Historical Analysis of Genetic Variation Reveals Low Effective Population Size in a Northern Pike (*Esox lucius*) Population

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ABSTRACT

Effective population size (N_e) of a natural fish population was estimated from temporal changes in allele frequencies at seven microsatellite loci. Use of a historical collection of fish scales made it possible to increase the precision of estimates by increasing the time interval between samples and to use an equation developed for discrete generations without correcting for demographic parameters. Estimates of N_e for the time intervals 1961–1977 and 1977–1993 were 35 and 72, respectively. For the entire interval, 1961–1993, the estimate of N_e was 48 when based on a weighted mean derived from the above two estimates or 125 when calculated from 1961 and 1993 samples only. Corresponding ratios of effective size to adult census size ranged from 0.03 to 0.14. An N_e of 48 over a 32-year period would imply that this population lost as much as 8% of its heterozygosity in that time. Results suggest the potential for using genetic methods based on microsatellite loci data to compare historical trends in N_e with population dynamic parameters. Such comparisons will help to evaluate the relationship between genetic diversity and long-term persistence of natural populations.

ALL finite populations undergo random genetic change, known as genetic drift. One of the most important consequences of this random change is that populations continuously lose genetic variation. An inverse relationship between population size and the rate of loss of genetic variation has long been established in population genetics theory. Loss of variation is not determined by the commonly measured census size; however, various demographic factors also play a role. Effective population size (N_e), a concept first developed by WRIGHT (1931, 1938), corrects for the influence of different demographic factors on genetic variation within a population.

In an ideal population N_e is equal to N; however, it is less than N in most real populations. Unequal sex ratio and nonrandom distribution of family size are the two factors that commonly reduce N_e below N. In addition, if N_e fluctuates over time, the appropriate value for all generations under consideration is the harmonic mean of N_e for each generation, the harmonic mean is then skewed toward the lowest values (HARTL and CLARK 1989). The result of all these demographic factors is that individuals of one generation do not contribute evenly to future generations and therefore only a limited amount of a population's genetic material is maintained. Effective size can be estimated if the above demographic information is known, but this is rarely the case, especially for natural populations.

The difficulty in estimating N_e directly from demographic data has led to the development of numerous methods for estimating it indirectly from molecular genetic data (WAPLES 1989). One such method, called the temporal method, is based on the logic that if N_e determines rates of change in genetic variation, then a measure of genetic change over time should allow N_e to be estimated. Despite the importance of N_e in determining genetic change, there has been little application of indirect genetic methods to natural populations. Most of the estimates using the temporal method in natural populations have been for species with short generation times, such as insects (NEI and TAJIMA 1981, based on data from KRIMBAS and TSAKAS 1971; BUTLIN and DAY 1989; TAYLOR *et al.* 1993) and plants (HUSBAND and BARRETT 1992); use of the temporal method to estimate N_e for fish populations has often focused on hatchery populations (*e.g.*, WAPLES 1990b).

The degree to which genetic diversity is important for sustainability of populations is a topic of considerable debate (DHONDT 1996). A fundamental assumption of conservation geneticists is that inbreeding and loss of genetic variability increase the risk of extinction. FRANKHAM (1995a) argued that there is ample evidence to support this assumption and that the genetic contribution to population declines has been underestimated. In contrast, CAUGHLEY (1994) suggested that even though this assumption has theoretical support, genetic impoverishment has rarely been shown to be a factor in the extinction of natural populations. A related issue for managers of exploited wild populations is the degree to which genetic diversity influences the long-term productivity of populations that are large enough that extinction is not an immediate threat.

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The debate over the role of genetic diversity remains unresolved, in large part, due to the difficulty in distinguishing genetic from nongenetic causes of population change. Making such a distinction requires some means of monitoring genetic characteristics of populations over time. For example, FRANKHAM (1995c) accounted for nongenetic factors and determined that the risk of extinction increased at a threshold level of inbreeding in laboratory populations of Drosophila and mice. More studies are needed to clarify the relationship between genetic diversity and the dynamics of natural populations.

This article presents the results of a study of historical genetic changes in a natural population of northern pike, *Esox lucius*. We examined genetic variation at three times over a period of 32 years by sampling fish scales from a historical collection. Variability was assessed from DNA extracted from the epithelial cells adhered to these unpreserved scales using PCR to amplify microsatellite DNA loci. Based on temporal changes in genetic variation, we estimated N_e for three time intervals. Our results show the potential for using genetic methods based on microsatellite data to compare trends in N_e, an indicator of genetic diversity, with population dynamic parameters in natural populations. Such comparisons will help to evaluate the importance of genetic diversity to the long-term persistence and productivity of natural populations and determine whether or not conclusions reached in studies of captive populations can be extended to populations in natural environments.

MATERIALS AND METHODS

Development of microsatellite markers: MILLER and KAPUSCINSKI (1996) reported the development of microsatellite markers from partial genomic libraries of northern pike DNA. We have since screened a second library using two modifications of our initial methods. First, we isolated 250–1000-bp DNA fragments from northern pike genomic DNA before inserting them into a vector. This step made it possible to avoid large inserts, which are difficult to sequence completely. Second, we screened clones with an AC · GT alternating copolymer (Pharmacia) rather than with an oligonucleotide. We labeled this copolymer with ^{32}P using a nick translation kit (Promega) and then proceeded with our original protocol. Use of the copolymer enhanced the signal given by positive clones because more than one radionucleotide could be incorporated into a single hybridizing strand.

