontario (Fig. 1, Table 1). The greatest distance between any two sites was 387 km, whereas the shortest distance was 82 km. Forty or more dried skins were preserved from each site by F. Schueler in 1971 (NONQ, CAMPB, MONTZ, HAPVY) or in 1979 (FAIRM) (Schueler 1981, 1982). These samples were collected in the fall after reproduction and thus correspond to sampling plan 1 of Nei and Tajima (1981; Waples 1989). These skins were deposited in the Canadian Museum of Nature (CMN; Ottawa, Ontario; see Table 1). We clipped a 1 cm square piece of dried skin from each individual and placed it into a 1.5 ml tube for DNA extraction. We returned to the same five localities in the summer of 2001. Current samples were collected by dip net and consisted of toe clips preserved by desiccation in 1.5 ml tubes filled with drierite desiccant (W. A. Hammond Drierite Co., Xenia, OH). We obtained tissue from 24 to 54 individuals per historic sample, and 22 to 46 individuals per current sample, with an average of 39 frogs/population/time period (Table 2). Additionally, we returned to FAIRM during the week of 23–30 April 2004 and conducted a search of the wetland area to count the number of egg masses to obtain a rough estimate of the census size of breeding adults.

For molecular analysis total genomic DNA was extracted following a standard phenol/chloroform technique (Sambrook et al. 1989). Five microsatellite loci designed from *R. pipiens* (Rpi100, Rpi101, Rpi103, Rpi106, Rpi108) were used under the PCR conditions described in Hoffman et al. (2003), and two microsatellite loci originally developed for *R. pretiosa* (RP193, RP415) were used under the PCR conditions in Hoffman and Blouin (2004a). All PCR product was visualized on a 2% agarose gel for verification of amplification and then run on an ABI 3100 capillary electrophoresis system for size scoring.

One concern about using historic samples for PCR is that low yields of poor DNA may lead to allele drop out or amplification of contaminants rather than from the intended DNA template. However, our extractions from the dried tis-

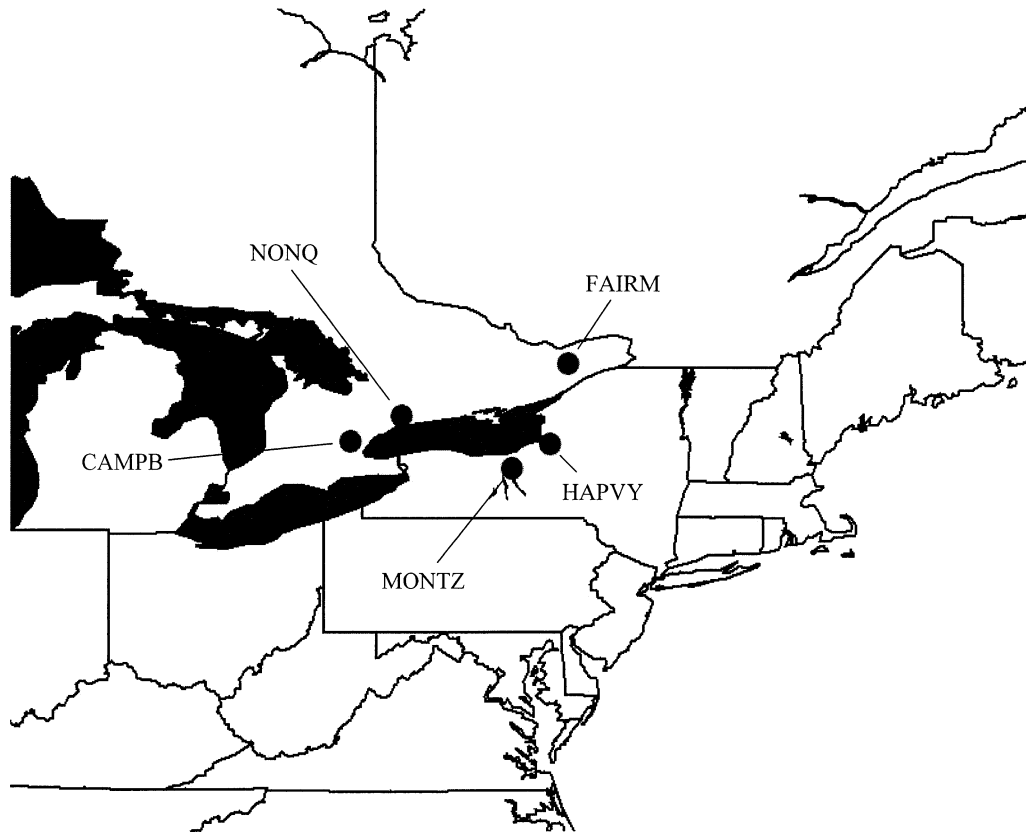


FIG. 1. Map of localities in New York and Ontario for which we obtained *Rana pipiens* DNA samples from two sampling periods 11–15 generations apart. See Table 1 for exact population location and date of sample collection.

sue produced high quality DNA. We also ran negative controls from both the extraction process and from PCR setup. These controls were run on the ABI 3100 gels along with our samples and consistently indicated that no spurious amplification occurred.

Statistical analyses

GENEPOP version 3.3 (Raymond and Rousset 1995) was used to estimate allele frequencies, observed numbers of alleles per locus, and expected and observed heterozygosities, and to test for Hardy-Weinberg equilibrium via exact tests (applying a sequential Bonferroni correction; Rice 1989). Sampling variances of expected heterozygosities were calculated following Nei (1987, pp. 180–181). For purposes of estimating genetic structure in each time interval, we included the 1979 sample from FAIRM with the other four historic

samples that were collected in 1971 (our conclusions are unchanged if we restrict our analysis to just the four samples from 1971). Genetic differentiation among all five populations within and between temporal periods, was estimated with θ (Weir and Cockerham 1984; calculated by the program F-STAT 2.9.1; Goudet 1995). To account for multiple comparisons, significance of these values was calculated at the 5% significance level using a Bonferroni correction for multiple comparisons. We tested for isolation by distance by computing the regression of $\theta/(1 - \theta)$ on geographic distance (Rousset 1997). For populations separated by Lake Ontario (see Fig. 1), we calculated distances around the lake. We assessed significance levels using a Mantel test (Mantel 1967) as implemented using the program ISOLDE in GENEPOP. Genetic distances among all populations were assessed using

TABLE 1. General data for populations used in this study (see Fig. 1) including location (map datum = WGS84), number of loci used to estimate N_e , and CMN catalog numbers of historic samples.

