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# Effective number of breeding adults in *Bufo bufo* estimated from age-specific variation at minisatellite loci

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**Abstract**

**Estimates of the effective number of breeding adults were derived for three semi-isolated populations of the common toad *Bufo bufo* based on temporal (i.e. adult-progeny) variance in allele frequency for three highly polymorphic minisatellite loci. Estimates of spatial variance in allele frequency among populations and of age-specific measures of genetic variability are also described. Each population was characterized by a low effective adult breeding number ($N_b$) based on a large age-specific variance in minisatellite allele frequency. Estimates of $N_b$ (range 21–46 for population means across three loci) were ≈ 55–230-fold lower than estimates of total adult census size. The implications of low effective breeding numbers for long-term maintenance of genetic variability and population viability are discussed relative to the species' reproductive ecology, current land-use practices, and present and historical habitat modification and loss. The utility of indirect measures of population parameters such as $N_b$ and $N_e$ based on time-series data of minisatellite allele frequencies is discussed relative to similar measures estimated from commonly used genetic markers such as protein allozymes.**

*Keywords*: age-specific variation, *Bufo*, effective population size, genetic variability, minisatellite loci

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## Introduction

There is a practical need in biological conservation for understanding the effects of demographic characteristics (e.g. population size, recruitment and mortality) on population genetic attributes. One parameter of particular importance is effective population size ($N_e$). Effective population size allows the estimation of the expected rate of loss of genetic variation, the rate of increase in inbreeding, and the strength of selection required to counter the effects of genetic drift (Crow & Denniston 1988). Unfortunately, estimates of $N_e$, the effective number of breeding adults ($N_b$), or other population parameters are difficult to obtain in many species due to complexities arising from their secretive habits, complex life histories, and varying dispersal abilities. Genetic and ecological methods have been employed to estimate $N_b$ and $N_e$ (see review by Nunney & Elam 1994). Due to the paucity of pertinent ecological data for many species, one genetic approach to estimating $N_e$ or $N_b$ which has received considerable attention involves the collection of population allele frequency data over generations or between successive cohorts, respectively (Nei & Tajima 1981; Pollack 1983; Waples 1989).

The utility of any genetic marker for population-level studies lies in the rate of evolutionary change within the particular segment of DNA. Many markers traditionally utilized for intraspecific genetic studies (e.g. allozymes or mitochondrial DNA) may not be appropriate to investigate phenomena within populations due to their relatively low levels of intrapopulation variation. This is particularly true for species which have experienced reductions in population size, or which have been subject to episodic extinction and recolonization. One set of markers which is currently receiving considerable attention is the highly polymorphic variable number of tandem repeat (VNTR) microsatellite

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(Bruford & Wayne 1993) and minisatellite (Bruford *et al*. 1992; Burke *et al*. 1996) loci.

Minisatellites are tandemly repeated (typically 1–20 kb) tracts of DNA which can exhibit extensive length variation due to differences in the number of copies of a typically 15–60 bp repeat. After the initial discovery of a large class of minisatellite DNA sequences in humans (Jeffreys *et al*. 1985a,b), these sequences were also shown to be widely dispersed in the genomes of other organisms (e.g. Jeffreys & Morton, 1987, Jeffreys *et al*. 1987; Burke & Bruford 1987; Bruford & Burke 1991). Minisatellite loci have been found to be highly variable and include the most heterozygous (occasionally $H > 98\%$) loci ever found (Wong *et al*. 1986, 1987). High rates of mutation (Jeffreys *et al*. 1988) and concomitant high levels of variation at specific minisatellite loci suggest that these loci may be extremely sensitive to changes in population breeding size, and thus may provide a powerful tool for addressing microevolutionary issues at the population level.

One group of organisms where minisatellite loci may be of particular utility due to these species' intrinsically low levels of genetic variation in populations from northern latitudes (Rafinski & Arntzen 1987; Arntzen & Wallis 1991) is the amphibians which inhabit semi-isolated ponds in Great Britain. There is particular concern over the fate of many amphibian species, including the common toad *Bufo bufo* which breed in ponds which are distributed within highly fragmented agricultural landscapes. Within the last 50 years amphibians have declined drastically in numbers due to loss of breeding sites (Beebee 1975; 1996), largely due to an increase in the conversion of pasture to arable land (Jones 1971). Pond loss was estimated at 28% per decade in the Leicestershire region of central Great Britain between 1967 and 1982 (Swan 1986).

Our objectives were to estimate allele frequencies and heterozygosity for *B. bufo* adults and progeny from each of several ponds and to use the age-specific variance in allele frequency to estimate the effective number of breeding adults.

## Materials and methods

This study was conducted on three permanent or semi-permanent ponds in the Leicestershire region of central Great Britain. Pond sizes were estimated as 0.3 ha (Osgathorpe), 1.0 ha (Osbaston), and 1.5 ha (Charnwood). Ponds were characterized by areas of emergent vegetation and were each immediately surrounded by small areas of deciduous woodland. The ponds were all separated by ≈ 5–15.5 km of agricultural land consisting predominantly of arable and pasture fields.

### *Sample collections*

Adult toads ($N = 40$ per location) were captured during March and April 1992 by using drift fences which completely (Charnwood) or intermittently (Osbaston) surrounded the pond or spotlighted during intensive searches within or immediately adjacent to each pond. All adults and juveniles were killed after anaesthesia using tricane methanosulphonate (MS222) and were stored in 100% ethanol until analysed. Juveniles (50 each from Osgathorpe and Osbaston and 40 from Charnwood) were sampled as metamorphs from terrestrial habitats immediately adjoining the ponds and stored in 100% ethanol.

Samples will provide realistic estimates of $N_e$ or $N_b$ only if they are representative of the entire population. Due to the prolonged period of sampling (throughout the course of the breeding season), and the coverage of areas surrounding each pond, the sampled adults were assumed to be a random and representative cross-section of the entire breeding population. All adults were assumed to be in breeding condition as *Bufo* occupy terrestrial habitats exclusively as adults except during the breeding season. Juveniles were also assumed to constitute a random and representative segment of the progeny produced. Larval clusters break up prior to metamorphosis (Eibl-Eibesfeldt 1949). Samples were of metamorphs actively dispersing from the ponds in terrestrial habitat, and were taken over a period of several days from all areas immediately surrounding each pond.