Population study site: This study focused on the northern pike population of Lake Escanaba, Wisconsin. Lake Escanaba is a 119-hectare lake located within the Northern Highlands Fishery Research Area of northern Wisconsin. Northern pike were introduced into Lake Escanaba in the late 1930's and early 1940's. The source population of the stocked fish is uncertain and more than one population may have been used [S. NEWMAN, Wisconsin Department of Natural Resources (WDNR), Woodruff, WI, personal communication]. No stocking has taken place since that time. It has an inlet and outlet at high water but fish migration is thought to be unlikely (KEMPINGER *et al.* 1975; KEMPINGER and CARLINE 1978). The WDNR has operated a check station since 1946 to monitor a compulsory permit system for fishing on the lake. The WDNR has also monitored the status of the population by netting spawning fish each spring. Scales were removed from all fish taken in nets or registered by anglers, and ages were determined by WDNR personnel.

Collection of samples: We obtained samples from the historical scale collection maintained at the WDNR check station. Unpreserved scales from each individual had been stored separately in envelopes that recorded information about the individual (*e.g.*, date sampled, length, age). Samples from 3 years were chosen based on two criteria: (1) a large number of individuals were available, and (2) many years (several generations) separated the samples. To meet these criteria, we took most of the spring spawning samples from 1961 (86 individuals) and 1993 (72), and a random subset (110) from the anglers' harvest in 1977.

Analysis of genetic variation at microsatellite loci: We determined sample allele frequencies at all loci following the procedures of MILLER and KAPUSCINSKI (1996). Briefly, microsatellite loci were amplified using PCR with epithelial cells adhered to fish scales as the source of DNA. A single scale was boiled in 200 μl of a chelating resin (5% w/v Chelex, Sigma Chemical) and 10 μl of the solution were added to a PCR reaction mix containing 25 pmol of each PCR primer, 0.2 mM dNTPs, 1 unit Taq DNA polymerase and 1× PCR buffer (Promega). After amplification in a thermal cycler, 4–8 μl of the PCR product were electrophoresed on an 8% non-denaturing polyacrylamide gel and visualized with UV light after staining with ethidium bromide. We excluded loci from further use if they were monomorphic after an initial screening of 20 individuals. All polymorphic loci were scored for all sampled individuals. We tested for agreement with Hardy-Weinberg (H-W) expectations using chi-square statistics. Observed heterozygosities were calculated as the number of heterozygotes observed divided by the total number in a sample. Expected heterozygosities were determined from sample allele frequencies by assuming H-W frequencies for the genotypes.

Estimation of effective population size: To estimate N_e, we used the following equation,

$$\hat{N}_e = t/[2(\hat{F} - 1/\tilde{S} + (1/N))], \quad (1)$$

or the following,

$$\hat{N}_e = t/[2(\hat{F} - 1/\tilde{S})], \quad (2)$$

depending on the type of sampling that was done. For intervals beginning with 1961, Equation 1 was appropriate because sampling was nonlethal and therefore individuals had the opportunity to contribute to future generations (plan I, WAPLES 1989). The appropriate equation for the interval 1977–1993 was not so evident. The sample from 1977 was collected lethally so these fish no longer contributed offspring (*i.e.*, Equation 2 following plan II, WAPLES 1989). Many of these fish were of reproductive age, however, and would have spawned in earlier years. For the interval 1977–1993, we estimated N_e using both equations and compared results. In addition to estimating the standardized variance of allele frequency change, (F), we needed to estimate census population size (N) at time 0 and the number of generations between samples (t). Sample sizes (S) were the harmonic means of sample sizes at time 0 and t (WAPLES 1989).

Estimation of F: Several methods of computing $\hat{F}$ have been proposed. WAPLES (1989) found that the measures $\hat{F}_c$ (NEI and TAJIMA 1981) and $\hat{F}_k$ (POLLAK 1983) led to similar results. We also found that the two methods led to similar results. We therefore present results based on F_k only.

POLLAK's measure for one locus is

$$\hat{F}_k = \frac{1}{(L-1)} \sum_{i=1}^{L} \frac{(X_{0i} - X_{ti})^2}{(X_{0i} + X_{ti})/2}, \quad (3)$$

where L is the number of alleles and the X's are the frequencies of allele i ($i = 1, 2, \ldots L$) at sampling times 0 and t. For multiple loci, we computed weighted means of single-locus $\hat{F}$ values as $\hat{F}_k = \Sigma(L_j - 1)F_{kj}/\Sigma(L_j - 1)$, where the j's index the different loci (NEI and TAJIMA 1984).

Estimation of N: Estimates of the adult census size (N) were based on data collected by fisheries managers at Lake Escanaba. Unfortunately, the needed population estimates were not made in all years. KEMPINGER *et al.* (1975) and KEMPINGER and CARLINE (1978) reported population estimates for fish 56 cm (hereafter designated N^*) and for fish of all sizes susceptible to the sampling gear (N) based on mark-recapture methods for the years 1958–1972. The WDNR made population estimates of N^* for all years since 1980. They also recorded harvests (N') of fish in all years including 1964–1972, when a 56 cm minimum size limit was in effect (S. NEWMAN, personal communication). We indirectly estimated N for all years by two methods. First, we applied the average ratio of N:N^* from the years 1958–1972 to records of N^* from 1980–1993. We then took the average N and assigned this value to the years during which no population estimates were made, 1973–1979. Next, we applied the average ratio of N:N' from the years 1958–1963 to records of N' from 1973–1993. Average N was then assigned to the years the size limit was in effect, 1964–1972. We used the averages from these two methods as population estimates of N for the years 1973–1993.