Pop ID	State/ Province	Latitude	Longitude	Museum Catalog Number	Number of loci used to estimate N_e
NONQ	Ont.	44.178 N	78.965 W	2456-1–2456-22; 24257-1–24257-22	7
CAMPB	Ont.	43.521 N	79.996 W	23890-1–23890-29; 22687-1; 22687-2	7
FAIRM	Ont.	45.069 N	75.653 W	24332-1–24332-39	7
HAPVY	NY	43.4679 N	76.01 W	23842-1–23842-15; 23851-1–23851-22; 23856-1–23856-17	6
MONTZ	NY	42.9893 N	76.7715 W	23876-1–23876-32; 23877-1–23877-10	6

TABLE 2. Summary statistics for seven loci including allele numbers (A), sample sizes (N), observed and expected heterozygosities (H_O and H_E) for both sampling dates at five populations of *R. pipiens*. Significant departures from Hardy-Weinberg equilibrium (after Bonferroni correction) are designated by $*$ ($P < 0.05$), marginally significant departures are designated by $*$ ($P = 0.05$).

Locus	A	NONQ		CAMPB		FAIRM		HAPVY		MONTZ	
		1971	2001	1971	2001	1979	2001	1971	2001	1971	2001
RP193	N	44	40	24	42	39	38	54	22	42	46
	A	11	11	8	10	12	12	13	10	14	15
	H_O	0.727	0.825	0.792	0.881	0.872	0.921	0.907	0.909	0.905	0.913
RP415	H_E	0.757	0.721	0.840	0.808	0.884	0.885	0.867	0.870	0.909	0.905
	N	43	40	22	42	39	38	54	22	42	46
	A	15	14	10	9	13	12	9	9	13	14
Rpi100	H_O	0.814	0.850	0.591	0.881	0.769	0.868	0.759	0.864	0.833	0.870
	H_E	0.888	0.921	0.867	0.884	0.895	0.894	0.849	0.866	0.885	0.866
	N	44	40	18	42	39	38	54	22	42	46
Rpi101	A	21	22	13	18	23	23	17	14	17	12
	H_O	1.00	0.975	0.944	0.905	0.949	0.974	0.889	0.955	0.857	0.783
	H_E	0.939	0.940	0.908	0.920	0.932	0.948	0.896	0.903	0.910	0.878
Rpi103	N	44	40	26	41	39	38	54	22	42	46
	A	16	14	13	15	11	11	16	9	13	17
	H_O	0.955	0.975	0.885	0.902	0.872	0.895	0.833	0.727	0.810	0.913
Rpi106	H_E	0.915	0.894	0.891	0.907	0.860	0.884	0.860	0.776	0.904	0.900
	N	44	40	25	41	39	38	54	22	42	46
	A	30	23	21	20	27	33	27	18	30	32
Rpi108	H_O	0.841	0.925	0.920	0.976	0.897	0.921	0.926*	0.909	0.881	0.870
	H_E	0.951	0.938	0.928	0.924	0.947	0.962	0.956	0.925	0.951	0.958
	N	44	40	14	42	39	38	54	21	37	46
Average across loci	A	26	29	16	35	23	23	33	20	29	29
	H_O	0.932	0.950	0.714	1.00	0.949	0.921	0.944	1.00	0.811*	0.957
	H_E	0.954	0.955	0.955	0.970	0.955	0.947	0.956	0.954	0.949	0.957
Average across loci	N	42	40	18	41	39	38	53	19	39	42
	A	23	22	13	20	12	11	18	8	17	23
	H_O	0.833	0.775	0.833	0.756	0.718	0.711	0.604**	0.579	0.538**	0.595
Average across loci	H_E	0.923	0.906	0.884	0.901	0.783	0.731	0.801	0.733	0.897	0.922
	H_E	0.904	0.896	0.896	0.902	0.894	0.893	0.884	0.861	0.915	0.912

TABLE 3. Above diagonal: θ values between pairs of contemporary samples collected in 2001. Below diagonal: θ values between pairs of historic samples. On diagonal (bold): θ values between pairs of populations across sampling periods. In all cases asterisks designate significance and statistics were calculated by *F*-STAT (Goudet 1995). Below the matrix are global θ values (95% confidence limits) for both sampling periods. Note that the contemporary and historic samples have overlapping 95% confidence limits.

Population	NONQ	CAMP	FAIRM	HAPVY	MONTZ
NONQ	0.0066*	0.0422*	0.0496*	0.0700*	0.0453*
CAMPB	0.0257*	0.0056	0.0464*	0.0616*	0.0245*
FAIRM	0.0411*	0.0487*	0.0000	0.0274*	0.0370*
HAPVY	0.0531*	0.0530*	0.0175*	0.0112*	0.0354*
MONTZ	0.0322*	0.0145*	0.0299*	0.0215*	0.0022
Contemporary samples	0.043 (0.022–0.050)				
Historic samples	0.034 (0.025–0.067)				

tools for population genetic analysis (TFPGA version 1.3; Miller (1997)) by building a UPGMA tree of Nei's unbiased distance (Nei 1978). Additionally, we used a Mantel test to determine whether matrices of genetic distance were similar between sampling periods.