Estimates of adult breeding population size (J. W. Arntzen and R. Oldham, unpublished data) were based on capture–recapture (Jolly 1965) of animals from the breeding pond (Osbaston), by direct-count censuses using spot-light searches of the pond and terrestrial habitats immediately adjacent during the breeding season (Osgathorpe), or using a Lincoln-index (Arntzen *et al*. 1995) based on captures of immigrating adults at the pond perimeter fence and of emigrating adults, also interrupted at the pond perimeter fence (Charnwood).

### *DNA extractions*

Approximately 0.25 g of muscle was excised from the hind leg of each adult for DNA extraction and analysis, while for juveniles one hind leg was removed and used. Samples from each individual were washed in distilled water and blotted dry to remove residual ethanol. Samples were digested overnight at 55 °C in 1.5-mL microfuge tubes containing 700 µL of extraction buffer [600 µL TNE (50 mM Tris, 100 mM NaCl, 25 mM EDTA, pH 7.5), 60 µL 1 M Tris HCl (pH 8.0), 10 µL 25% SDS, 25 µL proteinase K (20 mg/mL stock solution), and 5 µL of 1 M dithiothreitol (DTT)]. Samples were extracted twice using an equal volume of phenol/chloroform and once using chloroform/isoamyl alcohol. DNA was precipitated by adding 20 µL of 6 M NaCl and an equal volume of 100% isopropyl alcohol. The DNA was washed in 70% ethanol, dried and resuspended in 50 µL of TE (10 mM Tris, 1 mM

EDTA). The degree of degradation and DNA concentration was subsequently determined by electrophoresing 1 μL of each sample on 0.8% agarose gels along with lambda DNA of known concentration. No evidence of DNA degradation was noted for any samples.

Approximately 7 μg of DNA from each individual was completely digested overnight using the restriction enzyme *Mbo*I under conditions specified by the manufacturer (Boehringer Mannheim). Tests for complete digestion were conducted by running 5 μL of each sample on 0.8% agarose gels. Digested DNA samples were subsequently purified and reprecipitated by phenol/chloroform extraction followed by ethanol precipitation. Samples were resuspended in TE and DNA concentrations were determined by fluorometry. Five micrograms of digested DNA from each individual were used to run the minisatellite gels. To each DNA sample, 10 ng of an internal lane marker [8 ng of 1 kb ladder (Gibco BRL) and 2 ng of *Xho*I-digested lambda] was also added to facilitate fragment size estimations (see below).

Large (20 × 30 cm) agarose gels (0.8%) were run in Tris-Borate (0.089 M Tris, 0.089 M borate, 2 mM EDTA, pH 8.8) tank and gel buffers. Lambda DNA restricted with *Hin*dIII was run in the outermost lane and run times were determined with reference to the size marker standards. Gels were run for ≈ 1800–2000 volt-hours and stained with ethidium bromide (0.5 μg/mL) to determine marker band position. Gels were run so that all fragments greater than 1.0 kb in size remained on the gels.

### *Blotting*

Basic capillary blotting techniques were used as described in detail in Bruford *et al*. (1992, protocol 2). Gels were pretreated using two 8-min washes in 0.25 M HCl followed by two 15-min washes in 0.5 M NaOH, 1 M NaCl, and one 15-min wash in 3 M NaCl, 1 M Tris-HCl, pH 7.4. 20 × SSC (3 M NaCl; 0.3 M sodium citrate, pH 7.0) was used as the transfer buffer. DNA was blotted on to nylon membranes (Amersham: Hybond-Nfp) for 3 h using Quickdraw (Sigma) blotting sheets. Membranes were air dried and fixed using previously calibrated exposure to UV irradiation at 312 nm for 3 min.

### *Characterization of minisatellite loci*

Details concerning the cloning, identification, and characterization of the three minisatellite loci are presented in Scribner *et al*. (1994). Patterns of inheritance at each locus were tested using two pairs of amplexed adults and their respective offspring ($N$ = 25 offspring from each adult pair). Genotypes of progeny were consistent with those of their respective parents for each of the three loci. Loci were assumed to be unlinked.

### *Probing*

DNA inserts containing each of the *Bufo bufo* single-locus minisatellite sequences were isolated from low-melting-point agarose gels after digestion of recombinant charomid vectors with *Sau*3A1. Inserts were labelled with [$\alpha^{32}$P]-dCTP using standard oligo-labelling protocols (Bruford *et al*. 1992, protocol 3). Membranes were initially moistened in 3 × SSC and placed in prehybridization buffer [0.25 M Na-phosphate buffer; 1 mM EDTA; 7% SDS; 1% BSA (Sigma type V)] and incubated at 65 °C for 1–3 h. The labelled probe was then added directly to the buffer and the membrane was incubated overnight at 65 °C. Competitor DNA was added to the probing solution (for c*Bbu*MS3 only) as described in Bruford *et al*. (1992). Washing conditions varied greatly from locus to locus for the three *B. bufo* loci examined (for probe c*Bbu*MS1 – one wash for 10 min in 0.25 M Na-phosphate, 1% SDS followed by one 1-min wash in 2 × SSC, 0.1% SDS; for probe c*Bbu*MS2 – one wash for 10-min in 0.25 M Na-phosphate, 1% SDS followed by one wash for 10-min in 0.1 × SSC, 0.01% SDS; for probe c*Bbu*MS3 – one 10-min wash in 0.25 M Na-phosphate, 1% SDS followed by two 10-min washes in 0.1 × SSC, 0.01% SDS). All washing was conducted at 65 °C. After washing, filters were rinsed in 3 × SSC. Minisatellite fragments were revealed by autoradiography by placing filters in film cassettes for 2–3 days with one intensifying screen. Old probes were stripped using a 0.4 M NaOH wash at 45 °C for 15 min followed by a 45-min wash in 0.1 × SSC, 0.01% SDS at 45 °C. After single-locus probing was completed, filters were probed with labelled [$\alpha^{32}$P]-dCTP DNA from each of the two internal lane markers to reveal the marker size ladder in each lane to facilitate fragment (i.e. allele) size calculations (Scribner *et al*. 1994).