Estimation of the number of generations in an interval: Generation length for populations with overlapping generations is equal to the mean age of parents (HILL 1979). We approximated this through a weighted mean age of spawners. The number of females in each age class was weighted by a value to account for their relative fecundity. Because fecundity increases regularly with growth in northern pike (SPANOVSKAYA and SOLONINOVA 1984), the youngest spawners were assigned a value of one and each older age class was given a weighting value equal to its proportional increase in size, as determined by mean weight. Male contribution was assumed equal across age classes; thus all males were weighted equally. We then took the average of these weighted values from the three sampling years. Finally, we divided the number of years separating samples by the estimate of generation length to determine the number of generations in an interval.

Determination of confidence intervals: The $1 - \alpha$ confidence limits for F are

$$(1 - \alpha) \text{ Confidence interval for } F = \frac{nF}{\chi^2\alpha/2[n]}, \frac{nF}{\chi^2 1 - \alpha/2[n]}, \quad (4)$$

where n is the number of degrees of freedom associated with $\hat{F}$ [$n = \Sigma$(number of alleles per locus $- 1$)] and $\chi^2\alpha/2[n]$ is the critical $\alpha/2$ chi-square value for n degrees of freedom (WAPLES 1989). The confidence limits obtained from Equation 4 were used in place of $\hat{F}$ in Equation 1 or 2 to determine the confidence interval for N_e.

RESULTS

Microsatellite isolation: In a first round of genomic library screening, we isolated a total of 14 positive clones from ~4000 colonies. From these clones we developed nine pairs of primers for PCR amplification experiments (MILLER and KAPUSCINSKI 1996). In screening a second library, we isolated nine positive clones from 990 colonies and developed six new pairs of PCR primers.

Because this second library was size-selected we were able to estimate the number of AC*n* microsatellites in the northern pike genome. The average size of an insert was 625 bp. Thus, we screened ~990 × 625 = 619 kb, or 0.024% of the northern pike's estimated 2600 Mbp genome (BEAMISH *et al.* 1971). By assuming that the partial library was representative of the entire genome, we estimated that the northern pike genome contains 38,000 AC*n* microsatellites, or one approximately every 69 kbp if they are evenly distributed throughout the genome. The rate of occurrence of AC*n* microsatellites was threefold higher in brown trout (*Salmo trutta*) (every 23 kbp; ESTOUP *et al.* 1993) and over ninefold higher in Atlantic cod (*Gadhus morhua*) (every 7 kbp; BROOKER *et al.* 1994).

Seven (47%) of the 15 microsatellite loci that we studied were polymorphic in the northern pike population of Lake Escanaba (designated as Elu, for *E. lucius,* followed by a locus identification number: 19, 37, 51, 76, 78, 87, and 276). Core sequences and sequences of the PCR primers for these polymorphic loci are reported in Table 1 (with three of these repeated from MILLER and KAPUSCINSKI 1996). Comparisons of polymorphic and monomorphic loci showed that their core sequences had similar characteristics (Table 2). Each group had similar numbers in all repeat classes, as categorized by WEBER (1990). Polymorphic loci had greater average numbers of repeats than monomorphic loci (28 *vs.* 25 for the total number of repeats and 26 *vs.* 20 for the longest continuous run of repeats), but their ranges overlapped substantially.

We tested segregation at polymorphic loci using a pedigreed population maintained at the University of Minnesota. All tests that we could conduct indicated that the loci were inherited in a Mendelian fashion (data not shown). Using chi-square tests, we found agreement between observed and expected genotypic ratios of 20 offspring of parents who were variable at the Elu37, Elu76, and Elu276 loci (for each, $P > 0.05$). Although these results were encouraging, it should be noted that our small population limited our ability to conduct powerful tests for deviations from Hardy-Weinberg equilibrium. We previously reported that low microsatellite variability in the pedigreed population prevented us from conducting segregation analysis at loci Elu78 and Elu87 (MILLER and KAPUSCINSKI 1996). The Elu19 locus from the second library screening was also monomorphic in the pedigreed population. Analyses of additional pedigreed northern pike, when possible, will be useful for confirming the Mendelian inheritance of these loci.