We used three methods to obtain a temporal estimate of N_e in each population. The first two methods were the traditional moment estimator of Waples (1989; equations 9 and 12) and the pseudo-likelihood method of Wang (2001). Both methods assume that sample collection occurred according to plan 1 (Nei and Tajima 1981; Waples 1989) and that populations are closed to immigration. We almost certainly did not sample populations that are entirely closed to migration. However, if the effect of migration on allele frequencies is small compared to the effect of genetic drift, then migration should not greatly influence the estimate of N_e (Wang 2001). Rapid frogs tend to show isolation by distance, low gene flow, and geographically small genetic neighborhoods (e.g., Mosen and Blouin 2003, 2004), therefore we expect the effects of immigration to be minor. The third method jointly estimates N_e and the immigration rate, m , into the focal population during the sampling interval (Wang and Whitlock 2003). This approach requires data on temporal change in allele frequencies in the focal population and on allele frequencies in whatever source is sending migrants into the focal population and assumes that samples are collected according to plan 1 (Nei and Tajima 1981; Waples 1989). We do not know from what sites immigrants into each of our focal populations are most likely to originate. However, the Wang and Whitlock method was thought to be robust to violations of assumptions concerning this source population (Wang and Whitlock 2003). Therefore, to obtain the joint estimate of N_e and m for each population, we simply pooled allele frequencies from the four other populations (from both time periods) and used these to represent the allele frequencies in the source.

We used the program MNE 1.0 (Wang and Whitlock 2003) to estimate N_e via the methods of Wang and Whitlock (2003) and of Wang (2001). Both methods use a pseudolikelihood approach that is supposed to work nearly as well as full likelihood methods, but is less computationally intensive and is therefore ideal for highly polymorphic markers such as microsatellites (Wang 2001). Additionally, MNE requires the input of maximum N_e value allowed. We initially used 9000 (the maximum value for use with a standard computer). However, when we varied this value between 2000–9000 our es-

timates of N_e were unchanged. Waples' (1989) estimate was computed by hand. We used starting allele frequencies estimated from the historic samples, final allele frequencies estimated from the contemporary samples, and number of generations between sampling periods calculated using two years per generation (Ryan 1953; Leclair and Castanet 1987; Gilbert et al. 1994). Although it is known that average age of reproduction is geographically variable (Gilbert et al. 1994), two independent studies found average generation time for *R. pipiens* to be two years in the region of our populations (Leclair and Castanet 1987; Gilbert et al. 1994).

RESULTS

Genetic diversity within populations

After accounting for multiple comparisons, locus Rpi108 showed a homozygote excess in the historic samples from populations HAPVY₇₁ and MONTZ₇₁. No other locus by population combination was significantly out of Hardy-Weinberg equilibrium. These results suggest that there may be a low-frequency null allele at Rpi108 in some populations. All seven microsatellites showed high levels of variation in the populations sampled here. Allele frequencies for each locus in each population/sampling period are in the Appendix, available online only at: <http://dx.doi.org/10.1554/04-444.1.s1>. Numbers of alleles per locus ranged from 18 to 57 and H_E ranged from 0.721–0.970 (Table 2). These values did not change substantially between sampling periods (Table 2).

Genetic structure

Significant differentiation ($\theta > 0$) existed between all population pairs within each time period (see Table 3). Overall θ among populations within sampling periods was $\theta = 0.034$ (95% confidence limits from 0.022–0.050) for historic samples and $\theta = 0.043$ (95% confidence limits from 0.025–0.067) for contemporary (2001) samples. Global θ was not significantly different between the two sampling periods. There was a positive, significant correlation between genetic distance and geographic distance in both sampling periods (Fig. 2; Mantel test: historic, $P = 0.018$; 2001, $P = 0.017$ for probability (correlation > observed correlation)).

Pairwise genetic distances between populations were very similar across sampling times (Mantel test with 1000 permutations, $r = 0.94$, $P = 0.012$), indicating that the pattern of genetic differentiation among population did not change

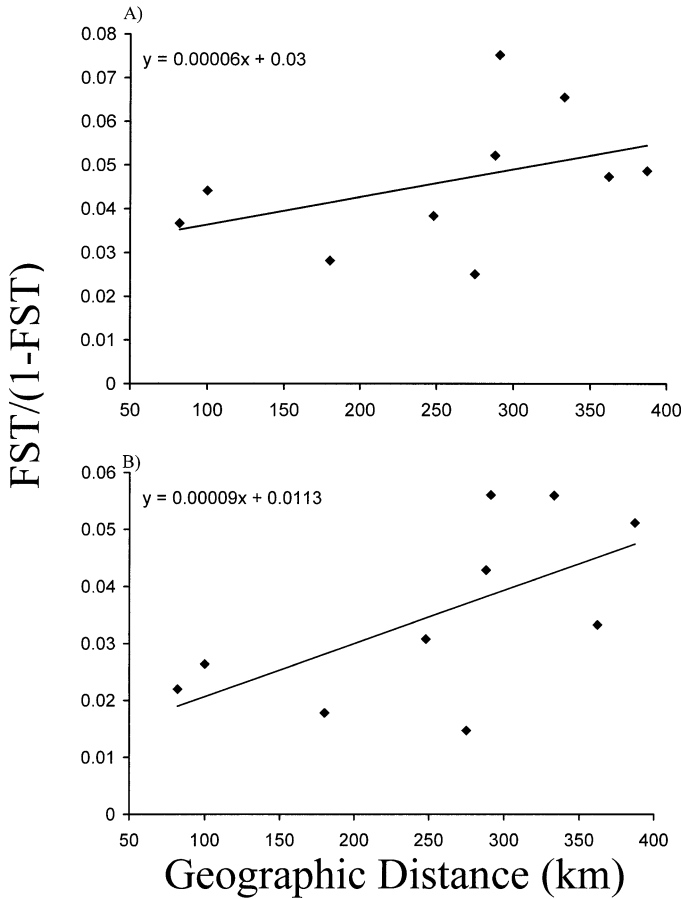


FIG. 2. Isolation by distance plots for pairwise comparisons from five *Rana pipiens* populations at two time intervals, contemporary (A) and historic (B).

over the 30 years separating collection periods. For example, the lowest pairwise θ in both sampling periods was between CAMPB and MONTZ (historic = 0.0145, contemporary = 0.0245) and the largest pairwise θ in both sampling periods was between NONQ and HAPVY (historic = 0.0531, contemporary = 0.0700). A UPGMA tree of Nei's (1978) unbiased genetic distance among sampling locations and time periods shows that for each population the most genetically similar population was the sample collected from the same location at the other sampling period (Fig. 3). Indeed, θ values between temporal samples from the same population are approximately an order of magnitude less than those values between different populations in the same time period (Table 3).