### *Allelic designations*

Allelic designations were based on fragment size (Scribner *et al*. 1994) and represent the midpoint of 'fixed' bins which were determined empirically on the basis of fragment mobilities relative to internal lane size standards. Fragments from each gel were initially grouped into 'fixed bins' of ≈ 100 bp in size (approximately one-tenth of the 1-kb fragment ladder interval). Forty-six individuals were arbitrarily selected and run on additional gels using the above conditions and a second size estimate was obtained for each individual's minisatellite fragments. Bin size was subsequently adjusted for loci *Bbu*MS2 and *Bbu*MS3. The mean difference between the first and second fragment size estimates plus twice the standard error (SE) were used as the final 'fixed' bin widths (mean ± SE for *Bbu*MS2 and *Bbu*MS3 were 65.1 ± 46.5 bp and 10.3 ± 13.9 bp, respectively; Scribner *et al*. 1994). Multiple fragment size

estimates focused on fragment size ranges of highest allelic diversity (e.g. 6200–8900 bp for *Bbu*MS2 and 3200–5100 bp for *Bbu*MS3). The wide distribution of fragment sizes for *Bbu*MS1 precluded the need for further characterization of this locus. Frequency histograms showing the distributions of fragment sizes for adults at each locus (across all three populations used in the present study) are shown in Scribner *et al*. (1994; Fig. 2).

### *Statistical analysis*

Allele frequencies, Wright's (1951) inbreeding coefficient (*F*), and estimates of genetic variability [number of alleles per locus and multilocus heterozygosity (both direct-count and Hardy–Weinberg expected)] for adults and juveniles were calculated for each of the three *Bufo* populations using the program BIOSYS (Swofford & Selander 1981). Estimates of the degree of spatial variation among populations ($F_{ST}$; assessed using adults only) and measures of gene diversity among individuals within each population ($F_{IS}$) are described in Scribner *et al*. (1994). Significance of *F* was tested as described by Li & Horvitz (1953). This test may be biased when observed and expected heterozygosities are estimated for loci with many alleles. However, tests may serve as a measure of major departures from random mating. Significance of allele frequency differences between adults and juveniles for each population was tested using *G*-tests (Sokal & Rohlf 1980). Nominal rejection levels for each locus were adjusted for multiple testing following Bonferroni procedures (e.g. Manly 1985).

Estimates of the effective number of breeding adults were calculated for each of the three *Bufo* populations based on the standardized variance ($F_c$, Nei & Tajima 1981) in allele frequency between adults and juveniles as described by Waples (1989; eqn 8). The model considers a diploid, randomly mating population of size *N*, from which samples of sizes $S_0$ and $S_t$ are drawn at two or more time intervals (0 and *t*, respectively). The population is assumed to be closed to migration, selection is assumed to be absent, and mutation over the time interval is considered unimportant. The adult–offspring sampling design and all subsequent calculations are based on Waples (1989; Plan I). In brief, the variance in adult–juvenile allele frequency can be estimated for each locus as:

$$F_c = \frac{1/K \,\Sigma\, (x_i - y_i)^2}{(x_i + y_i)/2 - x_i y_i} \qquad (1)$$

according to Nei & Tajima (1981), where $x_i$ and $y_i$ are the allele frequencies of the *i*th of *K* alleles for adults and juveniles, respectively. Effective breeding population size was subsequently calculated for each locus over $t = 1$ breeding cycle as:

$$N_b = \frac{t}{2[F_c - 1/(2S_0) - 1/(2S_t) + 1/N]} \qquad (2)$$

A mean $F_c$ across the three loci, weighted by the number of alleles at each locus, was taken as an estimate of the population mean $F_c$ and a mean $N_b$ was also estimated. Ninety-five per cent confidence intervals (CI) for $N_b$ were calculated as described by Waples (1989; eqn 16).

Age-specific variation in allele frequency could be particularly pronounced for loci with rare alleles. To examine the effect of rare alleles, estimates of $F_c$ (and $N_b$) were derived using both actual allele frequencies and by pooling all alleles present in frequencies < 5% in both adults and juveniles for a particular population.

For loci such as minisatellites, where allelic states are created by the binning of fragments of similar size, estimates of allele frequency could also be affected by scoring error. For each locus, differences in allele size greatly exceed empirically determined scoring error (comparisons of standard errors calculated as described above and allelic designation described in Table 1). However, several alleles at locus *Bbu*MS3 were of similar size (e.g. alleles 3251, 3305, 3356, 3403, and alleles 4924, 2964, and 5023; Table 1). As a test of the potential effect of scoring error in sizing fragments and of proper 'bin' assignment, the two groupings of alleles of the above sizes were combined for this locus and estimates of $F_c$ and $N_b$ were recalculated.

## Results

The minisatellite loci surveyed were highly variable. All loci were characterized by a large number of alleles per population (6–13; Table 1) of varying size (Fig. 1). Several alleles at each locus were present at moderately high frequency, while several rare alleles were consistently observed at frequencies < 5%. Levels of genetic variability were high for adults and juveniles (range $H_{DC}$ = 0.604–0.730; Table 1). Observed heterozygosities were consistently slightly lower than Hardy–Weinberg expected estimates, in the direction of heterozygote deficiencies. Differences between observed and expected estimates of heterozygosity are reflected in positive, although nonsignificant inbreeding coefficients (*F*; Table 1) and for adults, nonsignificant values of $F_{IS}$ (0.117 ± 0.089; $P > 0.05$ over the three loci; Scribner *et al.* 1994). Significant differences in allele frequency were observed among the three adult samples from these populations ($F_{ST}$ = 0.014 ± 0.004; Scribner *et al.* 1994). There was no significant variation between adults and juveniles in heterozygosity ($H_{DC}$) and number of alleles per locus (*A*; Table 1).