Genetic variation: We determined allele frequencies

TABLE 1

Characteristics of seven microsatellite loci found to be polymorphic in the Lake Escanaba northern pike population

Locus	Core sequence	PCR primer sequences (5′–3′)	Alleles (bp)
Elu19[a]	$(AC)_{23}Ag(AC)_3$ $AAT(AC)_3$	CATCATgAACATTCAgACgC gAgATgCTAATTCATCCACTg	155, 149, 147
Elu37[b]	$(AC)_{32}(AT)_5$	ggCTACTCCAgAACCTTCCC CAAATTTTATgACCAgCACC	150, 138
Elu51[c]	$(AC)_{16}$	gtgggCATTCAgCCgATATAgC CTgTCTCATTACTgCCTggCTC	125, 123
Elu76[a]	$(AC)_{17}$	ACCACATTCCACATCTgATgg AATCCCTTATTCTgACCCTgC	167, 165
Elu78[a]	$(AC)_{13}$	CTAgAgggggAAAACAAACC CACTgTCCATCATCACCCCTCTC	136, 132
Elu87[c]	$(AC)_{20}(N)_{14}$ $(AC)_4TT(AC)_5$	AgCACTgCCACACATgACgTg CCAgCTgCCTCAgATTgCTCCCC	161, 157, 153
Elu276[a]	$[(CT)_2(gTCT)_3]_4$ $(CT)_2(gTCT)_2$ $(CT)_2(gTCT)_4$	CTgTCACAgTTCAAAgATggC TCTTTAAACTgggggggAggAAAg	165, 149

[a] PCR conditions: annealing temperature = 58°, 30 cycles.
[b] Annealing temperature = 60°, 27 cycles.
[c] Annealing temperature = 63°, 25 cycles.

at the seven polymorphic loci for the three sampling dates (Table 3). Five of the seven loci had only two alleles each. The other two (Elu19 and Elu87) each had three alleles. We observed all of the alleles at each sampling date, *i.e.*, there were no new or lost alleles throughout the time period. The frequency of the most common allele at a locus ranged from 0.45 to 0.99, with most values (14 of 20) > 0.70. Alleles at the Elu51 locus could not be resolved in the samples from 1961 because of the poor quality of the PCR products. The bands on the acrylamide gels were often blurred, which made the similarly sized products (2 bp difference) difficult to distinguish. After attempting to score ~40 samples, of which the majority were blurred, we decided to discontinue use of Elu51 for this year's samples. Alleles that are difficult to resolve are not uncommon with microsatellites. Our findings suggest that sample age, or presumably DNA quality, can affect the ability to resolve alleles in a locus-specific manner.

Genotypes at all loci were in agreement with Hardy-Weinberg (H-W) expectations except for Elu276 in 1961. The Elu 276 locus included a homozygous genotype with zero observations and an expected value of 6.4 in the 1961 sample; for such a situation, the chi-square test may be inappropriate. Therefore we used bootstrap resampling (EFRON and GONG 1983), which yielded $P = 0.001$. This locus was in agreement with H-W expectations on the other two sampling dates.

When averaged across all sampling years, observed heterozygote frequencies ranged from 0.11 to 0.62 per locus. When averaged across all polymorphic loci within a sampling year (excluding Elu51 because the 1961 data were missing), there were changes among years. Average observed heterozygosity decreased between 1961 (0.41) and 1977 (0.32), but it increased slightly from 1977 to 1993 (0.33). Comparable expected heterozygosities, based on allele frequencies, were 0.35, 0.30, and 0.32.

Sample allele frequencies changed between years at all loci. We determined changes for three time intervals: 1961–1977, 1977–1993, and the entire interval, 1961–1993. Magnitudes of change varied greatly among alleles and often among time intervals for the same allele. In the intervals 1961–1977 and 1977–1993, we found

TABLE 2

Comparisons between polymorphic and monomorphic loci

Locus type	No. in repeat class			No. of repeats	
	Perfect	Imperfect	Compound	Total	Continuous
Poly	3	2	2	28 (13–56)	26 (13–56)
Mono	2	4	2	25 (15–44)	20 (6–44)

Repeat classes correspond to those described by WEBER (1990). Number of repeats is reported as the total number in the entire core sequence (total) and as the longest stretch of uninterrupted repeats (continuous), with range in parentheses.

TABLE 3

Observed allele frequencies and heterozygosities at seven microsatellite loci in the northern pike population of Lake Escanaba, Wisconsin

Locus	Allele (bp)	1961	1977	1993
n		86	110	72
Elu19	155	0.27	0.20	0.13
	149	0.24	0.25	0.24
	147	0.49	0.55	0.63
H. obs.		0.69	0.64	0.53
Elu37	150	0.85	0.87	0.86
	138	0.15	0.13	0.14
H. obs.		0.30	0.23	0.25
Elu51[a]	125		0.65	0.60
	123		0.35	0.40
H obs.			0.43	0.44
Elu76	167	0.85	0.99	0.96
	165	0.15	0.01	0.04
H obs.		0.24	0.03	0.08
Elu78	136	0.90	0.81	0.86
	132	0.10	0.19	0.14
H obs.		0.20	0.33	0.19
Elu87	161	0.10	0.08	0.06
	157	0.77	0.72	0.76
	153	0.13	0.20	0.18
H. obs.		0.45	0.45	0.40
Elu276	165	0.73	0.87	0.67
	149	0.27	0.13	0.33
H obs.		0.55	0.25	0.5
Average H[b]		0.41	0.33	0.34
Without Elu51		0.41	0.32	0.33

H obs., heterozygosities.

[a] We could not determine allele frequencies at locus Elu51 in 1961 because of difficulties in interpreting gels.

[b] Average heterozygosity does not include the nine loci that were monomorphic.

the greatest changes in the frequencies at the Elu276 locus (0.14 and 0.20, respectively), but an allele of the Elu19 locus had the greatest change from 1961–1993 (0.14). The smallest changes occurred in the interval 1961–1977 at a different allele of the Elu19 locus (0.01) and at this allele and those of Elu37 in the intervals 1977–1993 and 1961–1993 (0.01 for all). Many alleles changed frequency in one direction in the first time period but shifted back toward the earlier frequency during the second period. In fact, the average magnitude of change was greatest from 1961 to 1977, rather than over the longer interval of 1961–1993.