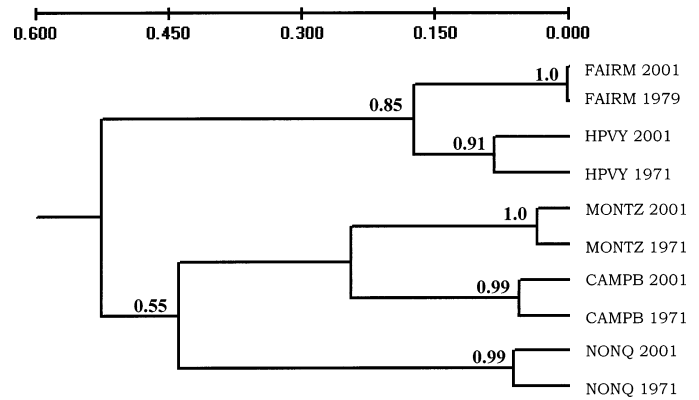


FIG. 3. UPGMA tree of Nei's (1978) unbiased distance. Nodal support was assessed from bootstrapping over loci (1000 permutations) and is given above the line for each node.

Effective size

We did not use Rpi108 to estimate N_e in HAPVY and MONTZ owing to departure from HWE at that locus in the historic samples (see Table 2). For all other populations, all seven loci were included in the N_e calculation.

The two pseudolikelihood-estimation methods produced strikingly different results from each other (Table 4). The method that jointly estimates N_e and m (Wang and Whitlock 2003) estimated N_e 's to be from 15 in HAPVY to 21 in NONQ, with immigration rates into these populations of 0.46 and 0.51, respectively. The pseudolikelihood-point estimates of m in the other three populations were 1.0 in FAIRM and MONTZ and 0.99 in CAMPB. In all populations the upper 95% confidence limit of m was 1.0. In contrast, the values of N_e estimated assuming isolated populations (Wang 2001) ranged from 102 for HAPVY to 469 for MONTZ. Likelihood surfaces in all populations exhibited similar topologies, with narrow but flat likelihood surfaces (Fig. 4). The moment estimates of N_e ranged from 410 for HAPVY, to 1820 for MONTZ, and were all approximately two to four times larger than the equivalent Wang (2001) estimate (Table 4).

During our surveys at FAIRM we found 82 egg masses over approximately one-half of the wetland area. If we double this number and assume an equal number of males and females, then we get a point estimate of the census number of breeders of 328. Although this is not a precise measure, it suggests that a reasonable guess at the census size of the FAIRM population is in the hundreds of frogs (i.e., it is certainly not in the many thousands).

TABLE 4. Effective sizes estimated by each method.

Population	Estimated N_e (95% confidence interval)			
	Waples (1989)	Wang (2001)	Wang and Whitlock (2003)	
			N_e	m
NONQ	588 (378–1355)	324 (230–488)	21 (17–26)	0.51 (0.3–1.0)
CAMPB	420 (245–837)	205 (150–295)	15 (13–18)	0.99 (0.57–1.0)
FAIRM	1019 (490–∞)	243 (165–395)	16 (14–19)	1.0 (0.6–1.0)
HAPVY	410 (222–940)	102 (71–152)	15 (13–17)	0.46 (0.25–1.0)
MONTZ	1820 (660–∞)	469 (313–786)	21 (18–24)	1.0 (0.56–1.0)