Considerable variation in allele frequency was observed between adults and juveniles for each of the three populations (Table 1). For the Osgathorpe population, seven alleles across the three loci showed frequency

**Table 1** Allele frequencies for three single-locus minisatellite loci and estimates of genetic variability for adult and juvenile (Juv) *Bufo bufo* samples from three ponds in Leicestershire, UK

| | | Population | | | | | |
|---|---|---|---|---|---|---|---|
| | | Osgathorpe | | Osbaston | | Charnwood | |
| Locus | allele[a] | Adult (40)[b] | Juv (50) | Adult (40) | Juv (50) | Adult (40) | Juv (40) |
| *Bbu*MS1 | 2203 | 0.128 | 0.170 | 0.115 | 0.184 | 0.103 | 0.026 |
| | 2575 | 0.000 | 0.000 | 0.038 | 0.000 | 0.000 | 0.000 |
| | 2645 | 0.064 | 0.100 | 0.154 | 0.102 | 0.128 | 0.231 |
| | 2934 | 0.013 | 0.000 | 0.013 | 0.041 | 0.000 | 0.000 |
| | 4072 | 0.269 | 0.350 | 0.218 | 0.276 | 0.256 | 0.385 |
| | 6100 | 0.000 | 0.000 | 0.000 | 0.020 | 0.000 | 0.000 |
| | 6842 | 0.295 | 0.210 | 0.244 | 0.245 | 0.192 | 0.141 |
| | 12210 | 0.179 | 0.160 | 0.218 | 0.133 | 0.308 | 0.218 |
| | 13370 | 0.051 | 0.010 | 0.000 | 0.000 | 0.013 | 0.000 |
| $\chi^2$ | | 7.40 (6)[c] | | 11.24 (7) | | 10.70 (5) | |
| *Bbu*MS2 | 3025 | 0.000 | 0.000 | 0.038 | 0.000 | 0.000 | 0.000 |
| | 3276 | 0.200 | 0.186 | 0.188 | 0.150 | 0.237 | 0.262 |
| | 3400 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.013 |
| | 3565 | 0.013 | 0.000 | 0.000 | 0.020 | 0.000 | 0.013 |
| | 3763 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.038 |
| | 6349 | 0.000 | 0.000 | 0.000 | 0.010 | 0.025 | 0.025 |
| | 6515 | 0.000 | 0.000 | 0.000 | 0.000 | 0.013 | 0.000 |
| | 6775 | 0.375 | 0.333 | 0.412 | 0.380 | 0.400 | 0.338 |
| | 6843 | 0.000 | 0.020 | 0.000 | 0.000 | 0.000 | 0.038 |
| | 6987 | 0.013 | 0.000 | 0.013 | 0.000 | 0.013 | 0.000 |
| | 7300 | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 7603 | 0.000 | 0.010 | 0.000 | 0.000 | 0.050 | 0.013 |
| | 7834 | 0.313 | 0.284 | 0.213 | 0.190 | 0.200 | 0.188 |
| | 8487 | 0.025 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 8966 | 0.050 | 0.147 | 0.138 | 0.130 | 0.050 | 0.050 |
| | 9376 | 0.000 | 0.000 | 0.000 | 0.070 | 0.013 | 0.025 |
| | 9565 | 0.000 | 0.000 | 0.000 | 0.050 | 0.000 | 0.000 |
| | 10480 | 0.013 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 |
| $\chi^2$ | | 12.70 (10) | | 17.63 (9)* | | 12.69 (12) | |
| *Bbu*MS3 | 2805 | 0.000 | 0.000 | 0.013 | 0.000 | 0.000 | 0.000 |
| | 3251 | 0.100 | 0.059 | 0.075 | 0.120 | 0.112 | 0.075 |
| | 3305 | 0.287 | 0.392 | 0.287 | 0.470 | 0.300 | 0.262 |
| | 3356 | 0.237 | 0.157 | 0.175 | 0.040 | 0.075 | 0.150 |
| | 3403 | 0.038 | 0.000 | 0.025 | 0.000 | 0.025 | 0.000 |
| | 3528 | 0.038 | 0.000 | 0.013 | 0.000 | 0.000 | 0.025 |
| | 4240 | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 4600 | 0.000 | 0.020 | 0.000 | 0.010 | 0.000 | 0.013 |
| | 4924 | 0.112 | 0.029 | 0.050 | 0.000 | 0.038 | 0.100 |
| | 4964 | 0.138 | 0.157 | 0.175 | 0.230 | 0.387 | 0.287 |
| | 5023 | 0.038 | 0.137 | 0.100 | 0.120 | 0.050 | 0.087 |
| | 5535 | 0.000 | 0.039 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 6757 | 0.000 | 0.000 | 0.000 | 0.000 | 0.013 | 0.000 |
| | 6942 | 0.013 | 0.000 | 0.013 | 0.000 | 0.000 | 0.000 |
| | 7076 | 0.000 | 0.000 | 0.038 | 0.010 | 0.000 | 0.000 |
| $\chi^2$ | | 27.92 (11)** | | 30.93 (11)** | | 13.08 (9) | |
| $H_{DC}$[d] | | 0.705 | 0.730 | 0.714 | 0.604 | 0.613 | 0.612 |
| $H_{HW}$[e] | | 0.783 | 0.772 | 0.800 | 0.764 | 0.760 | 0.779 |
| $A$[f] | | 8.00 | 7.67 | 8.33 | 7.33 | 7.67 | 8.00 |
| $F$[g] | | 0.088 | 0.045 | 0.096 | 0.201 | 0.183 | 0.204 |

[a] Allele size (bp) based on mobilities relative to internal lane molecular weight standards (see Methods for details).
[b] Sample size.
[c] Degrees of freedom for heterogeneity *G*-test.
[d] Direct-count observed heterozygosity.
[e] Hardy–Weinberg expected heterozygosity.
[f] Mean number of alleles per locus.
[g] Wright's (1951) inbreeding coefficient.
* $P < 0.05$; ** $P < 0.01$.