Effective population size: We made three estimates of effective population size based on temporal changes in allele frequencies observed in the first (1961–1977) and second (1977–1993) time intervals, and over the entire interval (1961–1993). We also estimated N_e for the entire interval based on the sum of $\hat{F}$'s for the first two intervals, weighted by the number of independent alleles contributing to each estimate. For this case, the sampling correction in the denominator includes the terms $1/S$ and $1/N$ (where appropriate) for both sampling periods. When the number of alleles is equal, this estimate is the harmonic mean of the two component interval estimates of N_e.

TABLE 4

Population estimates for northern pike in Lake Escanaba

Year	*n*	Year	*n*
1961[a]	250	1978	720
1962	1200	1979	680
1963	700	1980	420
1964	1100	1981	850
1965	1950	1982	690
1966	1900	1983	520
1967	1200	1984	220
1968	700	1985	100
1969	1200	1986	80
1970	2300	1987	110
1971	1800	1988	1170
1972	1500	1989	460
1973[b]	870	1990	330
1974	1110	1991	580
1975	780	1992	1210
1976	760	1993	2060
1977	770		

[a] Estimates for the year 1961–1972 are from mark-recapture studies (Kempinger *et al.* 1975, Kempinger and Carline 1978).

[b] Estimates for 1973–1993 were based indirectly on the number of fish harvested and estimates of fish greater than 56 cm.

$\hat{F}_k$: We estimated standardized variances of allele frequency change ($\hat{F}_k$) for each locus and each time interval. These values varied greatly, reflecting the variation in allele frequency changes. In the intervals 1961–1977 and 1961–1993, the Elu76 locus had the largest $\hat{F}$ values (0.237 and 0.127, respectively), but in the 1977–1993 interval, the Elu276 locus had the largest values (0.228). Locus Elu37 had the smallest values for all intervals (0.000–0.003). Weighted mean $\hat{F}_k$ was largest for the first interval (0.063), and almost equal for the second and the entire intervals (0.038 and 0.041).

N: Estimates of adult census size ranged from 80 to 2300 over the period 1961–1993 (Table 4). Estimates made by mark-recapture studies for the years 1961–1972 ranged from 250 to 2300 (KEMPINGER *et al.* 1975; KEMPINGER and CARLINE 1978). Estimates by our indirect method ranged from 80 to 2060. Census size fluctuated greatly with one relatively consistent series of low estimates in the mid 1980's.

t: The mean age of parents, estimated by the weighted mean age of spawners, averaged 4.0 years for the three sampling years. Annual values of weighted mean age were 4.4, 3.8, and 4.0 for 1961, 1977, and 1993, respectively (the average was taken before rounding off annual estimates). Samples were taken 16

TABLE 5

Estimated effective population size and 95% confidence intervals

Period	$\hat{F}_k$	t	S	N_0	$\hat{N}_e$ (95% CI) $\hat{F}$	$Ne:N$
1961–1977	0.063	4	97	250	35 (9–90)	0.03
1977–1993	0.038	4	88	767	72 (17–258)	0.11
1961–1993	0.041	8	79	250	125 (29–404)	0.14
61–77–93	0.099				48 (19–101)	0.05

We estimated N_e based on standardized variances of allele frequency change $\hat{F}_k$ (POLLAK 1983). t, time in generations; S, the harmonic mean of sample size; N_0, census size at time 0; and $N_e:N$, the ratio of effective size to the average adult census size over an entire interval. The estimate for the period 1961–1993 is from the samples taken in 1961 and 1993 while the estimate for 61–77–93 is based on the weighted sum $\hat{F}$ for the two inclusive periods. CI, confidence interval.

TABLE 6

Effect of removing a single locus from analysis and recalculating $\hat{N}_e$ based on remaining loci

Locus removed	1961–1977 $\hat{N}_e$	95% CI	1977–1993 $\hat{N}_e$	95% CI	1961–1993 $\hat{N}_e$	95% CI
None	35	9–90	72	17–258	125	29–404
19	27	5–75	61	12–229	159	26–745
37	31	7–80	62	13–217	106	22–345
51			65	14–233		
76	63	13–195	70	15–263	203	38–1070
78	36	8–96	67	14–246	112	23–374
87	29	5–79	55	11–194	104	18–366
276	42	9–115	516	53–∞	113	23–378

CI, confidence interval.

years apart, resulting in a t of 4.0 for the shorter sampling intervals and 8.0 for the entire interval, 1961–1993. By comparison, the unadjusted mean age of spawners was 3.7 years, which would result in an estimate of 4.3 generations for the 16 year intervals. Individual size may also affect the reproductive success of males. When fish of both sexes were weighted in the same manner, the estimated mean age was 4.2, resulting in a t of 3.8.