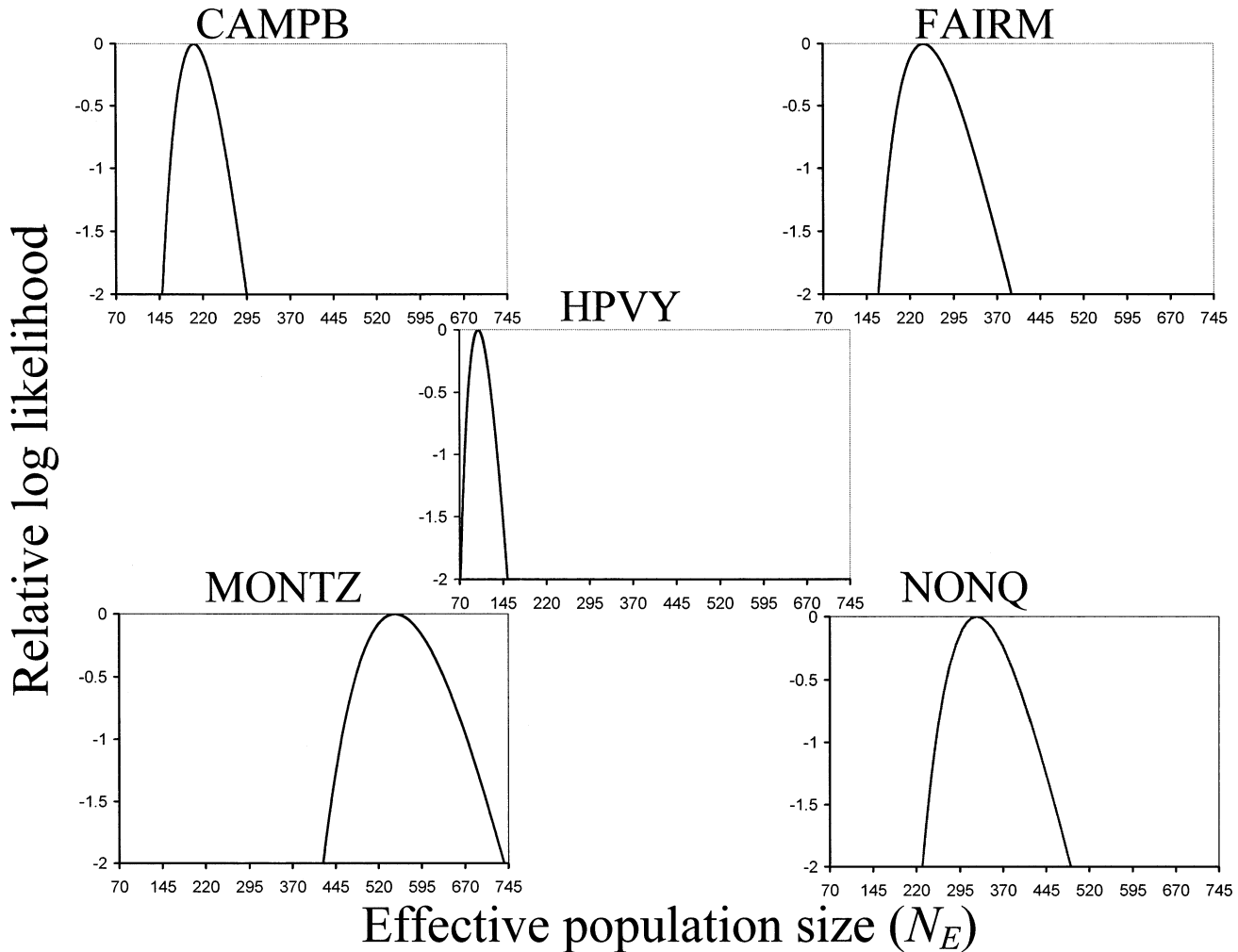


FIG. 4. Likelihood surfaces for estimates of effective population size according to Wang (2001) based on allele frequency change through time from microsatellite data. The 95% CI for the method implemented here can be calculated as the range of support associated with a drop of 2 in the log-likelihood (Wang 2001).

DISCUSSION

Genetic diversity

Within-population genetic variation (H_E) was high, averaging 0.86 to 0.92 per population (Table 5). Relatively few studies have investigated microsatellite variation in anurans, but those studies found relatively low levels of within-population diversity. Mean H_E ranged from 0.088–0.77 in studies reviewed by Newman and Squire (2001). More recently, mean H_E was calculated from microsatellite loci in *Hyla arborea* (0.52, Arens et al. 2000), *Rana lessonae* (0.57, Garner et al. 2000; Zeisset et al. 2000), *R. ridibunda* (0.59, Zeisset et al. 2000), and *R. temporaria* (0.76, Berlin et al. 2000). Moreover, a comparative review of (AC)_n dinucleotide microsatellite repeat variation among the five vertebrate classes found that amphibians had the lowest mean heterozygosity (Neff and Gross 2001).

Why might these populations of *R. pipiens* exhibit higher levels of H_E than other anuran species? Newman and Squire (2001) ascribe low H_e in some anurans to geographic and demographic features such as the colonization of glaciated

areas, and natural fluctuations in population size and local extinction followed by recolonization (attributed to pond breeding). These effects could cause natural founding and bottleneck events in anurans that may perturb the loci from a mutation-drift balance. Perhaps northeastern populations of *R. pipiens* are larger and/or more stable over time than populations of other anuran species that have been studied. Large, stable populations are consistent with the N_e estimates and stable genetic structure that we also observed in this study (see below).

Genetic structure

There is an acknowledged need to assess the accuracy and stability of population genetic structure estimates (e.g., Balloux and Lugan-Moulin 2002). Yet few studies have directly measured stability in spatial population genetic structure over time, and most of these studies were on salmonid fish (e.g., Hanson et al. 2002; Heath et al. 2002; Palm et al. 2003). Ours is the first study to test whether population genetic structure in an anuran was stable over time. Here *R. pipiens*

TABLE 5. Observed percentage change in heterozygosity between sampling times, and percentage change expected given N_e 's estimated by each of the three methods.

Population	Number of generations, t	Estimated H_E at time 0 (sampling variance)	Estimated H_E at time t (sampling variance)	Percentage change in H_E over time			
				Observed	Predicted from Waples' (1989) estimate of N_e	Predicted from Wang's (2001) estimate of N_e	Predicted from Wang and Whitlock (2003) estimate of N_e
NONQ	15	0.904 (.00016)	0.896 (.00042)	-0.9	-1.2	-2.3	-30.3
CAMPB	15	0.896 (.00056)	0.902 (.00025)	0.7	-1.8	-3.6	-39.9
FAIRM	11	0.894 (.00032)	0.893 (.00031)	-0.1	-0.5	-3.0	-29.5
HAPVY	15	0.884 (.00026)	0.861 (.0010)	-2.6	-1.8	-7.21	-40.0
MONTZ	15	0.915 (.00016)	0.912 (.00018)	-0.3	-0.4	-1.6	-30.3

showed temporally stable spatial genetic structure, at least over the 11–15 generations studied here. These data suggest that *R. pipiens* populations from this part of North America are not in a dynamic metapopulation with substantial extinction and recolonization occurring over a 22–30 year time frame. It appears that they more likely exhibit isolation by distance (Fig. 2) with restricted gene flow (Hoffman et al. 2004b), as is the case with most ranid frogs (Monsen and Blouin 2003, 2004).