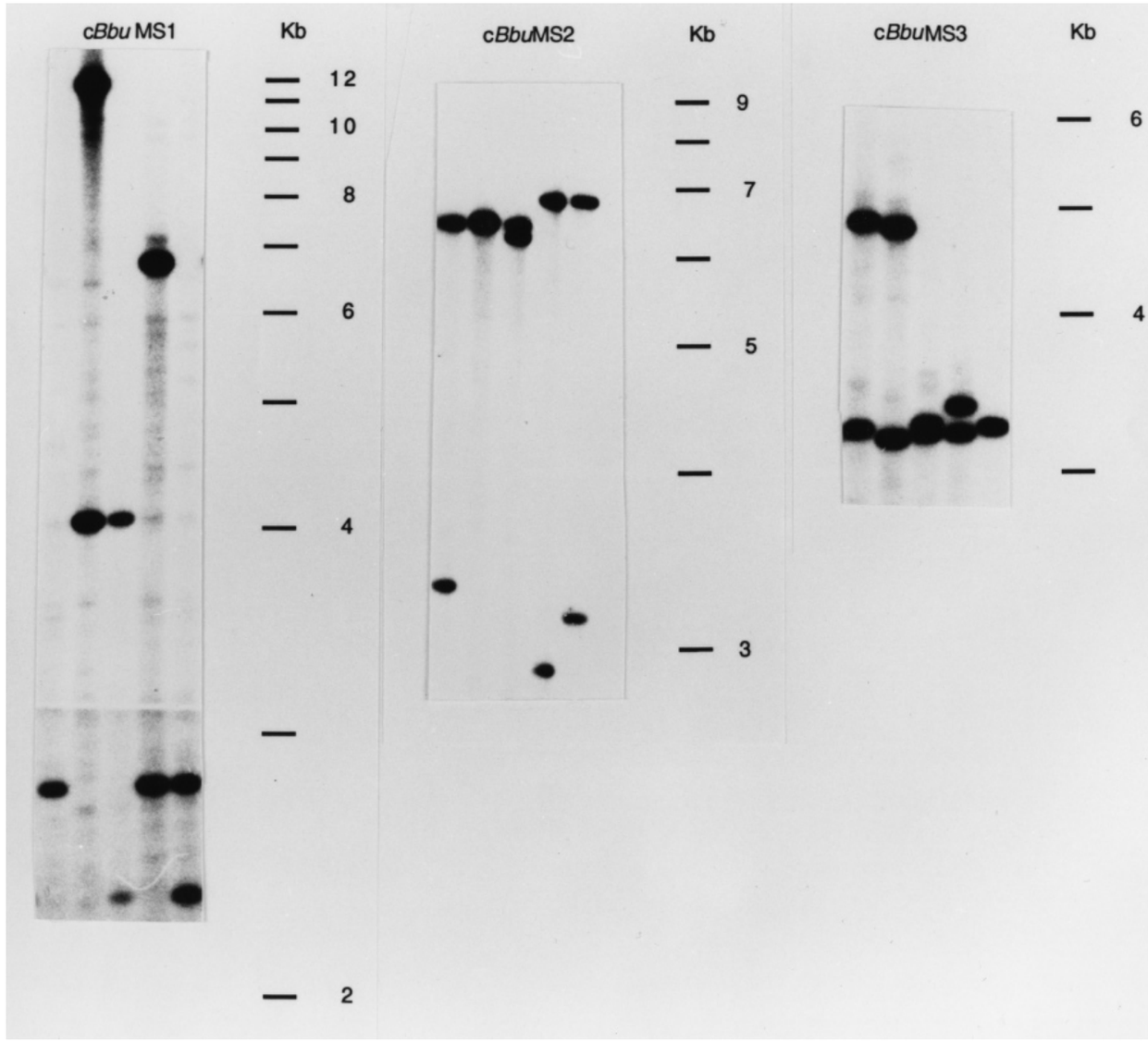


**Fig. 1** Genotypes for five *Bufo bufo* characterized using three single-locus minisatellite probes.

differences between adults and juveniles of 7–11%. Significant ($P < 0.05$) age-specific differences were observed at the *Bbu*MS3 locus. Thirteen alleles were observed in only one age class. In the Osbaston population, adult and juvenile allele frequencies differed by at least 7% for five alleles (including an 18.3% difference for the 3305 allele at the *Bbu*MS3 locus). Fourteen alleles were observed in only one age class. Significant ($P < 0.05$) age-specific variation was documented for the *Bbu*MS2 and *Bbu*MS3 loci. In the Charnwood population five alleles differed in frequency by > 7% between adults and juveniles, while 11 alleles were observed in only one age class. When Bonferroni procedures were used to adjust rejection levels, age-specific differences for the Osgathorpe and Osbaston populations at the locus *Bbu*MS3 remained statistically significant. The occurrence of many alleles in only one age class in each population suggests substantial drift in rare allele frequency.

Large age-specific variation in allele frequency for each of the three populations was reflected in low estimated effective breeding population size ($N_b$; Table 2). Effective breeding numbers for the Osgathorpe, Osbaston, and Charnwood populations were estimated to be 31, 21 and 46, respectively (means across three loci; Table 2). These estimates of $N_b$ were two orders of magnitude lower than the estimated total adult breeding population size (2500,

4000 and 4800 for Osgathorpe, Charnwood, and Osbaston, respectively; R. Oldham, unpublished data).

Rare alleles had little effect on estimates of effective population size (Table 2). Results, both in terms of $N_b$ estimates for specific loci and confidence intervals, were highly concordant in pooled and unpooled calculations. Pooling alleles of similar size for locus *Bbu*MS3 resulted in lower age-specific allelic variance and concomitantly in higher estimates of $N_b$. Estimates of $N_b$ changed from 16 to 35 for Osgathorpe and from 15 to 54 for Osbaston. For Charnwood, the sampling variance exceeded the estimates of age-specific variance in allele frequency.