N_e: Estimates of N_e are shown in Table 5. The $\hat{N}_e$ value shown for 1977–1993 (72) included N in its estimate (*i.e.*, Equation 1 for sampling plan I). Excluding the correction for N (*i.e.*, Equation 2 for sampling plan II), the estimate was quite similar (78), indicating that despite the uncertainty about the appropriate sampling plan, the results would not be greatly biased using either equation 1 or 2. The accuracy of the indirect estimates of N also was not critical. When we replaced the estimates of N with the minimum and maximum estimates of N (N = 80 and 2300), resulting estimates of N_e were 31 and 38 for 1961–1977 (original estimate = 35), 52 and 75 for 1977–1993 (72), and 99 and 141 for 1961–1993 (125). For the interval 1961–1993, the estimate of N_e based on $\hat{F}$ for the entire interval was ~2.5 times larger than the estimate based on the weighted combination of $\hat{F}$'s from the two shorter time intervals (125 *vs.* 48).

To examine the impact of each locus on $\hat{N}_e$, we removed one locus at a time and recalculated estimates based on the remaining loci (Table 6). The only changes in $\hat{N}_e$ > 25% were for the Elu76 and Elu276 loci. Removal of Elu76 led to increases of 80% and 60% in $\hat{N}_e$ for the first and the entire intervals, respectively. When we removed Elu276, the estimate for 1977–1993 increased over seven times to 516, and the upper bound of the confidence interval increased to infinity. Note, however, that this large impact did not occur when this locus was out of H-W equilibrium. There were only small changes (20% and 9%) in $\hat{N}_e$ for the intervals involving 1961, the year at which this locus was out of H-W equilibrium.

N_e/N: The ratios of effective size to adult census size ranged from 0.03 to 0.14 (Table 5). These values are based on $\hat{N}_e$ for an interval divided by the average population size of adults for all years in that interval because N_e estimates are harmonic means for all generations between samples. The highest ratio was for the estimate based on the interval 1961–1993 using the first and last sample data only. The ratio for this period based on weighted of the two inclusive intervals was only 0.05. Average ratios for all three time intervals were therefore 0.09 or 0.06, depending on the method used to estimate N_e for the interval 1961–1993, or 0.08 if all four ratios are included in the average.

DISCUSSION

The use of fish scales has given us the rare opportunity to examine historical trends in genetic diversity within a natural population. We estimated effective population size, a predictor of a population's rate of loss of neutral genetic variation, from temporal changes in allele frequencies at microsatellite loci. Use of a historical tissue collection provided two benefits: (1) it increased the precision of our estimates by allowing us to increase our sampling interval (WAPLES 1989) and (2) it let us apply an equation developed for discrete generations without correcting for demographic parameters (JORDE and RYMAN 1995). Our estimates indicated that the study population of northern pike has persisted at an N_e that was possibly as low as 48 over a 30 year period, which implies that it lost as much as 8% of its heterozygosity in that time. The relatively high levels of heterozygosity that we detected may be the remnants of high heterozygosity in the source population(s) used for stocking Lake Escanaba. This heterozygosity will, of course, continue to decline if the population remains closed. The importance of this loss to the sustainability of the population is uncertain. We suggest that the anal-

ysis of historical effective population size, made possible using procedures like ours, provides a means to compare population changes with rates of loss of genetic variation.

Certain fish species, especially freshwater types inhabiting lakes, may comprise a large part of the group of species for which this type of study is possible because of the ability to identify closed populations and the presence of historical tissue collections. Fisheries management agencies and research institutions have routinely collected fish scales as a means to age fish and examine growth characteristics. There may be no other taxa for which tissue from large numbers of individuals has been saved on a population basis. With the advent of PCR, however, tissue collection is often much easier, and wise sampling now (*e.g.*, bird feathers, hair samples; ELLEGREN 1992) may make long-term, PCR-based genetic studies possible in other species.

Advantages of historical sampling: For estimating effective population size by the temporal method, the ability to examine samples taken several generations apart provided us two important advantages. First, because of the larger number of generations between samples we were able to make estimates with reasonable precision (WAPLES 1991). Second, we could apply discrete generation formulas (Equations 1 and 2) without making demographic corrections as in JORDE and RYMAN (1995). With overlapping generations, JORDE and RYMAN showed that changes in allele frequencies between cohorts are a function of N_e and age-specific survival and death rates. Based on simulation results, however, they showed that N_e could be estimated directly from allele frequency changes with little bias when intervals exceeded one generation (Table 1, JORDE and RYMAN 1995).

Possible bias: An important source of potential bias is the estimate of generation length. Because generation length, t, appears alone in the numerator of Equations 4 and 5, bias in an estimate of N_e is directly proportional to the error in estimating t. For estimates of t, we weighted age frequencies of spawners by a measure of relative fecundity and then used the average from the three sampling dates. The weights certainly had some error associated with them. As shown in RESULTS, estimates of t would have been 4.3 if we did not weight ages, and 3.8 if we weighted all fish rather that just females. It is also likely that the average age of parents fluctuated among generations. Values for the three years we sampled ranged from 3.8 to 4.4. It is unlikely, however, that bias arising from estimates of t would significantly alter our conclusion that both N_e and N_e:N were low in this population. The greatest change resulting from the comparison values above would be a 10% increase in $\hat{N}_e$.

Differing estimates for the interval 1961–1993: Estimates of $\hat{N}_e$ for the interval 1961–1993 differed by a factor of 2.5, depending on whether the estimate was based on allele frequency changes from years 1961 to 1993 or on the combined changes observed from 1961 to 1977 and 1977 to 1993. Although the estimates of N_e differed, their confidence intervals overlapped considerably, with the lower 95% limit of the higher estimate (29) being less than the lower estimate (48).