Effective size

An interesting result of this study is that the estimates of N_e obtained by the three temporal methods were very different. The two methods that assume closed populations gave the most similar results, although estimates from Waples' method were consistently two to four times higher than estimates from Wang's method. The Wang and Whitlock method that jointly estimates m and N_e gave estimates that were an order of magnitude smaller. This leads to the question of which estimates more closely approximate the actual N_e 's within these populations. We doubt the results from the joint estimation method because the m estimates (some of which approached 1.0) are unrealistically high. There were significant allele frequency differences among populations and these changed little over time (e.g., Table 3). It seems unlikely that the populations experienced so much immigration without showing more appreciable allele frequency change. One possible explanation for the failure of the joint estimation method is incorrect source allele frequencies. The Wang and Whitlock (2003) model is based on a source-sink model with an infinitely large source population such that m (the immigration rate) unidirectionally influences the allele frequencies of the focal (sink) population. Because we did not know the true sources of potential immigrants to our populations, we simply used allele frequencies in the other four sites to represent the source. The joint estimation method may be more sensitive to choice of sources than was previously appreciated (Wang and Whitlock 2003). The two closed-population estimators suggest that N_e 's in these populations are in the range of a few hundred to fewer than 2000 individuals. Are these estimates reasonable given other information about the populations? We can begin by asking if the estimated N_e 's are compatible with the observed genetic diversities, with the expected changes in diversity over time, and with census sizes.

Expected loss of heterozygosity over time.—In a closed population we expect gene diversity (expected heterozygosities)

to decrease over time according to $(1 - 1/2N_e)^t$, where t is the number of generations. Four of five populations showed a decrease in heterozygosity over time, and the populations with smallest estimated N_e showed the largest decrease (Table 5). Interestingly, the observed changes in heterozygosities were close to those predicted by the N_e 's from Waples' estimator, while those predicted by the N_e 's from Wang's were too large (Table 4). These results suggest that the Waples' method estimates of N_e may be closer to the true values and that Wang's are slight underestimates. On the other hand, the moment estimates can be upwardly biased when based on loci having many rare alleles (Berthier et al. 2002; Wang, 2001). Also, any immigration would have slowed the loss of heterozygosity, making Wang's estimates of N_e appear to be underestimates. The change in heterozygosity predicted by the Wang and Whitlock estimates are grossly out of line with observed changes, again suggesting that this method produced substantial underestimates of true effective size.

Observed genetic diversities versus estimated N_e 's.—Average gene diversities in these populations ranged from 0.86 to 0.92 (Table 5). These diversities are higher than expected for completely closed populations having N_e 's in the hundreds. For example, under an infinite alleles model of mutation (Nei 1987, p. 375), and assuming mutation rates are between 10^{-3} and 10^{-4} (Goldstein and Schlotterer 1999), N_e 's of around 2200 to 22000 are required to maintain $H_E = 0.9$ in a closed population at drift-mutation equilibrium. Under a pure stepwise model of mutation (Nei 1987, p. 379), N_e 's of around 12,000 to 120,000 would be required. Microsatellite mutation is not purely stepwise, and it takes only a small proportion of large-step mutations to make allele frequency distributions resemble those of the infinite alleles model (Goldstein and Schlotterer 1999), therefore the most reasonable range is probably closer to that specified by the infinite alleles model. Nevertheless, even if mutation rates are close to 10^{-3} , closed populations in the thousands would still be required to maintain the observed heterozygosities over time. On the other hand, this result is not unique. In other studies in which short-term estimates of N_e were compared with heterozygosity-based (long-term) estimates, the long-term estimates were always much larger than the short-term estimates (Lehmann et al. 1998; Jorde and Ryman 1996; Balloux et al. 1998; Palm et al. 2003). This result probably reflects the fact that few populations are truly closed, and that it takes only a small amount of immigration to maintain genetic diversities over evolutionary time scales. So depending on the rate and mode of mutation, short-term N_e 's for *R.*

pipiens in the hundreds to low thousands could be quite compatible with the observed heterozygosities, provided there is occasional immigration. The estimates of N_e based on heterozygosity are probably best interpreted as estimates of the species-wide or metapopulation-wide N_e , not that of any local subpopulation.

Estimated N_e 's versus census sizes (N_c).—We do not have good estimates of census sizes for these populations. However, our egg mass counts at FAIRM suggested a census size of at least three to four hundred breeders at that site in 2004. Also, these populations appear to be typical *R. pipiens* populations, and mark-recapture data from similar sites give estimates of a few thousand total individuals (Merrell 1968; F. Schueler, pers. obs.). Thus, N_c 's in the range of hundreds to a few thousand are very likely, and are also consistent with our casual field observations at these and at similar sites. We can certainly be sure that N_e 's are *not* in the tens of thousands. Census sizes in the hundreds to a few thousand would imply that N_e/N_c ratios for these populations are somewhere in the range of 0.1–1.0. This range is consistent with typical values expected for wildlife populations (Nunney 1992). Thus, these data also suggest that our N_e estimates are not way out of line.

*N_e/N_c estimates in *R. pipiens* versus in other frog species*

We conclude that populations of *R. pipiens* in the New York/Ontario region have relatively stable populations, with N_e 's best estimated to be in the range of a few hundred to fewer than 2000 individuals. Our rough estimates of census size thus suggest that N_e/N_c ratios are at least 0.10, and probably higher. Thus, our estimates of N_e/N_c are much more in line with estimates from other ranid frogs than the tiny estimates obtained for toads (see Introduction). Unfortunately, there have been few attempts to estimate N_e in any frog species, and most of these were fairly crude. More data on effective size in other frog and toad species are needed before we can conclude what is "typical" for anurans, or if N_e/N_c ratios differ predictably among different groups of anurans.