## Discussion

Effective population size is one of the most important factors determining the rate of evolutionary processes at the population level. Knowledge of $N_e$ is particularly important for the formulation of conservation strategies for small populations as $N_e$ determines the rate of increase in inbreeding and of the loss of genetic variability in a population. Ecological and genetic approaches have been used to estimate effective population size [see Nunney & Elam (1994) and Waples (1991), respectively, for reviews]. Ecological methods are based on theory which relates particular ecological parameters (e.g. sex ratio, variance in life-time reproductive success, generation length) to changes in $N_e$. However, precise estimates of ecological parameters which are the basis of rigorous $N_e$ formulations necessitate extensive data which are often unavailable for natural populations. Due to the inherent difficulties in obtaining ecological data, a number of indirect approaches, including genetic methods based on temporal changes in allele frequency, have been used (see Waples 1989, 1991). Indirect estimates of effective population size which rely on temporal variance in allele frequency are based on the assumption that, for neutral markers sampled from closed, randomly mating populations of finite size, any variation in allele frequency over time is simply a function of the numbers of breeding individuals contributing gametes to the next generation and the variance due to sampling. Recent technical advances and the proliferation of new and highly polymorphic genetic loci (e.g. minisatellites and PCR-based microsatellites) may provide valuable data for the estimation of population parameters such as $N_e$ or $N_b$.

Regardless of the method employed, $N_e$ is estimated from variances (Nunney 1995). Larger variances translate into lower estimates of $N_e$. This dependence on variance, associated with (i) methodological issues related to assigning alleles to fragment size classes, (ii) sample sizes and the precision with which allele frequencies are estimated, and (iii) ecological factors which affect the transmission of gametes from one generation to the next, can effect estimates of $N_e$. Results from this study directly address each potential source of variance. Estimates of $N_b$ have revealed several interesting aspects of *B. bufo* ecology. From a technical perspective, we also address a number of issues related to the use of highly polymorphic minisatellite data in ecological genetic contexts, and of the assumptions underlying the use of genetic methods to estimate $N_b$ or $N_e$.

**Table 2** Estimates of temporal variance in minisatellite allele frequencies ($F_c$) and effective breeding population size ($N_k$) for three *Bufo bufo* populations in Leicestershire, UK. Estimates were derived with and without the pooling of rare (frequency < 0.05% in both adults and juveniles) alleles. $S_o$ and $S_t$ are the samples sizes for adults and juveniles, respectively

| | | | | | Rare alleles unpooled | | Rare alleles pooled | |
|---|---|---|---|---|---|---|---|---|
| Population | Locus | $n$[a] | $S_0$ | $S_t$ | $F_c$ | $N_k$[b] (95% CI) | $F_c$ | $N_k$ (95% CI) |
| Osgathorpe | *Bbu*MS1 | 7 | 39 | 50 | 0.0263 | 112 | 0.0263 | 112 |
| | *Bbu*MS2 | 11 | 40 | 50 | 0.0272 | 88 | 0.0284 | 73 |
| | *Bbu*MS3 | 12 | 40 | 50 | 0.0543 | 16 | 0.0487 | 19 |
| | All loci | 30 | | | 0.0378 | 31 (11–190) | 0.0348 | 38 (9–∞) |
| Osbaston | *Bbu*MS1 | 8 | 39 | 49 | 0.0343 | 41 | 0.0239 | 208 |
| | *Bbu*MS2 | 10 | 40 | 50 | 0.0421 | 25 | 0.0383 | 30 |
| | *Bbu*MS3 | 13 | 40 | 50 | 0.0555 | 15 | 0.0925 | 8 |
| | All loci | 31 | | | 0.0457 | 21 (9–63) | 0.0530 | 16 (5–53) |
| Charnwood | *Bbu*MS1 | 6 | 39 | 39 | 0.0546 | 17 | 0.0546 | 17 |
| | *Bbu*MS2 | 13 | 40 | 40 | 0.0255 | 333 | 0.0245 | 1000 |
| | *Bbu*MS3 | 10 | 40 | 40 | 0.0355 | 44 | 0.0291 | 98 |
| | All loci | 29 | | | 0.0350 | 46 (13–∞) | 0.0323 | 60 (12–∞) |

[a]Effective number of alleles.

[b]Estimates of $N_k$ (Waples 1989; eqn 12) and 95% confidence intervals (Waples 1989; eqn 16) were based on census breeding sizes of 1000 though only slightly different estimates were obtained using 10 000 as a breeding census size.

### *$N_b$ and estimates of genetic diversity within and among populations*

Estimates of genetic variability for juveniles (both in terms of heterozygosity and number of alleles per locus) were high, and in two of the three populations quite similar to levels documented in adults. Levels of heterozygosity were not significantly higher than expected given the large number of alleles (comparisons of direct-count and Hardy–Weinberg expected heterozygosities; Table 1). Estimates of $F_{IS}$ ($0.117 \pm 0.089$; $P > 0.05$; Scribner *et al*. 1994) for adults and values of $F$ from juveniles were not significantly different from zero, suggesting that it was unlikely that there were major departures from random mating within each population. These data are consistent with earlier findings based on multilocus fingerprinting which reported evidence of kin recognition and incest avoidance in another *Bufo* species (the congeneric American toad *Bufo americanus*; Waldman *et al*. 1992).

The estimates of $N_b$ were low (means across three loci = 21–46; Table 2). These results were somewhat unexpected given the relatively large estimates of adult census size ($N = 2500$, 4000 and 4800 for Osgathorpe, Charnwood and Osbaston, respectively; R. Oldham, unpublished data). These low estimates of effective breeding population size must be reconciled with the high heterozygosities. Juvenile heterozygosity was lower than that estimated for adults in the Osbaston population. However, the lack of age-specific differences in the other two populations, and the generally high levels of $H$ across all populations, suggests that either $N_b$ is not reflective of $N_e$ or that inbreeding and variance effective population sizes may differ considerably.