Use of the discrete generations model on a population with overlapping generations may have contributed to the difference in estimates. In simulations of populations with overlapping generations, JORDE and RYMAN (1995) found that the discrete generations model overestimated true N_e by ~50% when samples were taken one generation apart but by only 10% when the samples were three generations apart. Samples taken at longer intervals should produce estimates that more closely approach true N_e. Therefore, estimates based on an interval of eight generations should be less sensitive to population structure than estimates based on four generations.

Although the above argument favors the estimate based on the entire interval, 1961–1993, there is also support for the mean estimate of the shorter intervals. If the changes we observed at relatively few loci were by chance not typical of changes at all loci, then ignoring the intermediate sample resulted in loss of important information on the dynamics of allele frequency change. We may have been observing the real consequences of the lower estimated N_e over the short intervals and the stochastic drift of allele frequencies back toward their original values over the long interval.

The above considerations do not answer the question of which estimate to use for the entire time interval, but they do illustrate an important point. Even though the use of historical collections allows increased precision by increasing generation interval, the importance of examining a reasonable number of loci should not be overlooked. For some fish species (*e.g.*, several salmonids; MORRIS *et al.* 1996), the development of primers for many microsatellite loci does not make this a limiting factor. The expected magnitude of N_e is an important guide in determining the best study design. WAPLES (1989) has noted that the temporal method is best suited for the study of small populations. As N_e increases, the signal from sampling error quickly overwhelms that from drift. Therefore, if N_e is expected to be moderately large (several hundred), the number of loci, the number of generations between samples, and sample size should all be as large as possible. Researchers need to use caution in accepting lower numbers of loci and sample sizes in trade for easily increasing the generation interval by historical sampling.

Assumptions of the model: Assumptions of the temporal method are that the loci are selectively neutral, a closed population, no mutations during the sampling period, and random sampling of the population (WAPLES 1989). All assumptions were likely met in this study.

Neutral alleles: Microsatellite sequences generally appear to be selectively neutral. Using the Kolmogorov-Smirnov test, we tested the assumption of neutrality by comparing the distribution of $n\hat{F}/\hat{F}$ with the chi-square distribution with degrees of freedom equal to the number of loci (*e.g.,* HEDGECOCK *et al.* 1992). Finding a significant difference would suggest that drift alone could not explain the observed allele frequency changes. We found no significant differences for any of the three sampling intervals at the 0.05 level. The *P* value for 1977–1993 was close to significance (0.053) due to the large $\hat{F}$ found for the Elu276 locus. The Elu276 locus was also the only one found out of H-W equilibrium (in 1961). However, the only notable change in our results upon removing this locus (or any other) from the analysis was in the period, 1977–1993, when $\hat{N}_e$ increased to over 500. Additionally, the fact that this locus was in agreement with H-W expectations on the other two sampling dates suggests that there was no consistent force (*e.g.,* directional selection) acting to cause it to be out of H-W equilibrium. This does not rule out the possibility of some other form of selection acting on this locus. Selection of constant intensity has little effect on $\hat{F}$ if t/N_e is small (POLLAK 1983) but some forms of selection (*e.g.,* symmetric variable) bias N_e downward (MUELLER *et al.* 1985).

No mutation: Microsatellite loci may be subject to mutation rates at least as high as 10^{-3} per generation (WEBER and WONG 1993). While this rate is much higher than that for a typical allozyme locus, mutational change over the eight-generation interval examined was still unlikely. We never found an allele at a later date that was not detected in an earlier sample.

No migration: It was reasonable to assume that the Lake Escanaba population was closed. Although inlets and outlets can appear in periods of high water, migration of fish was thought to be unlikely (KEMPINGER *et al.* 1975; KEMPINGER and CARLINE 1978). There has been no official stocking of northern pike since the initial stocking efforts ended in 1941. It is unlikely that unrecorded stocking occurred because the lake is in a relatively remote region of northern Wisconsin on state-owned land and access is controlled at the WDNR check station.

Random sampling: Random sampling is always difficult to guarantee especially when sampling natural populations. Applying the temporal method to organisms with overlapping generations presents an additional difficulty in meeting this assumption: the sample must be random with respect to an entire generation. We believe that the sampling protocol approximately meets this assumption. The historical samples were collected by using fyke nets during spring spawning, which are routinely used to sample fish populations, and by angling. Age records indicate that a similar range of age classes, predominantly covering a 4-year (*i.e.,* one generation) span of 2–5-year-old fish, were sampled with both methods.