General conclusions

Overall, this study illustrates three findings that contribute to our general understanding of population genetics and evolution. First, this study represents an example of a species in which population genetic structure is temporally stable (over the 11–15 generations studied here). These data are the first to address the open question of the temporal stability of population genetic structure in anurans. It is important that more studies be conducted to determine the extent to which these results are found in other species. Second, this study indicates that the effective size of typical *R. pipiens* populations is in the range of hundreds to a few thousand frogs, giving N_e/N_c ratios between 0.10 and 1.0. Such ratios may be typical for ranid frogs, but not necessarily all anurans. Finally, the results of this study indicate that a promising approach to jointly estimating N_e and m (Wang and Whitlock 2003) should be used with caution. Improper designation of migrational source allele frequencies may lead to estimates of N_e that are inaccurate by up to an order of magnitude.

ACKNOWLEDGMENTS

We would like to thank M. Whitlock and J. Wang, and F. Cook for discussions of our results. The historic collections were supported by National Research Council of Canada grant A5999 to J. D. Rising at the Department of Zoology of the University of Toronto, and by the Herpetology Section of the National Museum of Natural Sciences (now CMN) under the direction of F. Cook. Thanks also to K. Monsen, R. Waples, F. Bonhomme, and one anonymous reviewer for helpful comments on this manuscript, and to M. Steigerwald for assistance in sampling the CMN collections. This research was funded by U.S. Geological Survey contract no. 00HQAG0027 to MB and Washington Department of Fish and Wildlife grant no. 58300726 to EH.

LITERATURE CITED

- Anderson, E. C., E. G. Williamson, and E. A. Thompson. 2000. Monte Carlo evaluation of the likelihood of N_e from temporally spaced samples. *Genetics* 156:2109–2118.
- Arens, P., W. V. Westende, R. Bugter, M. J. M. Smulders, and B. Vosman. 2000. Microsatellite markers for the European tree frog *Hyla arborea*. *Mol. Ecol.* 9:1944–1946.
- Balloux, F., and N. Lugan-Moulin. 2002. The estimation of population differentiation with microsatellite markers. *Mol. Ecol.* 11:155–165.
- Balloux, F., J. Goudet, and N. Perrin. 1998. Breeding systems and genetic variance in the monogamous, semisocial shrew, *Crocidura russula*. *Evolution* 52:1230–1235.
- Berlin, S., J. Merila, and H. Ellegren. 2000. Isolation and characterization of polymorphic microsatellite loci in the common frog, *Rana temporaria*. *Mol. Ecol.* 9:1938–1939.
- Berthier, P., M. A. Beaumont, J. M. Cornuet, and G. Luikart. 2002. Likelihood-based estimation of the effective population size using temporal changes in allele frequencies: a genealogical approach. *Genetics* 160:741–751.
- Breven, K. A., and T. A. Grudzien. 1990. Dispersal in the wood frog (*Rana sylvatica*): implications for genetic population structure. *Evolution* 44:2047–2056.
- Dole, J. W. 1965. Summer movements of adult leopard frogs, *Rana pipiens* Schreber, in northern Michigan. *Ecology* 46:236–255.
- Eastale, S. 1985. The ecological genetics of introduced populations of the giant toad *Bufo marinus*. II. Effective population size. *Genetics* 110:107–122.
- Garner, T. W. J., B. Gautschi, S. Rothlisberger, and H-U. Reyer. 2000. A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. *Mol. Ecol.* 9:2173–2175.
- Gilbert, M., R. Leclair, and R. Fortin. 1994. Reproduction of the northern leopard frog (*Rana pipiens*) in floodplain habitat in the Richelieu River, P. Quebec, Canada. *J. Herpetol.* 28:465–470.
- Goldstein, D. B., and C. Schlotterer. 1999. Microsatellites: evolution and applications. Oxford Univ. Press, Oxford, U.K.
- Goudet, J. 1995. FSTAT. (Vers. 2.9.3.: a computer program to calculate F -statistics. *J. Hered.* 86:485–486.
- Hansen, M. M., D. E. Ruzzante, E. E. Nielsen, D. Bekkevold, and K. D. Mensberg. 2002. Long-term effective population sizes, temporal stability of genetic composition and potential for local adaptation in anadromous brown trout (*Salmo trutta*) populations. *Mol. Ecol.* 11:2523–2535.
- Harris, R. B., and F. W. Allendorf. 1989. Genetically effective population size of large mammals: an assessment of estimators. *Conserv. Biol.* 3:181–191.
- Heath, D. D., C. Busch, J. Kelly, and D. Y. Atagi. 2002. Temporal change in genetic structure and effective population size in steelhead trout (*Oncorhynchus mykiss*). *Mol. Ecol.* 11:197–214.
- Hoffman, E. A., and M. S. Blouin. 2004a. Historical data refute recent range contraction as cause of low genetic diversity in isolated frog populations. *Mol. Ecol.* 13:271–276.
- . 2004b. Evolutionary history of the northern leopard frog:

- Reconstruction of phylogeny, phylogeography, and historical changes in population demography from mitochondrial DNA. *Evolution* 58:145–159.
- Hoffman, E. A., W. R. Ardren, and M. S. Blouin. 2003. Nine polymorphic microsatellite loci in the northern leopard frog (*Rana pipiens*). *Mol. Ecol. Notes* 3:115–116.
- Jorde, P. E., and N. Ryman. 1995. Temporal allele frequency change and estimation of effective size in populations with overlapping generations. *Genetics* 139:1077–1090.
- . 1996. Demographic genetics of brown trout (*salmo trutta*) and estimation of effective population size from temporal change of allele frequencies. *Genetics* 143:1369–1381.
- Krimbas, C. B., and S. Tsakas. 1971. The genetics of *Dacus oleae*. V. Changes of esterase polymorphism in a natural population following insecticide control: selection or drift? *Evolution* 25:454–460.
- Leclair, R. Jr., and J. Castanet. 1987. A skeletochronological assessment of age and growth in the frog *Rana pipiens* Schreber (Amphibia, Anura) from southwestern Quebec. *Copeia* 1987:361–369.
- Lehmann, T., W. A. Hawley, H. Grebert, and F. H. Collins. 1998. The effective population size of *Anopheles gambiae* in Kenya: implications for population structure. *Mol. Biol. Evol.* 15:264–276.
- Leonard, W. P., K. R. McAllister, and R. C. Friesz. 1999. Survey and assessment of northern leopard frog (*Rana pipiens*) populations in Washington State. *Northwest. Nat.* 80:51–60.
- Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Res.* 27:209–220.
- Merrell, D. J. 1968. A comparison of the estimated size and the “effective size” of breeding populations of the leopard frog, *Rana pipiens*. *Evolution* 22:274–283.
- Miller, M. P. 1997. Tools for population genetic analysis (TFPGA) 1.3. A Windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author at <http://bioweb.usu.edu/mpmbio/tfpga.asp>.
- Monsen, K. J., and M. S. Blouin. 2003. Genetic structure in a montane ranid frog: restricted gene flow and nuclear-mitochondrial discordance. *Mol. Ecol.* 12:3275–3286.
- . 2004. Extreme isolation by distance in a montane frog *Rana cascadae*. *Conserv. Genet. In press.*
- Neff, B. D., and M. R. Gross. 2001. Microsatellite evolution in vertebrates: inference from AC dinucleotide repeats. *Evolution* 55:1717–1733.
- Nei, M. 1978. Estimates of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89:583–590.
- . 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York.
- Nei, M. and F. Tajima. 1981. Genetic drift and estimation of effective population size. *Genetics* 98:625–640.
- Newman, R., and T. Squire. 2001. Microsatellite variation and fine-scale population structure in the wood frog (*Rana sylvatica*). *Mol. Ecol.* 10:1087–1100.
- Nunney, L. 1992. Estimating the effective population size and its importance in conservation strategies. *Trans. Western Section Wildl. Soc.* 28:67–72.
- . 1996. The influence of variation in female fecundity on effective population size. *Biol. J. Linn. Soc.* 59:411–425.
- Palm, S., L. Laikre, P. E. Jorde, and N. Ryman. 2003. Effective population size and temporal genetic change in stream resident brown trout (*Salmo trutta*, L.). *Conserv. Genet.* 4:249–264.
- Pope, S. E., L. Fahrig, and H. G. Merriam. 2000. Landscape complementation and metapopulation effects on leopard frog populations. *Ecology* 81:2498–2508.
- Raymond, M., and F. Rousset F. 1995. GENEPOP (vers. 3.3): population genetics software for exact tests and ecumenicism. *J. Hered.* 86:248–249.
- Rice, W. R. 1989. Analyzing tables of statistical tests. *Evolution* 43:223–225.
- Rousset, F. 1997. Genetic differentiation and estimation of gene flow from *F*-statistics under isolation by distance. *Genetics* 145:1219–1228.
- Ryan, R. A. 1953. Growth rates of some ranids under natural conditions. *Copeia* 1953:73–80.
- Sambrook, J., F. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning, a laboratory manual*. Cold Springs Harbor Laboratory Press, Cold Springs Harbor, NY.
- Schueler, F. W. 1981. Preserving anuran skins by drying. *Herpetol. Rev.* 12:10–11.
- . 1982. Geographic variation in skin pigmentation and dermal glands in the northern leopard frog, *Rana pipiens*. *Publications in Zoology, National Museum of Natural Sciences, National Museums of Canada* 16:1–80.
- Scribner, K. T., J. W. Arntzen, and T. Burke. 1997. Effective number of breeding adults in *Bufo bufo* estimated from age-specific variation at minisatellite loci. *Mol. Ecol.* 6:701–712.
- Seburn, D. C., and Seburn, C. N. L. 1997. Northern leopard frog survey of northern Ontario: report on a declining amphibian. Unpubl. report to the Wildlife Assessment Unit of the Ontario Ministry of Natural Resources, Thunder Bay, Ontario, Canada.
- Seppa, P., and A. Laurila. 1999. Genetic structure of island populations of the anurans *Rana temporaria* and *Bufo bufo*. *Heredity* 82:309–317.
- Sjogren, P. 1991. Genetic-variation in relation to demography of peripheral pool frog populations (*Rana lessonae*). *Evol. Ecol.* 5:248–271.
- . 1994. Distribution and patterns within a northern metapopulation of the pool frog, *Rana lessonae*. *Ecology* 75:1357–1367.
- Tessier, N., and L. Bernatchez. 1999. Stability of population structure and genetic diversity across generations assessed by microsatellites among sympatric populations of landlocked Atlantic salmon (*Salmo salar* L.). *Mol. Ecol.* 8:169–179.
- Wang, J. 2001. A pseudo-likelihood method for estimating effective population size from temporally spaced samples. *Genet. Res.* 78:243–257.
- Wang, J., and M. C. Whitlock. 2003. Estimating effective population size and migration rates from genetic samples over space and time. *Genetics* 163:429–446.
- Waples, R. S. 1989. A generalized approach for estimating effective population size from temporal changes in allele frequency. *Genetics* 121:379–391.
- . 2002. Effective population size of fluctuating salmon populations. *Genetics* 161:783–791.
- Weir, B. S., and C. C. Cockerham. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* 38:1358–1370.
- Whitlock, M. C. 1992. Temporal fluctuations in demographic parameters and the genetic variance among populations. *Evolution* 46:608–615.
- Williamson, E. G., and M. Slatkin. 1999. Using maximum likelihood to estimate population size from temporal changes in allele frequencies. *Genetics* 152:755–761.
- Wood, J. W. 1987. The genetic demography of the Gainj of Papua New Guinea. 2. Determinants of effective population size. *Am. Nat.* 129:165–187.
- Wright, S. 1931. Evolution in Mendelian populations. *Genetics* 16:97–159.
- Zeisset, I., G. Rowe, and T. J. C. Beebe. 2000. Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae*. *Mol. Ecol.* 9:1173–1174.