The large variance in allele frequency (Table 1) and disparity between census and effective breeding population size suggests a high degree of variance in reproductive success, as has been documented for this species elsewhere (Kuhn 1994). Unequal sex ratios and large male and female reproductive variance could dramatically reduce effective breeding numbers and effective population size (see Lande & Barrowclough 1987 for review). *Bufo* populations typically exhibit male biased operational sex ratios (typically two to five males per female; Gittins *et al*. 1980; Hemelaar 1988). Approximately 70% of breeding adults returning to Charnwood were males (J. W. Arntzen, unpublished data). Females are highly fecund, typically laying several thousand eggs at a time, but variance in reproductive success is high (Banks & Beebee 1986; Reading 1986), as are rates of mortality at the egg, larval, and metamorph stages. Merrell (1968) in a study of populations of leopard frogs *Rana pipiens* compared the number of egg-strings produced with adult census counts and estimated that across a series of breeding ponds, adult reproductive success varied greatly (1–67%).

Low $N_b$ estimates may also be the result of few males breeding with a disproportionately large proportion of the females. Males intercept females and amplexed pairs are formed either terrestrially immediately adjacent to the pond or in the pond itself. If there are preferred habitats for pair formation, and if dominant males occupy these sites, few other males may successfully reproduce (Davies & Halliday 1979; Loman & Madsen 1986; Høglund & Saterberg 1989).

Low $N_b$ could also result from restricted gene flow. *B. bufo* breed in widely separated ponds in intensively farmed agricultural landscapes. Increased pond loss and fragmentation of breeding sites could effectively decrease gene flow. Loss of dispersal corridors coupled with high propensity for natal homing (Berven & Grudzien 1990) and low dispersal rate (Reading *et al*. 1991) could effectively restrict genetic exchange and subject local populations to greater stochastic change (Merrell 1968; Halley *et al*. 1996). Adult allele frequencies differed significantly among populations ($F_{ST} = 0.014 \pm 0.005$; $P < 0.01$; Scribner *et al*. 1994) suggesting some degree of reproductive isolation. However, breeding pond density within this region is high. Analyses for a larger ($N = 20$) number of populations have revealed significant autocorrelation of minisatellite allele frequencies at inter-pond distances of < 2 km (J. W. Arntzen, unpublished data). While estimates of $N_b$ may reflect breeding conditions at each pond, all ponds are not independent. Population boundaries may therefore encompass several breeding locales.

### *Extrapolation of $N_e$ from $N_b$ and $N_e/N$ ratios*

Jorde & Ryman (1995) showed that for species with overlapping generations, temporal shifts in allele frequency alone are not sufficient for estimating $N_e$. An age-structured population does not constitute a homogeneous breeding group, but rather is composed of discrete cohorts, which may differ in allele frequency. The $N_e$ estimator advocated by these authors is identical to the discrete generation estimator used in the present study except that it contains the generation interval ($G$) and a correction factor ($C$) that is determined by the age-specific survival ($l_i$) and birth ($b_i$) rates of each population.

In temperate Atlantic climates *B. bufo* breed at ages 2–4 years (males) and 4–6 years (females; Hemelaar 1988; Kuhn 1994; Halley *et al*. 1996). Generation length can be approximated as 4 years. Data necessary for the estimation of ($C$) are not generally available for these populations. Given the number of overlapping generations which breed and that some gene flow occurs among breeding ponds in close proximity, $N_e$ would be expected to be higher that $N_b$. However, considering that males and females reproduce over few seasons, and that adult survival is generally low [< 5% for males and females at Charnwood; J. W. Arntzen, unpublished data; < 15% in four populations

studied by Kuhn (1994)], $N_b$ as estimated from one reproductive period may generally reflect population $N_e$. The available demographic and behavioural data (see above) suggest that estimates of $N_e$ would be substantially lower than the adult census size.

In a recent comprehensive survey of the wildlife literature, Frankham (1995) described fluctuating population size, variance in family size, and unequal sex ratios as important parameters related to $N_e/N$ ratios. Each of these parameters has been shown to be particularly variable in anurans. The ratio of effective to census size ($N_e/N$) would be expected to be within the range reported for other anurans (0.016–0.088, *Bufo marinus*, Easteal & Floyd 1986; 0.01–0.67, *Rana pipiens*, Merrell 1968; 0.44, *Rana sylvatica*, Berven & Grudzien 1990) and for wildlife populations in general (mean 0.10, Frankham 1995). The $N_b/N$ ratios estimated for the three *B. bufo* breeding ponds (0.007–0.012) were in the low range of $N_e/N$-values reported for other anurans and considerably less than $N_e/N$ ratios of most organisms cited in Frankham (1995). Differences between estimates of the present study and those of other organisms could be due to differences between $N_b$ and $N_e$ (as described above) and to the accuracy and precision with which adult *B. bufo* population sizes were estimated. Different techniques were used for each of the three breeding ponds. Only one population (Charnwood) was completely enclosed by a drift fence and some measure of precision of the population estimate was possible (4000 ± 1000; J. W. Arntzen, unpublished data). Breeding population sizes for all breeding ponds could be less than the point estimates.

Despite the inherent difficulties in relating estimates of $N_b$ to $N_e$ based on data from one reproductive cycle, this sampling strategy still provided more insight into effective population size than is possible with other studies. Low effective breeding numbers for species such as amphibians should be viewed with some degree of concern. Generation intervals are short. These species are highly philopatric and are directly tied to specific breeding habitats for reproduction. Aquatic habitats can be quite ephemeral, and during prolonged drought may be absent for considerable periods of time. Given the high rate of annual adult mortality, drift in allele frequencies over a single reproductive cycle could easily translate into substantial genetic change over few generations. This study has shown that the potential for such genetic change in populations is far greater that the surveyed population sizes might suggest.

### *Robustness of assumptions and utility of hypervariable single-locus minisatellites*

The use of indirect estimators such as time-series data of minisatellite allele frequency change require certain assumptions which warrant comment.