Comparable N_e:N ratios: Do our low estimates of N_e indicate a downward bias in our methods? In a review of published estimates of N_e for a wide variety of organisms, FRANKHAM (1995b) found that the average N_e:N ratio was 0.11 when only comprehensive estimates were considered. Comprehensive estimates were those that considered all factors that cause N_e to differ from N (*i.e.,* unequal sex ratio, nonrandom variance in family size, fluctuations in N_e). The most important of these factors was temporal fluctuations in population size. The temporal method produces a comprehensive estimate because it measures the consequence of all these factors, including population fluctuations because it estimates the harmonic mean N_e over the interval. Comprehensive estimates yielded N_e:N ratios that were as low as 10^{-6} for an oyster, *Crassostrea gigas,* (HEDGECOCK *et al.* 1992) and as high as 0.59 for a wombat, *Lasiorhinus krefftii* (A. C. TAYLOR, personal communication cited in FRANKHAM 1995b). One outlier estimate was 0.90 for a hatchery population of rainbow trout, *Oncorhynchus mykiss,* a population in which breeding structure was artificially manipulated in a way that increased N_e (BARTLEY *et al.* 1992). The lowest value for a fish population was 0.01 for chinook salmon, *Oncorhynchus tshawytscha,* and the only estimate for a natural fish population was 0.04, also for chinook salmon (BARTLEY *et al.* 1992). In our study, when confidence intervals are taken into consideration, estimates of N_e:N ranged from 0.01 to 0.09 for 1961–1977, from 0.03 to 0.37 for 1977–1993, and from 0.04 to 0.43 for the entire period 1961–1993. This range of values provides further evidence that low N_e:N ratios are common across varied taxa.

It should be noted that the combination of harmonic and arithmetic averaging methods in itself affects the estimated N_e:N ratio. Because a harmonic mean is skewed toward lower values, a ratio for a period of generations will tend to be lower than the mean of ratios for individual generations. We used the arithmetic mean census size, consistent with FRANKHAM (1995b), because it is more biologically intuitive. The impact of averaging, however, must be considered when comparisons are made among N_e/N ratios estimated over different time periods.

Genetic diversity and sustainability: The degree to which genetic diversity is important for sustainability of populations is a topic of considerable debate (DHONDT 1996). FRANKHAM (1995a) convincingly argued that loss of diversity increases extinction rates. There was a threshold relationship between the level of inbreeding and rate of extinction in an analysis of four captive populations (FRANKHAM 1995c). He found that the degree of inbreeding, rather than the rate, determined the threshold. Effective population size, then, would not by itself indicate risk of extinction in relation to a threshold. Smaller effective population sizes, however,

produce greater rates of increase in inbreeding and loss of variation and thus hasten the approach to a threshold level, if all populations have such a threshold.

It should be noted that estimates of N_e by the temporal method are not strictly applicable to predicting increases in inbreeding. Estimates produced by this method are called the variance effective size, $N_e(v)$, which determines the variance of the change in allele frequencies or the loss of heterozygosity. A second N_e, called the inbreeding effective size, $N_e(i)$, determines the rate of increase in inbreeding. These numbers will be the same in a population of constant size. When population size changes, they will differ because $N_e(i)$ is determined by the parents of a given generation, while $N_e(v)$ is determined by the offspring. Over time, however, the differences will be less important because $N_e(v)$ and $N_e(i)$ are related but have a generational lag as the offspring of one generation become the parents in the next (CROW and KIMURA 1970).

Use of PCR-based historical analyses of genetic variation will help us evaluate the importance of genetic diversity to the long-term persistence and productivity of natural populations. Analysis of historical effective population size provides a means to compare changes in population dynamic parameters (*e.g.*, population size and growth rate, harvested yield) with rates of loss of genetic variation. Much of the focus of conservation geneticists has been on the question of persistence, especially the persistence of small populations (CAUGHLEY 1994). Several approaches might be taken to address this question using historical analyses of N_e. Rapidly declining or even extinct populations may have historical samples available to allow analysis of whether or not a relationship between genetic variability and extinction exists for natural populations. Retrospective estimates could be used to determine if N_e was small for a long enough time that inbreeding had built up to a threshold level. Trends in demographic indicators of population sustainability (*e.g.*, number of mature adults, survival and reproductive rates) could be compared to trends in N_e. Finally, populations with low and high N_e could be compared for measures of genetic instability (*e.g.*, fluctuating asymmetry) that result from the detrimental effect of inbreeding on developmental processes (LEARY *et al.* 1985). This would be especially interesting if populations could be found that have similar N but largely different N_e's.

Similar approaches might be taken to address the relationship between genetic variability and measures of population productivity. From a conservation viewpoint, genetic concerns are seldom raised when populations are large (*i.e.*, hundreds to thousands) (*e.g.*, CAUGHLEY 1994). For at least two reasons, however, decreases in parameters of productivity upon the loss of genetic variation might occur in populations of relatively large size. First, there is empirical evidence for a positive relationship between heterozygosity and fitness (ALLENDORF and LEARY 1986), which suggests that fitness related traits may decline upon loss of variation at any population size. Second, our results support the findings of FRANKHAM (1995b) that the ratio of N_e:N is often quite low (0.15 or less), which indicates that populations are often more susceptible to loss of genetic variability than census records would indicate. To evaluate the relationship between genetic variability and population productivity, trends in N_e could be compared to a different set of population parameters (*e.g.*, production rates, or, especially in fisheries, yield and catch per unit effort) than those compared above.

For all such comparisons between genetic diversity and population dynamics a single generation estimate of N_e could be deceiving because genetic variation is determined by the history of the population. Because N_e over time is based on a harmonic rather than arithmetic mean, overall N_e will be skewed toward low values that may have occurred at some time in the past. By contrast, even if N_e is currently low, most heterozygosity is retained if N_e was high in the past and the population recovers quickly. Even where appropriate historical samples do not exist, the use of PCR-based techniques allows simplified sampling for genetic monitoring so that concurrent analyses could be used to test hypotheses about the relationship between genetic diversity and population dynamics.

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