*Discrete generations.* Hill (1979) showed that $N_e$ estimates obtained using a discrete-generation model were robust if populations were demographically stable. Here we use adult-progeny data from a single reproductive cycle. While the adults are potentially represented by individuals from several earlier brood years, genetic data from adults and their offspring from a single mating event unambiguously capture the signature of the sampling process (i.e. drift) within a breeding pond. However, difficulties arise when converting estimates of the effective number of breeders to estimates of effective population size for species with overlapping generations (Waples 1991; Jorde & Ryman 1995; see above). Year to year fluctuations in population numbers are typical for *B. bufo* (R. Oldham and D. Latham, personal communication) and for many other amphibian species (Pechman *et al.* 1991).

*Neutral alleles.* Selection may cause $F_c$ to be either smaller or larger than expected under genetic drift alone (Waples 1989). Selection could have occurred during the larval stage, prior to metamorphosis. Samollow (1980) in a study of temporal and age-specific variation in allozyme allele frequency in *Bufo boreas* suggested that large inter- and intragenerational differences in allele frequency were due to nonrandom processes. However, minisatellite loci are generally found within noncoding regions, and in the absence of linkage to nonneutral coding regions, should be transparent to the effects of selection. Further, selection of constant intensity has been shown to have little effect on $F_c$ if $t/N_b$ is small (Nicholas & Robertson 1976; Pollack 1983), which is the case in the present study.

*No mutation.* Concerns have been raised over the use of VNTR loci to address population genetic questions [see Shriver *et al.* (1993) and Forbs *et al.* (1995) for discussions of minisatellite data and Goldstein *et al.* (1995) and Slatkin (1995) for similar discussions regarding microsatellite data]. Assumptions of largely unknown mutational-drift processes are critical when drawing inferences from population allele frequency distributions. Mutation rates for VNTR loci have been shown empirically to be orders of magnitude higher than those documented for other loci (Jeffreys *et al.* 1988). Over relatively short periods of time (e.g. single breeding cycles or few generations) estimates of $N_b$ or $N_e$ obtained from highly polymorphic VNTR loci would not be expected to be confounded by high rates of mutation.

*No migration.* Multigenerational estimates of $N_e$ may be strongly biased by migration between subpopulations. However, use of parent–offspring data negates any potential bias. Further, amphibians are highly philopatric to breeding areas (Berven & Grudzien 1990) and thus little gene flow would be expected between ponds separated by

distances exceeding nonbreeding home ranges (1–2 km for *B. bufo*; Reading *et al*. 1991).

*Characteristics of minisatellite loci.* Several properties of minisatellite loci may confound estimates of $N_b$ or $N_e$. Estimates of the variance in $N_e$ may be problematic when the magnitude of $F_c$ is small relative to $[1/2(S_0) + 1/2(S_t)]$, as temporal differences in allele frequency can simply be explained by sampling error without invoking genetic drift (Waples 1989). Minisatellite loci (as described in this study) are typically highly polymorphic and large sample sizes may be required to adequately characterize population allele frequencies.

Waples (1989) provides valuable insights into sampling strategies to maximize the precision of $N_e$ estimates given time and resource constraints. Using computer simulations, Waples showed that precision increased by about the same degree whether sample size, number of alleles surveyed, or number of generations between samples increased. The large number of alleles typically resolved from minisatellite (e.g. Table 1) or microsatellite loci suggests that estimates obtained using VNTR loci should be more precise than estimates obtained using a comparable number of other marker loci (such as allozymes). For example, in *B. bufo*, only six of 46 allozyme loci were found to be variable in Great Britain and only three loci had more than two alleles (Scribner *et al*. 1994).

Populations surveyed using minisatellite loci are typically characterized by many alleles, often in low or moderate frequency. Rare alleles can upwardly bias estimates of $N_b$ (R. S. Waples, personal communication). In this study many rare minisatellite alleles were observed in one of the three populations and in only the adult age class (Table 1). However, all alleles were observed in other populations surveyed ($N$ = 26; J. W. Arntzen, unpublished data). One means of countering potential bias would be to combine rare alleles. Indeed, combining rare alleles (frequency < 0.05) had little affect on estimates of $N_b$ (Table 2).

Scoring errors can also effect estimates of $N_b$. VNTR loci are characterized by the number of repeat units they contain. For minisatellites, measurement error may exceed the repeat length, and estimates of fragment mobility may not correspond directly to repeat copy number. The continuous distribution of fragment sizes often necessitates the 'binning' of fragments of comparable size, and as such demands that considerable rigor be employed to empirically assess the potential for scoring error.

To determine the effects of precision of fragment size estimates (and thus bin assignment) on estimation of $N_b$, a series of alleles of similar size for the *Bbu*MS3 locus was pooled. Four alleles (3251, 3305, 3356 and 3403) were combined into one composite allele and alleles 4924, 4964 and 5023 were combined into a second composite allele. Estimates of $N_b$ based on these criteria resulted in slight changes in estimates of $N_b$ for two populations and a large adjustment for the third population. However, several factors suggest that scoring error was not a significant factor. Firstly, differences in allele size greatly exceeded the empirically determined scoring errors for alleles in this size range for *Bbu*MS3 and the other loci. Secondly, equal numbers of individuals from each population were run on each gel and it is difficult to conceive that scoring bias for individuals from Charnwood (the population showing the largest change in $N_b$ estimates with allele pooling) would not have been evident for the other populations as well. Thirdly, frequencies of pooled alleles were higher in the two populations (Osgathorpe and Osbaston) where estimates of $N_b$ were not dramatically changed by pooling. Finally, differences in allele sizes for *Bbu*MS2 and *Bbu*MS1 were more pronounced than for *Bbu*MS3 (i.e. less prone to scoring error) and yet single-locus estimates of $N_b$ were comparable across all loci.

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Data highlighted in this paper represent one component of a highly integrated ecological and genetics study of amphibians inhabiting fragmented habitats in central Great Britain. The study was the result of a fruitful collaboration between the laboratory of Professor H. C. MacGregor and Dr T. Burke at the University of Leicester and R. Oldham at DeMontfort University. Drs Scribner and Arntzen were postdoctoral researchers and were responsible for the laboratory and field ecology segments of the study, respectively. The authors share interests in using genetic markers to draw ecological and evolutionary inferences from natural populations.

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